Universidade Federal de Minas Gerais
Instituto de Ciências Biológicas
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Modelos animais de disfunção colinérgica: papel da acetilcolina na cognição

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Universidade Federal de Minas Gerais

DECLARAÇÃO

DECLARAMOS para os devidos fins, que **Xavier De Jaeger** concluiu o Doutorado em Ciências Biológicas: Farmacologia Bioquímica e Molecular da Faculdade de Medicina desta Universidade, tendo defendido sua tese intitulada "**Modêlos animais de disfunção colinérgica: papel da acetilcolina na cognição**", aprovada em 06/12/2010. Esta declaração será válida até o dia 31/03/2011, período correspondente ao prazo que o aluno tem para entregar o exemplar definitivo de sua tese a este Programa de Pós-graduação. London, Canadá, 06 de dezembro de 2010.

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“Discovery consists of seeing what everybody has seen and thinking what nobody has thought.”

Albert Szent-Gyorgyi

“If something goes wrong at the plant, blame the guy who can't speak English.”

Homer Simpson

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Abstract

The release of the neurotransmitter acetylcholine (ACh) depends on the vesicular loading by the vesicular acetylcholine transporter (VAChT). ACh is important for different cognitive functions such as learning, memory and attention. We have generated mice with distinct expression levels of VAChT and consequently ACh release to probe for functional roles of synaptic vesicle filling in cholinergic activity and cognitive function. We provided evidence that the reduction of ACh release or injection of muscarinic receptor antagonist blocked object recognition long term memory (LTM) without affecting remote memory. Using a second mouse line in which we selectively eliminated VAChT in the basal forebrain with the cre-loxP method, we suggest that the cholinergic projections of the basal forebrain neurons are crucial for LTM.

The introduction of a Tk-Neo resistance cassette added approximately 1.5 kb downstream from the VAChT stop codon caused a major reduction of VAChT expression in the brain, but relatively preserved peripheral function. We studied the consequence of the reduction of VAChT with two different strains of mice, and observed an increase of locomotor activity without changes in anxiety. We also demonstrated that genetic removal of the Neomycin resistance cassette rescues VAChT expression and the hyperactivity phenotype.

Striatal activity plays important roles in controlling motor functions and reward-related behaviours and it is the major brain region affected in several motor diseases such as Parkinson's disease and Huntington's disease. Dopamine-acetylcholine balance in the striatum is believed to be critical for proper motor function and behaviour. To understand the specific contribution of the cholinergic neurons in the striatum, we also developed a mouse line in which we selectively eliminated VAChT in the striatum. We found surprisingly that this mice do not show changes in motor performance and motor learning. However, decreased cholinergic function in the striatum seems to specifically improve the response of mice in the forced-swimming task, suggesting that striatal VAChT might be a novel target for treatment of depression associated with Parkinson's disease.

Resumo

A liberação do neurotransmissor acetilcolina (Ach) depende do transporte vesicular pelo transportador vesicular de acetilcolina (VAChT). A acetilcolina é importante para funções cognitivas diferentes como aprendizado, memória e atenção. Nós geramos camundongos com diferentes níveis de expressão de VAChT e, conseqüentemente, liberação de acetilcolina, para buscar os papéis funcionais do transporte de ACh em vesículas sinápticas nas atividades colinérgicas e funções cognitivas. Nós demonstramos que a redução de liberação de ACh ou injeções de antagonistas do receptor muscarínico bloquearam a memória de longo prazo (LTM) de reconhecimento de objetos sem afetar a memória remota. Usando uma linhagem diferente de camundongos, em que eliminamos o VAChT seletivamente do prosencéfalo basal com o método cre-loxP, sugerimos que as projeções colinérgicas dos neurônios do prosencéfalo basal são essenciais para a LTM.

A introdução do cassete de resistência a neomicina, adicionado aproximadamente 1.5 kb anteriormente ao "stop codon" do gene para o VAChT, causou uma redução da proteína principalmente no cérebro, com a expressão desse transportador preservada na periferia. Nós estudamos a conseqüência da redução de VAChT utilizando duas linhagens diferentes de camundongos, e observamos que a diminuição do VAChT promove um aumento na atividade locomotora sem afetar a ansiedade. Nós também demonstramos que a remoção genética do cassete de resistência à neomicina restaura a expressão de VAChT e o fenótipo de hiperatividade.

A atividade do estriado tem um papel importante no controle das funções motoras e do comportamento associado à recompensa. Essa é a principal região do cérebro afetada em diversas doenças motoras como as doenças de Parkinson e Huntington. Acredita-se que o balanço dopamina-acetilcolina no estriado seja essencial para a função motora e o comportamento. Para entender a contribuição específica dos neurônios colinérgicos do estriado, desenvolvemos uma linhagem de camundongos que tem uma deleção seletiva de VAChT nos neurônios do estriado. Nós observamos que esses camundongos não apresentam alteração no desempenho motor e aprendizagem motora. Porém, a diminuição colinérgica no estriado parece melhorar a resposta dos camundongos no teste de nado forçado, sugerindo que o VAChT no estriado poderia ser um novo alvo para o tratamento de depressão associada a doença de Parkinson.

Sumário

Introdução

I- Acetilcolina

a) Descoberta

Acetilcolina, o principal alvo deste estudo, tem papel de neurotransmissor no sistema nervoso, o que foi descoberto em 1920 por Otto Loewi. Loewi demonstrou que a transmissão nervosa entre o nervo e o coração é mediada quimicamente. Quando estimulado, o nervo vago do coração de uma rã produziu uma substancia que se difundiu e permitiu que, quando o líquido de perfusão tivesse com o coração de uma outra rã, alterasse a atividade do segundo coração. Como resultado, a atividade do coração da rã receptora foi diminuída através da ação da "substância do vago", denominada, por Loewi, de "vagusstoff" revisto por (Loewi, 1924; Brown, 2006; Bear et al., 2007).

Essa substância, que é essencial tanto para o controle da atividade cardíaca, bem como para a contração da musculatura esquelética, foi renomeada como acetilcolina (ACh). A acetilcolina é liberada na junção neuromuscular e participa diretamente na contração muscular. O bloqueio da ação da ACh na junção neuromuscular pode ser letal provocando paralisia e deficiência respiratória. A neurotransmissão colinérgica é também essencial para o funcionamento do sistema nervoso central. Sabemos que a acetilcolina participa do controle central e periférico do movimento, do funcionamento do sistema nervoso autônomo, da regulação do sono e de múltiplos processos cognitivos como memória, atenção e aprendizado (Gold, 2003; Sarter and Parikh, 2005; Hasselmo, 2006).

O papel crucial do sistema colinérgico nas funções cognitivas foi demonstrado através de estudos onde foi observado que antagonistas dos receptores muscarínicos, como a escopolamina e a atropina, diminuíam as habilidades cognitivas em vertebrados. (Deutsch, 1971; Drachman, 1977). Recentemente foi demonstrado que o transportador vesicular de acetilcolina (VAChT), presente nas vesículas sinápticas, é essencial para a liberação da ACh na sinapse (Prado et al., 2006; de Castro et al., 2009a). Os mesmos autores demonstraram que a liberação da ACh é crucial para sobrevivência, uma vez que os camundongos *Knockout* para VAChT, os quais não liberam ACh, não conseguem sobreviver após o nascimento (de Castro et al., 2009b).

b) Sinapse colinérgica

A neurotransmissão química clássica requer os seguintes passos: 1-Síntese do neurotransmissor no citosol do terminal pré-sináptico; 2-Armazenagem do neurotransmissor em vesículas sinápticas; 3-Liberação regulada de neurotransmissor na fenda sináptica; 4-A presença de receptores específicos para o neurotransmissor no terminal pós-sináptico; 5-Uma forma de terminar a ação do neurotransmissor (revisto por Taylor e Brown, 1999).

A ACh é sintetizada pela enzima colina acetiltransferase (ChAT), a partir dos substratos colina e acetato. A ChAT é um marcador específico para os neurônios colinérgicos, e catalisa a transferência de um grupo Acetil, proveniente de uma molécula de Acetil-CoA, para uma molécula de colina. A Acetil-CoA é gerada através da enzima citosolica ATP-citrato liase que catalisa a formação de Acetil-CoA e oxaloacetato a partir de CoA e citrato, com a hidrólise de ATP em ADP e fosfato (Beigneux et al., 2004). A colina é provida principalmente da alimentação, tanto que a ingestão de colina influencia diretamente a sua disponibilidade no cérebro assim como a síntese de ACh (revisto por Fernstrom, 1981). A fosforilação de ChAT pode modificar a atividade catalítica da enzima e modificar a síntese e a liberação de ACh, de tal forma a ChAT é uma enzima chave na transmissão colinérgica (revisto por Dobransky and Rylett, 2005).

A ACh é posteriormente transportada para o interior de vesículas sinápticas pelo transportador vesicular de acetilcolina (VAChT) (Whittaker et al., 1964; Diebler and Morot-Gaudry, 1981). VAChT é uma proteína com 12 domínios transmembrana com uma regiao N-e C-terminal voltada para o citosol. O gene do VAChT de *C. elegans* (*unc-17*) foi inicialmente clonado por Alfonso e colaboradores (1993). Eles mostraram que esse gene possui homologia com os transportadores vesiculares de monoaminas (VMATs) e propuseram que o produto desse gene poderia codificar um transportador vesicular. Posteriormente, foram identificados o homólogo de *unc-17* no rato e demonstrado in vitro que essa proteína é um transportador vesicular de acetilcolina funcional (Erickson et al., 1994). O VAChT é capaz de realizar o transporte da ACh utilizando um gradiente de prótons gerado por uma ATPase presente na vesícula. Para acumular Ach nas vesículas, o transportador troca dois prótons por uma molécula da ACh (Nguyen et al., 1998). A expressão de um tipo particular de transportador nas vesículas é provavelmente o maior determinante do tipo de neurotransmissor usado por um neurônio. No entanto essa regra geral tem exceções importantes: o transportador glutamatérgico (VGLUTt3) foi encontrado em alguns neurônios que não são considerados como glutamatérgicos, como os neurônios colinérgicos do corpo estriado (Fremeau et al., 2002; Gras et al., 2002; Schafer et al., 2002; revisto por Sudhof, 2004). A coexpressão do

transportador glutamato e colinérgico, pode conferir a esse grupo de neurônio a possibilidade de liberar diferentes neurotransmissores.

O gene VAChT tem a particularidade de ser incorporado no primeiro intron do gene para ChAT. Essa organização do lócus colinérgico foi demonstrada em *Caenorhabditis elegans* assim com em rato e drosófila (revisto por Mallet et al., 1998). VAChT e ChAT possuem promotores distintos e comuns que geram múltiplas espécies de RNA mensageiro (Mallet et al., 1998). Deste modo, em determinadas circunstâncias VAChT e ChAT podem ser regulados concomitantemente ou de forma independente.

Vesículas sinápticas formam “pools” funcionais distintos. Elas podem formar um “pool” de reserva (≈80%) e um “readily releasable pool” (20%). As vesículas sinápticas, prontas para serem liberadas, encontram-se em uma região subjacente à membrana sináptica, onde são ancoradas em uma zona denominada zona ativa. Nessa região, elas se tornam responsivas às mudanças na concentração intracelular de cálcio. Quando um potencial de ação atinge o terminal nervoso, canais de cálcio ativados por voltagem são abertos. As ondas de cálcio resultantes induzem a fusão de vesículas sinápticas com a membrana e a conseqüente liberação de ACh (revisto por Van der Kloot, 2003; Sudhof, 2004). Após a sua liberação, a ACh pode interagir com os receptores muscarínicos e nicotínicos pré e pós-sinápticos (Figura 1).

A hidrólise da ACh é realizada pela enzima Acetilcolinesterase (AChE), assim a ACh é metabolizada em acetato e colina (Soreq and Seidman, 2001; Silman and Sussman, 2005). A AchE é uma enzima chave para a regulação da concentração de ACh na fenda sináptica. A inibição da AChE é capaz de aumentar a duração de ação do neurotransmissor (revisto por Silman and Sussman, 2005). E a inibição da AchE pode ser induzida através de diferentes drogas como galantamina ou pyrostigmina, aumentando a ação do neurotransmissor (ACh) (Darvesh et al., 2003).

A colina, proveniente da hidrolise da Ach, é recaptada pelo transportador de colina de alta afinidade (CHT1) para neurônio pré-sináptico e utilizada para a síntese de novas moléculas de ACh (Prado et al., 2002; Ribeiro et al., 2006). Considerando-se que os neurônios não produzem colina suficiente e que a maior parte da colina necessária para a síntese de ACh provem da dieta, sua recaptação para o interior neuronal é essencial (Zeisel, 1981). Este transporte, realizado através do transportador de alta afinidade da colina (CHT1), é específico de neurônios colinérgicos e inibido por baixas concentrações da droga hemicolinium-3 (HC-3). Existe também um transporte de colina que está presente em todas as células e é inibido por altas doses de HC-3 (Yamamura and Snyder, 1973).

Na doença de Alzheimer (DA), a principal característica patológica é classicamente representada pela presença de depósitos extracelular do peptídeo β-amilóide, que é derivado de uma clivagem anormal da proteína precursora de amilóide (APP) nas placas senís, e pela formação intracelular de emaranhados neurofibrilares, contendo formas anormalmente fosforiladas da proteína Tau associada aos microtúbulos (revisto por Martorana et al., 2010). Embora haja um declínio generalizado de vários neurotransmissores nós corpos celulares na fase final da DA, as perdas mais consistentes ao longo da progressão da doença de Alzheimer são observadas em neurônios com projeções compridas, dentre eles, os neurônios colinérgicos do prosencéfalo basal (revisto Por Mufson et al. 2008). Os neurônios colinérgicos do prosencéfalo basal (RN) e do complexo banda diagonal septal são a principal fonte de inervação colinérgica do córtex cerebral e hipocampo, respectivamente, e acredita-se que desempenham um papel fundamental na memória e atenção (revisto por Mufson et al., 2008).

Os pacientes com DA têm uma alteração das faculdades cognitivas, manifestando-se inicialmente por alterações da memória episódica. Estes déficits amnésicos agravam-se com a progressão da doença, e são posteriormente acompanhados por déficits de orientação espaciais e de linguagem (Winkler et al., 1998). Foi descoberto em 1970 o déficit da ChAT (Perry et al., 1977) e a perda dos neurônios colinérgicos (Davies and Maloney, 1976) na doença de Alzheimer, seguido da descoberta da diminuição da recaptação da colina (Rylett et al., 1983; Nilsson et al., 1986; revisto por Francis et al., 1999). Baseado nessas observações, e nós primeiros estudos do papel da ACh na aprendizagem e na memória, foi desenvolvido a hipótese colinérgica da DA (Drachman and Leavitt, 1974). Essa hipótese levou ao desenvolvimento do tratamento com inibidor da AchE em pacientes com Alzheimer. Esse tratamento demonstrou em alguns estudos uma melhora das funções cognitivas em um grupo de pacientes (Sevush et al., 1991; Rogers et al., 1998). As drogas atualmente utilizadas são Galantamina, Rivastigmina e Donepezil (Lleo et al., 2006).

Além disso, a disfunção da transmissão glutamatérgica, que anteriormente era subestimada por ter-se como foco principal a cascada amilóide e a hipótese colinérgica, foi identificada como um processo típico precoce e importante na DA (Parsons et al., 2007; Ondrejcak et al., 2010). Por tais motivos, uma droga que atua na transmissão nervosa glutamatérgica, a memantina (antagonista do receptor NMDA não competitivo) também é utilizada no tratamento de demência (Martorana et al., 2010).

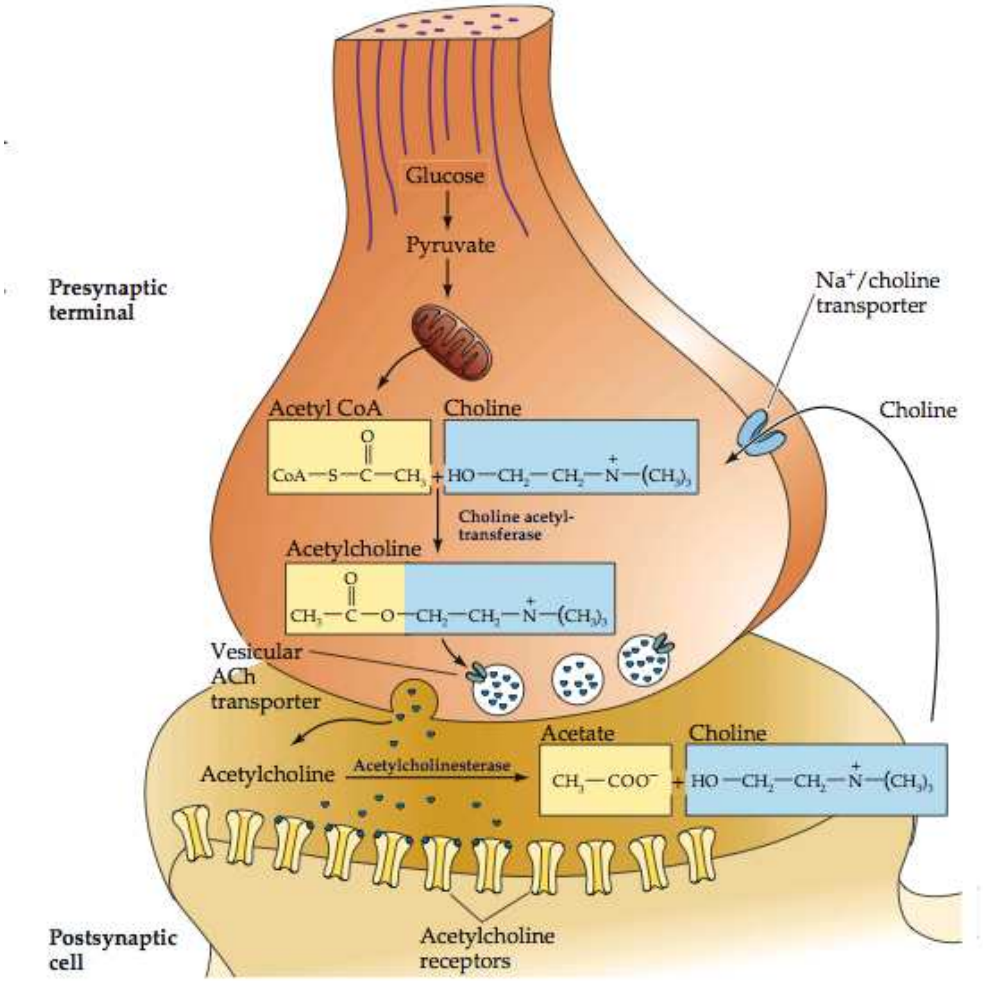

Figure 1. Desenho esquemático representando a neurotransmissão colinérgica (Purves and Williams, 2001).

c) Distribuição dos neurônios colinérgicos.

Na figura 2 estão demonstrados os principais grupos de neurônios colinérgicos: os neurônios colinérgicos do prosencéfalo basal compostos do septo medial, banda horizontal, núcleo basal e da substância inominada inerva uma grande parte do cérebro como o isocórtex, córtex peririnal (PRh) mas também algumas regiões mais profundas como o hipocampo, a amídala e o hipotálamo (revisto por Woolf, 1991). Os neurônios colinérgicos dessa região parecem estar envolvidos na memória e na atenção (revisto por Gold, 2003; McKinney and Jacksonville, 2005; Niewiadomska et al., 2009; Fadel and Burk, 2010).

O tronco encefálico agrupa o núcleo tegmental pedonculopontino (PPTg) e núcleos de neurônios motores como os neurônios motores faciais (Mo5) e os neurônios trigêmeos (7N). O núcleo peduncolopontino contém neurônios colinérgicos que são maiores que no

prosencefalo basal. Esses neurônios se projetam amplamente em vários alvos do tálamo, substância negra, prosencefalo basal e gânglio basal. Os neurônios colinérgicos do núcleo tegmental pedonculopontino parecem ter papel nós movimentos rápido dos olhos, na aprendizagem, no sono e nas função sensoriais e motor (Kobayashi and Isa, 2002; Wang and Morales, 2009). Muitos neurônios positivos para ChAT são encontrados nós núcleos dos nervos craniais 5, 7 e 12 (Mo5, 7N). Esses neurônios motores somáticos enervam a musculatura orofacial, incluindo os músculos superficiais da cabeça, do pescoço, da língua e da mandíbula (revisto por Woolf, 1991).

O estriado é composto do núcleo caudado, putamen e inclui o núcleo acumbens. O corpo estriado contém uma rica rede de interneurônios colinérgicos (revisto por Woolf, 1991). O caudato e o putamen são similares em sua morfologia, eles são considerados como um complexo único denominado neostriatum. Essa região recebe “input” de todas as regiões do córtex (revisto por Woolf, 1991). Acredita-se que os neurônios colinérgicos dessa região estão envolvidos no comportamento locomotor, assim como em mecanismos envolvidos com a ação de drogas psicoativas como a cocaína (revisto por Williams and Adinoff, 2008).

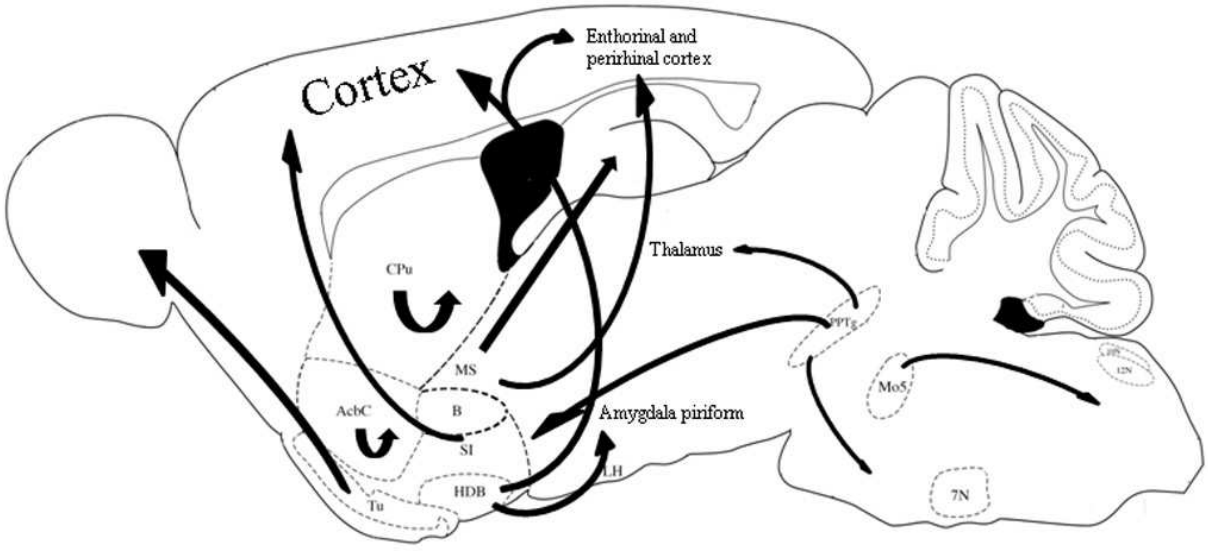

Figura 2. Representação dos principais neurônios colinérgicos e as conexões eferentes em um corte parasagital.

III- Papel da acetilcolina na cognição

Vários trabalhos têm demonstrado a influência da modulação da ACh em várias funções cognitivas como a memória, locomoção, ansiedade, depressão (Smythe et al., 1996; Chau et al., 2001; Hikida et al., 2001; Gold, 2003; Kitabatake et al., 2003). A seguir, faremos um pequeno resumo do estado da arte do papel da ACh no cérebro.

a) Memória

O aprendizado é o processo que permite obter informações, enquanto a memória é o processo que permite armazenar essas informações. A memória é definida como a aquisição, formação, conservação e a evocação de informações. Existem várias classificações para a memória, de acordo com sua função, com o tempo de duração e com o seu conteúdo (Izquierdo et al., 2002).

Em relação ao tempo de duração podendo ser classificada de memória de curta duração, memória de longa duração e memória remota. Geralmente a memória de curto prazo é considerada como a memória que mantém a informação por algum horas, o tempo necessário para que a memória de longa duração seja consolidada (McGaugh, 1966; Dudai, 2004). A memória de curta duração é lábil, ela pode ser alterada por estímulos externós e vários (Duncan, 1949; Izquierdo, 1989; McGaugh, 2000). A memória de longo prazo não é tão lábil, mas precisa de um tempo maior para ser codificada na rede neural. O tempo de consolidação para a memória de longa duração é estimado por no mínimo 6 até 12 horas. A maior diferença entre essas duas memórias não reside no conteúdo, mas nós mecanismos de cada uma. Os mecanismos moleculares envolvidos mostram claramente que esses dois tipos de memória são mecanismos separados, paralelos e independentes (Izquierdo et al., 1999; Izquierdo et al., 2002). A consolidação permite que a informação lábil sensível nas perturbações seja fixada na rede neural e estabilizada. Ela necessita vários mecanismos como a transcrição de genes ou a síntese de nova moléculas (Davis and Squire, 1984; Freeman and Young, 1999; Guzowski, 2002; Alkon et al., 2005). De fato, foi comprovado que, durante essa fase, a administração de vários inibidores de enzimas, antagonistas dos receptores podem impedir a fixação da memória (Izquierdo et al., 2006).

Existe também a memória remota para conservar as informações por mais tempo, essa memória vai ao longo do tempo tornar-se independente do hipocampo e parece envolver regiões mais corticais. Foi observado no labirinto radial, um aumento do metabolismo do córtex e uma diminuição do metabolismo do hipocampo quando os animais foram testados 25

dias após o treino (Bontempi et al., 1999). Resultados similares foram observados em humanós. Por exemplo, o paciente E.P. tem lesão bilateral extensiva do lobo temporal medial, incluindo o hipocampo, ele apresenta uma grande dificuldade para reconhecer um examinador mesmo após várias visitas. No entanto esse paciente consegue lembrar-se muito bem da cidade onde ele morou 50 anós atrás, mostrando assim que as memórias remotas estão conservadas em região independente do hipocampo (Teng and Squire, 1999). O processo de consolidação que permite a transferência da informação do hipocampo ao córtex é chamado consolidação sistêmica, enquanto o processo que leva a formação da memória de longo prazo é chamado consolidação celular. A estabilização das representações internas na memória de longo prazo envolve uma consolidação sináptica, que é atingida dentro de minutos a horas. Em paralelo, ou como uma conseqüência, o processo de consolidação do sistema é iniciado, caracterizando-se por ter uma cinética temporal mais lenta (Dudai, 2004).

Nader em 2000, demonstrou que mesmo a memória consolidada volta ser lábil após a sua reativação, e que para conservar a consolidação, uma nova fase de síntese protéica era necessária (Nader et al., 2000). Ele mostrou que mesmo a memória remota voltava ser dependente da síntese protéica após a reativação. Ambas, a consolidação e a reconsolidação, tem uma janela temporal durante a qual o bloqueio das proteínas afeta os processos (Artinian et al., 2008). Mesmo sendo a síntese protéica após a reativação bem estabelecida (revisto por Dudai, 2006), não há consenso se a reconsolidação é apenas uma repetição de consolidação ou um processo distinto (Alberini, 2005). Por exemplo foi mostrado que a consolidação na região CA3 do hipocampo no teste do labirinto aquático necessita duas ondas de síntese protéica enquanto a reconsolidação precisa só de uma (Artinian et al., 2008).

A importância da ACh nas funções cognitivas, particularmente na aprendizagem e na memória, foi demonstrada através de estudos farmacológicos. Estudos recentes mostram que uma disfunção colinérgica pode influenciar a memória alterando a consolidação desta. A injeção de drogas que afetam a função colinérgica após treino como antagonistas dos receptores colinérgicos perturba a consolidação da memória (Izquierdo et al., 1992; Kopf et al., 1998). Por outro lado, a memória melhora com injeção de agonistas dos receptores muscarínicos (Dalmaz et al., 1993; Barros et al., 2002; Power and McGaugh, 2002). Esse papel da ACh na consolidação foi demonstrado em diferentes tarefas como a memória do gosto (Gutierrez et al., 2003), ou ainda no labirinto radial, onde foi observado que um aumento da ACh durante a consolidação da memória permite diminuir a quantidade de erro dessa tarefa. Para estimular a liberação de ACh os autores colocaram um objeto na caixa após

o treino, a exploração desse objeto provocou um aumento da liberação da ACh no hipocampo (Degroot et al., 2005).

A memória também pode ser classificada quanto ao seu conteúdo. Elas podem ser dividas em memória de trabalho, procedural ou implícita e declarativa ou explícita (Squire, 1987). A memória de trabalho é aquela que mantém a informação por segundos ou minutos, para ser usado num momento imediatamente posterior a sua aquisição para a realização de uma tarefa ou tomada de decisão (Baddeley, 2000). A memória de trabalho serve por exemplo para lembrar um número de telefone o tempo necessário antes discá-lo. Um bom teste para acessar a memória de trabalho muito utilizado na clínica, é o da lembrança de número (digit spam). Depois de alguns segundos um indivíduo normal consegue lembrar entre sete ou oito "dígitos", enquanto um paciente com a doença de Alzheimer avançado consegue lembrar só um ou dois.

A memória procedural se refere as capacidades e habilidades motoras ou sensoriais, como tocar piano ou andar de bicicleta (Squire, 1987). Em 1962, Milner treinou o paciente H.M. (paciente com lesão bilateral do hipocampo) em uma tarefa de desenho no espelho. Esta tarefa envolve o rastreamento de alguma figura no papel onde só se vê a imagem espelhada do desenho. Inicialmente as pessoas normais tinham uma grande dificuldade nessa tarefa, mas desenvolveram certa habilidade com o treinamento. H. M. tinha um padrão de aprendizagem normal para esta tarefa (Corkin, 1968; Squire, 2009), sugerindo que o hipocampo não intervem na memória procedural. Resultados similares foram observados em pacientes com a doença de Alzheimer onde esses conseguem melhorar a suas performances nessa tarefa (Gabrieli et al., 1993).

As principais estruturas implicadas para a aprendizagem de tarefa motora são o estriado, cerebelo e regiões motoras corticais (Doyon et al., 2003). Em ratos foi observado que a memória procedural não envolve o hipocampo, mas o estriado se revelou importante. A tarefa do labirinto em T, pode ser resolvida usando uma estratégia corpórea (egocêntrica) ou de lugar (alocêntrico) (Chang and Gold, 2003). Ratos foram treinados para ir do braço sul até o braço leste e foi observado que a ACh aumenta no hipocampo no início do treino quando o animal usa mais uma estratégia de lugar associada na memória espacial, enquanto no estriado ocorre um aumento da ACh gradual seguindo a mudança de uma estratégia espacial para uma estratégia corpórea, envolvendo a memória procedural (revisto por Gold, 2003). Uma tarefa classicamente utilizada para avaliar a memória procedural nós roedores é o "rotarod" (cilindro giratório) acelerado. Nessa tarefa o papel da ACh estriatal parece ser importante (Carta et al., 2006), embora camundongos nocaute para receptores nicotínicos não apresentaram déficit

nessa tarefa (Marubio and Paylor, 2004). Essa contradição mostra a importância de realizar estudo complementar sobre o papel da ACh na memória procedural.

A memória declarativa ou explicita é consciente e composta por fatos, eventos ou conhecimento (Squire, 2004). As memórias declarativas dividem-se em episódicas e semânticas referindo-se, respectivamente, aos acontecimentos passados na biografia de um indivíduo e aos conhecimentos gerais do significado das coisas, adquiridos no decorrer da vida. A memória episódica se relaciona aos eventos que participamos ou vivemos, como as últimas férias ou os rostos dos amigos. Essas lembranças são associadas à um lugar e à um momento (por exemplo, todos conseguem lembrar muito bem do momento e o lugar onde uma injúria grave ocorreu). A memória declarativa necessita de uma integração de informações multimodais, essa memória lida de forma contínua com três componentes: objeto, espaço, tempo ou o que, quando, onde. Os estudos sobre a memória declarativa começaram a partir da descrição do paciente H.M. no anós 1957 (Scoville and Milner, 1957). Esse paciente, com uma epilepsia refratária, tornou-se amnésico após a cirurgia bilateral do lobo temporal medial. Os testes neuropsicológicos mostraram que, embora HM teve uma grande perda de memória anterógrada, ele ainda poderia adquirir habilidades motoras e cognitivas implicitamente, mas não conseguiu lembrar o contexto dessa aprendizagem. H.M. não conseguia reconhecer as pessoas encontradas depois da operação, mais ele conseguia aprender tarefas como tocar piano.

Um grande desafio foi desenvolver um modelo animal com amnésia do lobo medial temporal, a partir daí surgiu o “paired-comparison” um teste de preferência espontânea por novidade desenvolvido em primata (revisto por Clark and Squire, 2010). Esse teste foi adaptado para roedores por Ennaceur, e ao contrário dos testes com primata, os ratos exploram fisicamente os objetos. Este teste é denominado *Teste de reconhecimento do novo objeto* (OR) (Ennaceur and Delacour, 1988; Bevins and Besheer, 2006).

1. Reconhecimento de objeto

Nós primatas não humanós o teste de reconhecimento de objetos é geralmente testado no “delay nonmatching-to-sample” (revisto por Clark and Squire, 2010). Nessa tarefa, o examinador apresenta uma imagem que o macaco tem que memorizar, após um intervalo duas imagem são apresentadas, a mesma e uma nova. O macaco tem que escolher a imagem nova que não correspondente a que ele viu anteriormente (nonmatching). O “delay nonmatching-to-sample” foi desenvolvido para acessar a memória declarativa nós animais e para reproduzir o mesmo déficit de memória declarativa observado no paciente H.M. (Mishkin and Delacour,

1975; Mishkin, 1978; detais em Clark and Squire, 2010). Tem sido proposto que o teste do reconhecimento espontâneo de objetos apresenta analogia com testes de memória de reconhecimento utilizados em humanós para caracterizar síndromes amnésicas, como o teste "delay nonmatching-to-sample", que fornece um índice acurado do grau de severidade geral de prejuízos da memória declarativa (Reed and Squire, 1997; Dix and Aggleton, 1999). Por essas razões, o teste de reconhecimento de objeto é um excelente teste para acessar a memória declarativa dos roedores. Baseia-se na exploração espontânea e diferencial de objetos novos e familiares, através do tempo gasto na exploração nós objetos.

Nós roedores foi demonstrado que várias regiões do cérebro podem estar relacionadas com o reconhecimento de objetos, como o hipocampo (Rossato et al., 2007; Broadbent et al., 2010), o córtex perirhinal (Abe and Iwasaki, 2001; Abe et al., 2004; Winters and Bussey, 2005a, b; Winters et al., 2010), e o lobo temporal (Balderas et al., 2008). Recentemente foi também demonstrado que o teste de reconhecimento de objeto era capaz de induzir modificações sinápticas e promover potenciação de longa duração no hipocampo (Clarke et al., 2010). Além de provocar LTP, a exploração de objeto novo provoca um aumento da liberação de ACh no hipocampo (Degroot et al., 2005).

A intervenção da ACh nesse tipo de memória foi demonstrada com vários estudos farmacológicas em ratos e camundongos. O déficit de memória provocada por injeção de escopolamina (antagonista muscarínicos), pode ser revertida com injeção de galantamina (inibidor de acetilcolinesterase) (Ennaceur and Meliani, 1992; Abe et al., 2004; de Bruin and Pouzet, 2006). Os receptores nicotínicos participam também da memória, quando são estimulados por agonistas, permitem melhora das capacidades cognitivas no teste de reconhecimento do objeto (Puma et al., 1999; For more detail see Dere et al., 2007). As projeções colinérgicas do prosencéfalo basal para o córtex perirhinal parecem particularmente importantes na formação da memória de reconhecimento de objeto. O fato de remover o input colinérgico do córtex perirhinal matando neurônios do prosencéfalo basal com a injeção da imunotoxina 192 IgG-saporin no córtex perirhinal, perturba a memória de objeto (Winters and Bussey, 2005c). Outros estudos mostram a participação dos receptores muscarínicos nessa região do cérebro nós mecanismos de memória do reconhecimento de objeto (Abe and Iwasaki, 2001; Warburton et al., 2003; Abe et al., 2004; Winters et al., 2010). A ACh, no córtex perirhinal, e prefrontal, é essencial para formar memória espacial dos objetos (Barker and Warburton, 2009). Esses resultados fornecem evidências sobre a implicação da ACh no componente do conteúdo e do lugar da memória declarativa. O teste de reconhecimento de objeto permite também acessar o componente temporal da memória declarativa, e de novo a

ACh participa na formação da memória. Os camundongos mutante knock-down (KD) para VAChT apresentam um déficit de memória no teste temporal de reconhecimento de objeto, e esse déficit pode ser revertido com inibidor de acetilcolinesterase (de Castro et al., 2009a). Apesar de o papel da ACh ser bastante estudado, o uso de camundongo mutante para VAChT permite aumentar esse conhecimento, pois fornece informações relacionadas a mecanismos fisiológicos envolvidos com o aumento da transmissão colinérgica, devido a alterações na expressão e função desse transportador.

2. Habituação

A habituação é uma aprendizagem não associativa em que ocorre uma diminuição automática da intensidade de uma resposta comportamental a um estímulo repetitivo (Bear et al., 2007). Um exemplo de habituação é o espantalho para assustar as aves, depois de várias dias ele não vai mais assustar as aves pois estas habituam-se com a presença do espantalho.

A habituação é muito importante ao longo da evolução dos organismos, esse mecanismo de memória não-associativa permite a economia de energia e o desenvolvimento de uma resposta adaptada, evitando respostas comportamentais inadequadas. Nós roedores, a habituação foi freqüentemente estudada através do comportamento exploratório em um ambiente novo (Ericson et al., 1991; Leussis and Bolivar, 2006). A atividade locomotora medida pela distância que um animal anda reflete a exploração ao um contexto novo. Nós avaliamos a habituação em um contexto novo quantificando o comportamento ambulatório dos camundongos em um campo aberto.

De acordo com a teoria do mapa cognitivo (O'Keefe and Nadel, 1978), um roedor, quando colocado em um novo ambiente inicia a formação de uma representação espacial interna do ambiente. Essa formação é realizada com o reforço das sinapses. Uma vez que o reforço for suficiente e que o mapa hipocampal do espaço for formado, a exploração ao ambiente é reduzida e tem-se a habituação. O papel do hipocampo na habituação foi demonstrado com injeção intra-hipocampal de diferentes drogas (Vianna et al., 2000) e com hipocampectomia (Wright et al., 2004). Entretanto, estudos recentes demonstram que o hipocampo não é a única estrutura envolvida nesse mecanismo, também foram demonstradas a participação da amídala (Daenen et al., 2001; Hale et al., 2008) e do núcleo acumbens (Schildein et al., 2000, 2002).

A participação do sistema colinérgico na habituação é sugerida por diferentes abordagens. A administração de escopolamina após a aquisição, interfere na habituação entre sessões enquanto a injeção de nicotina promove a retenção da habituação (Platel and Porsolt,

1982). Da mesma maneira, a administração de nicotina, diretamente no núcleo acumbens, imediatamente após a primeira exposição ao campo aberto, foi capaz de aumentar a habituação entre as sessões. Entretanto, uma injeção de escopolamina na mesma região bloqueia a aquisição da habituação se fosse injetado logo após o treino ou 5 hora depois (Schildein et al., 2000, 2002). A importância do momento da injeção, revela que a habituação como outros tipos de aprendizagem, necessita de uma fase de consolidação.

O papel do sistema colinérgico foi inicialmente estudado com estudo de lesões colinérgica. A indução de lesões em neurônios colinérgicos do septum medial com o uso de imunotoxina 192 IgG-saporina produziu déficits na habituação intra-sessão (Lamprea et al., 2003). Medições in vivo dos níveis de ACh, através da técnica de microdiálise, revelam um aumento na liberação hipocampal de ACh seja na apresentação ao novo ambiente, seja na reexposição (Thiel et al., 1998). A ACh desempenha também um papel no processo de consolidação da memória no hipocampo. O bloqueio de receptores muscarínicos colinérgicos com escopolamina por injeção intra-hipocampal imediatamente após a tarefa de habituação causa amnésia retrógrada em ratos (Izquierdo et al., 1992; Rosat et al., 1992). Portanto, ainda são necessários estudos com novas abordagens para determinar de maneira mas específica as regiões onde a ACh participa da habituação a um novo ambiente .

b) Atividade Locomotora (Campo aberto)

O teste do campo aberto é usado para medir a atividade locomotora em roedores e também podem servir como um bom teste inicial para determinar déficits motores, ansiedade, bem como efeitos de drogas e manipulação genética. A atividade locomotora é medida através da determinação do montante da distância percorrida e observações de vários comportamentos no sentido horizontal e vertical. A ansiedade é determinada pelo padrão de exploração no campo aberto (centro versus periferia).

No sistema central, as injeções sistêmicas de agonista dos receptores nicotínicos o de antagonista dos receptores muscarínicos, aumenta a locomoção dos animais (Fink and Morgenstern, 1980; Clarke and Kumar, 1983). A escopolamina induz um aumento da atividade locomotora nós camundongos selvagens ou *Knock-out* para M5 sugerindo que o efeito da escopolamina é independe dos receptores M5 (Chintoh et al., 2003). Os mesmos autores conseguem observar um aumento da atividade dos animais com injeção bilateral de oxotremorina-M na área tegumental ventral, eles sugerem que o aumento de atividade locomotora é causado pela ativação dos receptores nicotínicos. Injeção intraventricular de uma droga tóxica para os neurônios colinérgicos (mu p75-SAPORIN) provoca uma perda

desses neurônios no septal medial e nós núcleos basais respectivamente de 82% e 55% (Moreau et al., 2008). Os camundongos tratados com essa toxina apresentam um aumento da atividade locomotora sugerindo de novo a importância da ACh no sistema central para o controle da atividade locomotora. Esses experimentos, no entanto, são complexos de serem interpretados devido as ações não específicas de toxinas, que podem afetar outros tipos de neurônios alem dos neurônios colinérgicos. Além disso, várias das drogas utilizadas podem ter efeitos fora dos alvos escolhidos.

A dopamina é um outro neurotransmissor que tem um papel importante na locomoção e nas funções cognitivas (Amara and Kuhar, 1993; Giros and Caron, 1993). O sistema dopaminérgico se relaciona com movimento e atividade locomotora em roedores. A cocaína inibe recaptação de dopamina através do bloqueio do transportador de dopamina (DAT) (Kuhar et al., 1991). A locomoção induzida por cocaína aumenta com altas doses desta (Jones et al., 1993) e camundongos DAT KO apresentam hiperlocomoção espontânea (Giros et al., 1996; Ralph et al., 2001). Essas evidências demonstram uma relação próxima entre o sistema dopaminérgico e a atividade locomotora. Além disso, a cocaína parece ser particularmente adequada para estimular o sistema dopaminérgico.

O sistema colinérgico influencia também a atividade locomotora como vimos anteriormente. A interação entre acetilcolina e dopamina dever ser detectável no processo de locomoção, uma vez que os dois neurotransmissores podem interferir na locomoção. Camundongos com neurônios colinérgicos removidos do núcleo accumbens apresentaram uma resposta maior na hiperlocomoção provocada por cocaína no campo aberto (Hikida et al., 2001). No entanto, o uso de toxinas leva novamente a interações complexas que dificultam a interpretação de resultados. No caso dos neurônios colinérgicos do corpo estriado, alem de liberarem ACh, eles podem liberar glutamato, ja que expressam o VGLUT3. Portanto, a degeneração desses neurônios abole uma forma de sinalização muito mais complexa, e não apenas o controle pelo sistema colinérgico.

Muitos artigos científicos demonstram evidências da interação entre a acetilcolina e a dopamina na locomoção espontânea (De Parada et al., 2000; Avale et al., 2008). Parte do trabalho descrito nessa tese teve como objetivo estudar a interação dos sistemas colinérgico e dopaminérgico utilizando novos modelos animais com alterações específicas e seletivas no sistema colinérgico para compreender em profundidade o papel da ACh em funções do sistema nervoso central.

c) Ansiedade

A ansiedade é a sensação que antecede momentos de perigo real ou imaginário, marcada por sensações físicas desagradáveis, tais como uma sensação de vazio no estômago, coração batendo rápido, medo intenso, aperto no tórax, transpiração etc. A ansiedade é frequentemente confundida com o estresse, porque têm mecanismos e sintomas muito similares, e podem até ter a mesma causa. Uma das principais diferenças entre o estresse e a ansiedade é que o estresse geralmente tem uma causa identificável, enquanto ansiedade pode ser um sentimento mais amplo de medo sobre o que poderia acontecer no futuro, sem causa definida. O estresse, por outro lado, nunca é qualificado como um transtorno mental, é geralmente transitório e pode ser controlado limitando-se a fonte do estresse (Cecil et al., 2008).

Para medir a ansiedade nós camundongos o teste da cruz elevada é o mais usado. Esse ensaio testa a capacidade do animal para lidar com um ambiente ansiolítico. Ele foi descrito pela primeira vez por Pellow e colaboradores em 1985, que demonstraram que a quantidade de entrada e o tempo no braço aberto refletia a ansiedade do animal (Pellow et al., 1985). Nós braços abertos o animal estaria mais exposto aos predadores, então o tempo que o animal fica nós braços abertos pode ser usado como variável que reflete o comportamento não-ansiolítico. A validade desse teste foi realizada com diferentes drogas anxiogênicas (yohimbine, amfetamina) e antidepressivas (Imipramine, Mianserine), concluindo-se que a cruz elevada permitia investigar os efeitos ansiolíticos e anxiogênicos de fármacos, drogas e hormônios (Pellow et al., 1985).

O campo aberto é um outro teste popular para estudar a ansiedade e o fenótipo dos animais. Os roedores preferem espontaneamente regiões periféricas e evitam a parte central do ambiente utilizado para avaliar a atividade locomotora (Prut and Belzung, 2003). Andar perto da parede é um comportamento chamado tigmotatismo. Uma grande quantidade de drogas ansiolíticas foi testada nesse teste (para detais Prut and Belzung, 2003), mas o papel da ACh na ansiedade foi pouco estudado. Por enquanto, foi mostrado que injeção de escopolamina (antagonista muscarínico) tem efeito anxiogênico nós ratos em um teste diferente da cruz elevada (Smythe et al., 1996). Também, camundongos KO para a sub-unidade beta 4 ou beta 3 do receptor nicotínico apresentam um comportamento menós ansioso que os selvagem (Salas et al., 2003a; Booker et al., 2007). Mais recentemente foi mostrado em camundongo que injeção i.p. de PNU-282987, um agonista do receptor nicotínico alpha7, aumenta o tempo no centro do campo aberto, portanto a estimulação desse receptor diminui a ansiedade (Vicens et al., 2010). A investigação da ansiedade e do estresse, em animais que

demonstram aumento de atividade locomotora pode ser importante para explicar alteração observada da atividade locomotora no campo aberto. Por exemplo, os camundongos KO heterozigotos para o CHT apresentam um aumento de atividade locomotora mas eles não têm diferença em relação aos camundongos selvagem na cruz elevada (Bazalakova et al., 2007). Para ampliar o conhecimento da relação da ACh e do estresse, diferentes animais mutantes para VAChT que apresentam déficit colinérgico específico e seletivo foram testados na cruz elevada e observados no campo aberto.

d) Depressão

A depressão é um problema médico caracterizado por diversos sinais e sintomas, dentre os quais dois são essenciais: humor persistentemente rebaixado, apresentando-se como tristeza, angústia ou sensação de vazio e redução na capacidade de sentir satisfação ou vivenciar prazer. Esse problema afeta entre 8-18 % da população (Andrade et al., 2003), dependendo do país.

A depressão é o distúrbio psiquiátrico mais comum na doença de Parkinson (DP) (Schneider et al., 2008; Lees et al., 2009). Essa doença é caracterizada por uma desordem progressiva do movimento devido à neurodegeneração dos neurônios secretores de dopamina nós gânglios da base. Recentemente foi demonstrado que algumas disfunções cognitivas na DP são devidas a um desarranjo do equilíbrio ACh/DA (Calabresi et al., 2006). Kim 2003 não achou correlação entre um transtorno serotoninérgico e os sintomas depressivos nós pacientes com DP (Kim et al., 2003; for review see Schneider et al., 2008). Isso sugere que neurotransmissores diferentes da serotonina poderiam intervir nós mecanismos da depressão do paciente com a doença de Parkinson.

Um modelo animal para testar medicamentos antidepressivos em animais foi introduzido por Porsolt em 1977. O teste de nado forçada ou “forced swimming test”, é baseado na observação que quando um rato é obrigado a nadar em uma situação que ele não tem escapatória, após período de atividade, ele vai deixar de se mover fazendo unicamente o mínimo esforço para manter a cabeça fora da água. Esse comportamento de imobilidade mostra um estado de desespero onde o rato apreendeu que não havia saída e ele se resigna (Porsolt et al., 1977). Apesar de o papel da ACh na depressão ter sido pouco estudado, um estudo mostra que o bloqueio do receptor M1 no núcleo acumbens diminui o tempo de imobilidade. Como conseqüência, os animais ficam mais tempo procurando uma saída e, ao contrário, injeção de arecoline o galantamine aumenta o tempo que os animais ficam parados (Chau et al., 2001). Com essa falta de estudos sobre a importância da ACh na depressão, nós

decidimos estudar a depressão e as respostas para diferentes drogas antidepressivas, nós camundongos KO para VAChT no estriado.

IV- Modelos de animais de hipofunção colinérgica

O uso de drogas direcionadas ao sistema colinérgico (*e.g*. antagonistas muscarínicos e nicotínicos e inibidores da acetilcolinesterase) trouxe valiosas contribuições para a compreensão do papel da ACh nas funções cognitivas. Porém, para se avaliar o papel de uma região específica do cérebro, técnicas complexas como a implantação de cânulas, são necessárias para a administração da droga. A implantação de cânulas permite que as injeções sejam realizadas no momento desejado, porém, requer a anestesia e pode causar lesões em regiões do cérebro dos animais. Além disso, as injeções podem promover um aumento da tensão intracranial e o estresse causado pelas injeções pode alterar os resultados. Uma outra abordagem, em que não se tem o controle do momento exato da alteração do sistema colinérgico, foi realizada através do uso de excitotoxinas, mas a falta de seletividade contra neurônios colinérgicos limitou as conclusões obtidas a partir desses estudos (Hepler et al., 1985). O desenvolvimento de toxinas seletivas para neurônios colinérgicos veio com o intuito de sanar tais limitações. Primeiramente, a toxina 192IgG-saporin foi testada em ratos (Wiley et al., 1991; Schliebs et al., 1996) e recentemente, a toxina p75 IgG-saporin foi testada em camundongos (Moreau et al., 2008). A interpretação dos resultados ainda é controversa, uma vez que os neurônios colinérgicos podem liberar glutamato conjuntamente com a ACh, pelo menós no estriado. Uma outra maneira de estudar o sistema colinérgico é o uso de animal mutante para um gene específico.

O nocauteamento de genes é uma ferramenta que possibilita o estudo da função de proteínas específicas em vivo. Dessa maneira, alguns camundongos mutantes servem de modelo para patologias humanas. Mas de novo essa técnica tem algumas limitações, por exemplo, o nocautamento de gene pode perturbar o desenvolvimento ou levar a morte do animal antes de nascer.

O interesse em neurotransmissores e a possibilidade de criar camundongos geneticamente modificados levou os pesquisadores a se interessarem primeiramente pelos receptores, pelo fato dos mesmos serem ótimos alvos farmacológicos. Para estudar participação da neurotransmissão colinérgica em processos comportamentais, vários

camundongos knockout foram desenvolvidos para os receptores colinérgicos, tanto para os receptores muscarínicos (Gomeza et al., 1999a; Gomeza et al., 1999b; Yamada et al., 2001a; Yamada et al., 2001b; Fisahn et al., 2002) como para os receptores nicotínicos (Salas et al., 2003b). Seguindo esses mutantes outras proteínas envolvidas na transmissão colinérgica foram estudadas tais como a AchE (Xie et al., 2000), o transportador de Colina de alta afinidade - CHT (Ferguson et al., 2004) e a Colina acetyltransferase - CHAT (Brandon et al., 2003). Nósso grupo de pesquisa tem estudado a biologia molecular e as vias celulares utilizadas na função do VAChT, proteína-chave que controla a armazenagem de ACh nós neurônio. Nósso grupo gerou, através de técnicas de recombinação homóloga, diferentes linhagens de camundongos.

a) VAChT KD mice

O primeiro modelo de animais geneticamente modificados desenvolvido em nósso laboratório foi o de camundongo com uma redução da expressão de VAChT (Knock-downs). Nósso grupo desenvolveu os camundongos VAChT KD^{HET} e VAChT KD^{HOM} que apresentam respectivamente uma redução em torno de 40% e 70% na expressão do VAChT comparados aos camundongos selvagens; e como consequência eles apresentam uma redução nós níveis extracelulares de acetilcolina no sistema nervoso central (Prado et al., 2006). Esses animais foram amplamente estudados e descritos por nósso grupo, mostrando que os animais HET apresentam um déficit cognitivo sem os déficits periféricos presente nós animais HOM (Prado et al., 2006; de Castro et al., 2009a). Em efeito, os VAChT KD^{HOM} apresentam uma grande fraqueza e fadiga muscular (Prado et al., 2006). Observamos que os VAChT KD^{HET} possuem um déficit de memória declarativa no teste temporal de reconhecimento de objeto assim como um déficit de memória procedural no teste do rotarod. O mais intrigante foi que os mesmos animais conseguiram apreender a tarefa do rotarod e atingiram o mesmo nível em sua performance quando foram submetidos a vários dias de treino (de Castro et al., 2009a). Estudo detalhado desse modelo animal permite entender como a liberação fisiológica de ACh regula funções críticas do sistema nervoso.

b) VAChT$^{del/del}$

Para continuarmos investigando as funções da proteína VAChT decidimos desenvolver uma linhagem de camundongos com a perda total da expressão do gene (nocautes) $VAChT^{WT/DEL}$ e $VAChT^{DEL/DEL}$. Os camundongos homozigotos são natimortos como o outro modelo de nocautes para proteína do sistema colinérgico. Os camundongos

homozigotos nocaute para CHT1, ChAT e VAChT são de aparência normal, eles apresentam tamanho e peso similares aos selvagens. No entanto observa-se que logo após o nascimento eles não movem e morrem provavelmente por incapacidade de contração do diafragma (Brandon et al., 2003; Ferguson et al., 2004; de Castro et al., 2009b). O problema de sobrevivência é o maior problema dos camundongos nocaute para proteínas envolvidas com a síntese e liberação de ACh, por isso nós decidimos usar estratégias alternativas para estudar a ação da liberação de ACh no sistema nervoso central.

Novos modelos animais foram produzidos por nósso grupo para estudar a função colinérgica no sistema nervoso central. Esses novos animais estão descritos à seguir assim como, a minha contribuição para a caracterização dos mesmos e a compreensão do papel da ACh na regulação da cognição são descritas para cada um dos trabalhos.

Objetivos

O objetivo geral deste trabalho é investigar o papel da armazenagem de acetilcolina pelo VAChT em funções cognitivas. Nós hipotetizamos que a ACh tem papel seletivo em vários processos cognitivos, no entanto devido a inexistência de modelos animais com alterações seletivas na liberação de ACh o papel desse neurotransmissor ainda não se encontra esclarecido.

I- Objetivos específicos

- Avaliar as funções motoras em camundongos com redução na expressão do VAChT, utilizando o teste do campo aberto, rotarod e teste de força.

- Avaliar a memória declarativa em camundongos com redução na expressão do VAChT, utilizando o teste de reconhecimento de objeto.

- Avaliar a memória não-associativa em camundongos com redução na expressão do VAChT, utilizando o teste de habituação ao campo aberto.

- Avaliar o estresse e a depressão em camundongos com redução na expressão do VAChT, utilizando o teste da cruz elevada e o teste de nado forçado.

II- Lista de publicações

Artigo 1: Reduced expression of the vesicular acetylcholine transporter causes learning deficits in mice

O objetivo desse trabalho foi de avaliar o efeito de uma diminuição da liberação de ACh sobre a memória. Minha contribuição foi testar a memoria procedural dos animais VAChT KD^{HET} através do teste do rotarod ilustrado nas figuras 2 e 3.

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Reduced expression of the vesicular acetylcholine transporter causes learning deficits in mice

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Storage of acetylcholine in synaptic vesicles plays a key role in maintaining cholinergic function. Here we used mice with a targeted mutation in the vesicular acetylcholine transporter (VAChT) gene that reduces transporter expression by 40% to investigate cognitive processing under conditions of VAChT deficiency. Motor skill learning in the rotarod revealed that VAChT mutant mice were slower to learn this task, but once they reached maximum performance they were indistinguishable from wild-type mice. Interestingly, motor skill performance maintenance after 10 days was unaffected in these mutant mice. We also tested whether reduced VAChT levels affected learning in an object recognition memory task. We found that VAChT mutant mice presented a deficit in memory encoding necessary for the temporal order version of the object recognition memory, but showed no alteration in spatial working memory, or spatial memory in general when tested in the Morris water maze test. The memory deficit in object recognition memory observed in VAChT mutant mice could be reversed by cholinesterase inhibitors, suggesting that learning deficits caused by reduced VAChT expression can be ameliorated by restoring ACh levels in the synapse. These data indicate an important role for cholinergic tone in motor learning and object recognition memory.

Keywords: Acetylcholine, learning, memory, mutant mice, synaptic vesicle

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Maintenance of cholinergic tone is directly linked to the capacity of nerve endings to effectively synthesize and release acetylcholine (ACh) (Ribeiro *et al.* 2006). Synthesis of ACh depends on the activity of a high affinity choline transporter (CHT1) that is necessary to supply choline for ACh synthesis. Choline provided by CHT1 is used to generate ACh in the cytosol, in a step catalyzed by the enzyme choline acetyl transferase (ChAT) (Dobransky & Rylett 2005). ChAT is likely to be in kinetic excess, therefore moderate changes on its activity or expression may not affect ACh synthesis (Brandon *et al.* 2004). The final key player in cholinergic nerve-endings is the vesicular acetylcholine transporter (VAChT), a protein that takes up cytosolic ACh into synaptic vesicles and thereby provides vesicular neurotransmitter for exocytotic release in response to calcium influx (Edwards 2007; Parsons 2000).

In contrast to ChAT and CHT1, VAChT activity plays a rate-limiting role in ACh release (Prado *et al.* 2006). Mice lacking VAChT (VAChT knockout) do not survive (Prado, de Castro, Caron and Prado, unpublished data), however, mice with reduced levels of VAChT (VAChT knockdown, VAChT KD mice) are viable and were instrumental to demonstrate a role for this transporter '*in vivo*'. These mutant mice present distinct behavioral deficits, suggesting that the ability to efficiently pack ACh in synaptic vesicles is fundamental for certain physiological functions (Prado *et al.* 2006).

Our initial investigation detected several physiological and behavioral deficits in heterozygous and homozygous VAChT mutants that are consistent with decreased cholinergic tone. In particular, homozygous VAChT KD (KD^{HOM}) mice showed severe muscle fatigue consistent with a myasthenic phenotype as well as selective cognitive alterations. In contrast, heterozygous VAChT KD (KD^{HET}) mice displayed seemingly normal neuromuscular function, although they also showed cognitive alterations similar to those found in VAChT KD^{HOM} mice. This suggested that central cholinergic synapses are more sensitive to decreased VAChT levels than motor end-plates. These VAChT KD^{HET} mice showed object and social recognition memory deficits and also presented impairments in performance on the rotarod. However, the precise contribution of reduced cholinergic tone for these behavioral alterations has not been investigated. Hence, deficits in object recognition memory may potentially relate to the inability of

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mutant mice to acquire or to retrieve information. In addition, alteration in rotarod performance could be explained by altered gait and equilibrium or by deficits in motor learning.

Here we used VAChT KD^{HET} mice to address the question of how reduced VAChT levels affect cognitive processing related to these previously described behavioral abnormalities. We found that most cognitive deficits observed in VAChT KD^{HET} mice are related to deficiency in learning, rather than retrieval of information. We suggest that targeting mechanisms that can facilitate information encoding could help to ameliorate the consequences of cholinergic dysfunction because of reduced VAChT levels.

Material and methods

Animals

VAChT mutant mice were previously described (Prado *et al.* 2006). These mice were generated by targeting the 5′ untranslated region of the VAChT gene by homologous recombination in a mixed 129S6/SvEvTac (129S6) x C57BL/6J background and were backcrossed to C57BL/6/Uni [imported to Brazil from Zentralinstitut fur Versuchstierzcht, Hannover, Germany and maintained at the University of Campinas (UNICAMP)] for only three generations (N3), as further backcrossing into this strain caused infertility (data not shown). Only male mice were used in this study. Heterozygous mice were intercrossed to generate all the genotypes used in these experiments and littermate wild-type mice were used as controls.

Animals were housed in groups of three to five per cage in a temperature-controlled room with a 12:12 light-dark cycles in microisolator cages. Food and water were provided *ad libitum*. Mouse colonies were maintained at the Federal University of Minas Gerais, Brazil, in accordance with National Institutes of Health (NIH) guidelines for the care and use of animals. Experiments were performed accordingly to approved animal protocols from the Institutional Animal Care and Use Committees at the Federal University of Minas Gerais and PUC-RS. To minimize the number of animals used, naïve mice were used for the object recognition as well as for the Y maze task and then they were used for one of the following tasks: evaluation of rotarod performance, assessment of locomotor activity or for the Morris water maze (MWM). An interval of at least 1 week was observed between two distinct tasks.

Immunofluorescence

Adult VAChT KD and wild-type (WT) mice were anesthetized with ketamine/xilazine (70/10 mg/kg) i.p. and transcardially perfused with ice-cold phosphate-buffered saline (PBS) pH 7.4 for 10 min, followed by ice-cold 4% paraformaldehyde (PFA) in PBS for 10 min. Perfused brains and spinal cords were immediately postfixed in 4% PFA in PBS overnight at 4°C. Following cryoprotection in 4% PFA with 10% sucrose, tissues were rapidly frozen in isopentane over dry ice and kept at −80°C. Serial sections (40 μm thick) were cut on a Cryostat (Micron) and immersed in ice-cold PBS. Sections from control and test mice were processed simultaneously for all experiments.

Brain and spinal cord slices were permeabilized in 1.2% Triton/PBS and rinsed in PBS. Tissues were blocked for 1 h in 10% normal goat serum/PBS and immunonstained with primary antibodies VAChT (rabbit polyclonal, 1:250, Sigma Chem. Co., São Paulo, Brazil), CHT1 (rabbit polyclonal 1:250, kindly provided by R. Jane Rylett, University of Western Ontario, London, Canada) in incubation buffer (2% normal goat serum; 0.2% Triton; PBS) for 48 h at 4°C. After washing three times with PBS for 20 min each, tissues were incubated with Alexa Fluor 488 goat anti-rabbit (1:500, Invitrogen, SP, Brazil) in incubation buffer for 1 h. Slices were rinsed again and incubated with DAPI (1:1000) for 10 min at room temperature. Tissue sections were then mounted and coverslipped using ProLong® Gold antifade reagent (Invitrogen, SP, Brazil). Images were acquired using an Axiovert 200M using the ApoTome system or a LEICA SP5 confocal microscope to obtain optical sections of the tissue. Objectives used were 20× dry, 40× water immersion (1.2 Numerical aperture [NA]) and a 63× oil immersion (1.4 Numerical aperture). Fluorescence intensity analyses were carried out with the (Image J, http://rsbweb.nih.gov/ij/). Images were obtained in grayscale and a threshold was applied based on the mean value of fluorescence intensity of the whole image for VAChT staining automatically obtained using Image J. The total fluorescence intensity for VAChT was then detected automatically by the software and normalized for the fluorescence intensity of a image from a sequential Cryostat section stained with anti-CHT1 antibody that was submitted to the same procedure (i.e. automatic threshold and detection of fluorescent intensity as well). The normalized fluorescence for VAChT staining was divided by the area of the image and data from WT mice provided values for 100%.

Behavioral procedures

All experiments were conducted during the light phase of the cycle. All efforts were made to minimize any suffering and the number of animals used.

Temporal order task

There are several tasks that can be used to assess object recognition memory and here to evaluate whether information encoding is affected in these mutants, we used a temporal order task for recency memory (Bevins & Besheer 2006; Dere *et al.* 2005). In this task rodents need to remember the order in which two distinct objects were presented, what is shown by increased exploration of the first object presented in a sequence (the less familiar object).

All animals were given a single 10-min habituation session, with no objects in the open-field arena (50 × 30 cm) which was maintained inside an illuminated larger box with controlled illumination. Twenty-four hours later, in the first sample phase (S1), the animals were allowed to explore two copies of an identical object for a total of 10 min. In the second sample phase (S2), with a delay of 1 h, other two copies of an identical object were used and animals were let to explore these objects for 10 min. One hour after S2, in the test session (T), animals were allowed to explore, during 10 min, one object identical from S1 and another identical from S2. The objects were Lego toys that presented similar textures, colors and sizes, but distinct shapes. Between trials objects and arena were cleaned with 70% alcohol and air dried. Exploration time was defined as sniffing or touching the object with nose and/or forepaws (Prado *et al.* 2006). The experiments were recorded and an experimenter blind to the genotype scored time exploring the object for each mice.

If object recognition memory is intact, the subjects will spend more time exploring the object from S1 compared with the object from S2 in the test session. The results are expressed as percentage of exploration for each object and the exploration times (in seconds, mean ± SEM) in S1 (WT = 36 ± 3; KD^{HET} = 38 ± 3; $t_{(104)} = 0.6034$) and S2 (WT = 35. ± 3; KD^{HET} = 34 ± 3; $t_{(97)} = 0.2705$) between the two genotypes were not statistically different. We noticed no alteration in these times after pharmacological manipulations.

To evaluate for statistical significance for the object recognition memory, the percentage exploration time for the two objects in the test session were compared by One-sample *t*-test to evaluate if exploration was significantly larger than chance (50% of the time). The null hypothesis was that the mice did not remember which object was presented less recently and explored the two objects equally.

Morris water maze

The water maze was a black circular pool (120 cm in diameter) conceptually divided in four equal imaginary quadrants for the purpose of data analysis. The water was made opaque with nontoxic white tempera and the temperature was 21–23°C. One centimeter beneath the surface of the water and hidden from the mouse's view was a circular platform (9 cm in diameter), with a rough surface, which allowed mice to climb onto it easily. The swimming path of the animals was recorded using a video camera mounted above the center of the pool and analyzed using an in house video tracking and analysis system. The water maze was located in a white room with

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several posters and other distal visual stimuli hanging on the walls to provide spatial cues. A curtain separated the water maze room from the room where the computer was set up and where the animals were temporarily housed during the behavioral sessions. Training in the hidden platform (spatial) version of the MWM was carried out during 5 consecutive days as previously described (Rossato *et al.* 2006). On each day, mice received six consecutive training trials during which the hidden platform was kept in a constant location. A different starting location was used on each trial, which consisted of a swim followed by a 30-s platform sit. Any mouse that did not find the platform within 60 s was guided to it by the experimenter. The intertrial interval (ITI) was 30 s. During the ITI, mice were carefully dried with a towel by the experimenter. Memory retention was evaluated in a 60-s probe trial carried out in the absence of the escape platform 24 h after the last training session. Difference of performance between genotypes was assessed using the *t*-test.

Y maze

Immediate working memory performance was assessed by recording spontaneous alternation behavior during a single session in a Y maze (Hughes 2004; Pych *et al.* 2006). Each mouse, new to the maze (30 cm long by 6 cm wide by 20 cm high), was placed at the end of one arm and allowed to move freely through the maze during an 8-min session. The series of arm entries was recorded visually. Alternation was defined if mice entered different arms three times in succession from the results of consecutive arm entering. The number of overlapping entrance sequences (e.g., ABC, BCA) was defined as the number of alternations. The effect was calculated as percentage alternation according to the following formula: [total alternation/(total arms entered $-$ 2)] $\times$ 100. Therefore, the following hypothetical sequence of arms entered by a mice: A, C, A, B, C, A, C, B, A, C would yield an alternation score of 75% ([6 alternations/(10-2) arms entered] $\times$ 100). A generic triad sequence has $3 \times 2 \times 2 = 12$ possibilities, because arms cannot be repeated in adjacent observations but may appear twice on the same triad sequence. However, the number of possibilities, if arms are not allowed to repeat themselves in a triad sequence, would be $3 \times 2 \times 1 = 6$. Thus, the index of 50% means random selection of goals arms. Animals statistically reach more than 50% alternation, indicating no random arms selection. By this criterion, we would see 100% alternation score only if the animal had run consistently clockwise or anticlockwise, however, this kind of behavior was not observed for any of the animals, independently of the genotype. To determine if alternation scores were significantly above the chance (50%), we used one-sample *t*-test. The differences between groups were compared using Student's *t*-test.

Locomotor activity

Locomotor activity was measured using an automated activity monitor (Accuscan Instruments, Inc., Columbus, OH, USA) (Sotnikova *et al.* 2004). Experiments were performed between 10:00 and 16:00 h. Mice were allowed to explore the locomotor activity chamber (20 $\times$ 20 cm) for 1 h. After that, they received either saline or galantamine (1 mg/kg, i.p.) and locomotor activity was monitored for additional 2 h. Activity (converted from beam breaks to cm) was measured at 5-min interval. Measurements of total activity were obtained and statistical significance was assessed by *t*-test when the data passed a normal distribution test. Otherwise, we used a Mann–Whitney Rank Sum Test.

Rotarod

To assess procedural learning we used an accelerating rotarod and investigated motor skill learning during several days. The rotarod apparatus was made of gray plastic (rotating cylinder diameter: 5 cm, width: 8 cm, height: 20 cm) with an automatic fall recorder (Insight Equipaments, Ribeirão Preto, Brazil). To characterize improvement in rotarod performance, mice were trained during eight consecutive days. In order to evaluate retained performance, mice were re-exposed to the same task 10 days later. Before daily training, mice were allowed to accommodate on the rod for 5 min. The animals were placed on the rotating roller and the rotation was increased from 5 to 35 r.p.m. over a 5-min period. For each day, mice were subjected to ten trials with a 30-s interval between trials. Statistical analysis was performed with analysis of variance (ANOVA) (repeated measure) when the data passed a normality test, or a Friedman repeated measures ANOVA on Ranks otherwise.

Pharmacological procedures

In the pretraining galantamine dose–response study, either saline or galantamine (0.5, 1 and 3 mg/kg, i.p.) was injected 30 min prior to S1. In the second study, saline or galantamine (1 mg/kg i.p.) was injected 30 min before the test session (T). The choice of dose of galantamine (1 mg/kg) was based on the findings from the dose–response study and by the fact that at this dose there is no alteration in locomotor activity. In some experiments we injected saline or donepezil (0.5 mg/kg, i.p.) 30 min before S1.

Results

Expression of VAChT in nerve terminals is reduced in VAChT KD mice

To visualize how decreased VAChT expression influences transporter levels in nerve terminals, we used indirect immunofluorescence in cryostat sections obtained from cortex (M1 motor cortex region, layer V), hippocampus (CA1 region), striatum (caudate) and spinal cord (lamina IX, ventral edge of the ventral horn on the cervical region). Sections from WT mice present robust and widespread punctated labeling of nerve endings detected with a VAChT antibody and this labeling is similar to that obtained with other integral synaptic vesicle proteins (Fig. 1). Staining with this VAChT antibody is specific, as it is suppressed in sections from VAChT KO mice (data not shown). The punctated labeling was decreased both in heterozygous (VAChT KD^{HET}) and homozygous (VAChT KD^{HOM}) mice in different areas of the brain and also in the spinal cord (Fig. 1a–c). Quantification of immunofluorescence labeling in hippocampal sections indicates that immunoreactivity is decreased by 40% by reduction of VAChT in VAChT KD^{HET} mice. As a control experiment we also used sections from VAChT KD^{HOM} mice, in which we could detect even further reduction (70%). No decrease in immunoreactivity was detected in sequential cryostat sections from hippocampus stained with an antibody against CHT1 (Fig. 1d) (Ribeiro *et al.* 2005).

Motor skill learning is affected in VAChT KD mice

We previously found that VAChT KD^{HET} mice present a deficit in motor skill performance, whereas VAChT KD^{HOM} mice are unable to spend prolonged amounts of time on the rotarod, likely because of reduced physical capacity (Prado *et al.* 2006). Although this observation suggests decreased coordination in VAChT KD^{HET} mice, it is also possible that they may have alterations in learning of this motor skill. Therefore, in order to test the hypothesis that motor skill learning is affected in VAChT KD^{HET} mice, we used experimental protocol involving accelerating rotarod that allows maximum motor skill acquisition over the period of days for WT mice (Fig. 2). In this experimental protocol, both WT and VAChT KD^{HET} mice showed an improvement in performance both within sessions (intrasession performance on day 1,

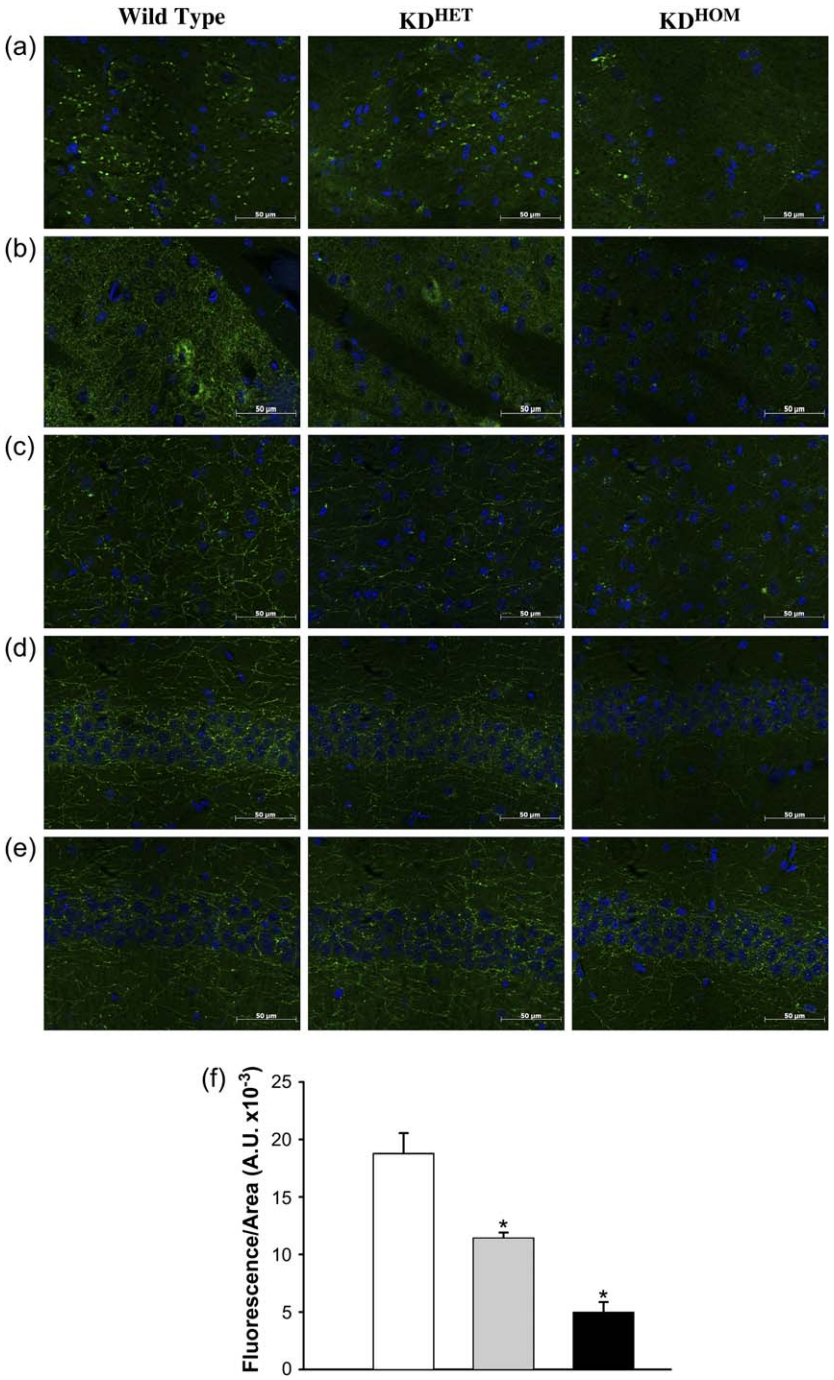
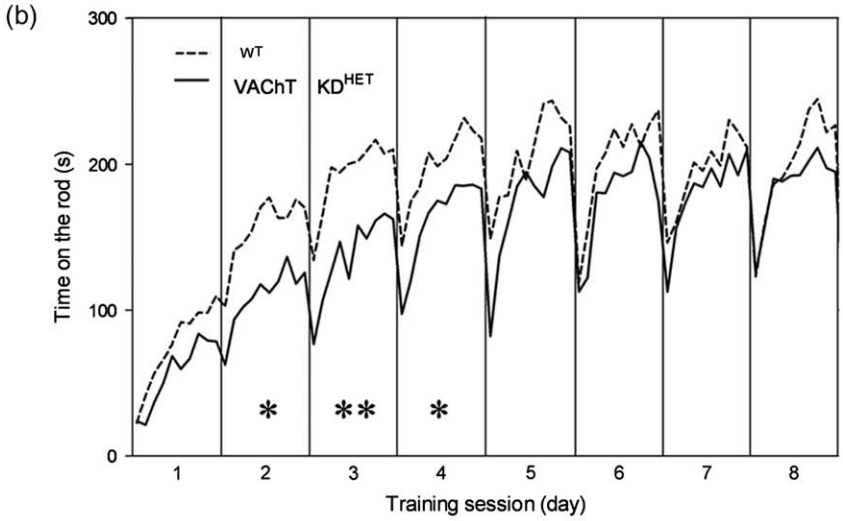


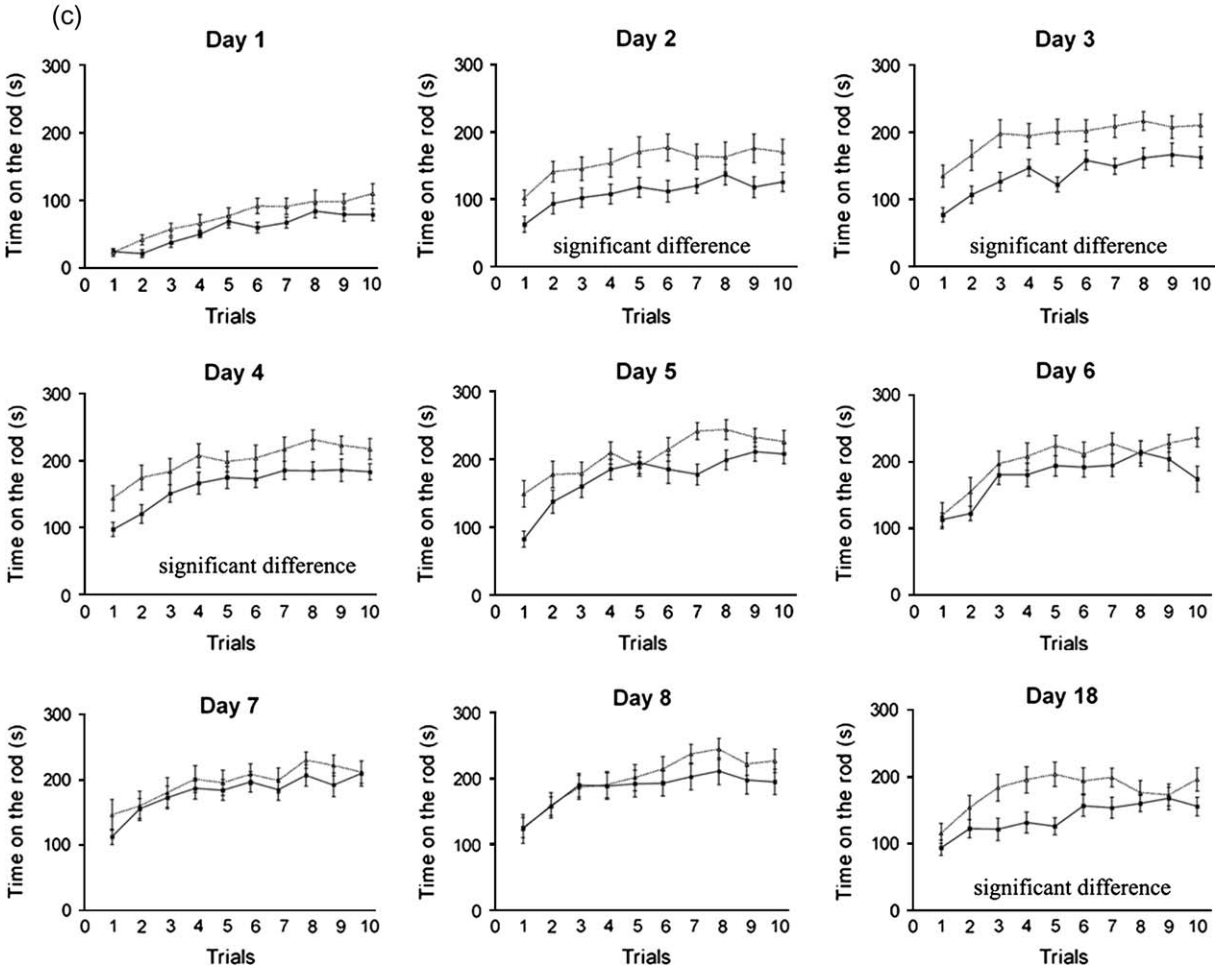
Figure 1: VAChT immunoreactivity is decreased in nerve endings. Representative optical sections of central nervous system regions stained with a VAChT (a–d) or CHT1 (e antibodies (green). (a) ventral horn of the spinal cord at cervical level, (b) Striatum, caudate region, (c) Layer V, motor cortex-M1 region, (d) hippocampus-CA1 region and (e) CHT1 immunoreactivity in the CA1 region of the hippocampus. Blue labeling corresponds to nuclei stained with DAPI. (f) Quantification of fluorescence. Fluorescence of CHT1 was used for normalization (see *Material and Methods*). The results were expressed as mean ± SEM. (*) Indicates statistical significant difference (one-way ANOVA, with Bonferroni *post hoc*; $F_{(2,12)} = 33.19$, $P < 0.0001$).

a Friedman Repeated Measures Analysis of Variance on Ranks show a significant difference for WT, Chi-square = 71.850, $P < 0.001$ and for VAChT KD^{HET} Chi-square = 157.650, $P < 0.001$) and between sessions [intersession performance, two-way repeated measure ANOVA shows effect of day $F_{(7,203)} = 59.381$, $P < 0.001$); Figs 2 and 3], indicating that both genotypes are able to learn the motor skill. A main effect of genotype just failed statistical significance ($F_{(1,270)} = 4.064$, $P = 0.05$) as did the genotype × day interaction ($F_{(7,203)} = 2.020$, $P = 0.054$). Because the data indicated a clear trend for statistical difference, we also analyzed the performance for individual days. This analysis showed a significant effect of genotype on days 2, 3 and 4 [two-way repeated measure ANOVA genotype effect for days 2: $F_{(1,270)} = 5.019$; $P < 0.05$; day 3: $F_{(1,270)} = 8.089$; $P < 0.01$; and day 4: $F_{(1,270)} = 7.35$; $P < 0.05$], suggesting that VAChT KD^{HET} mice performed worse than WT mice on these days (Fig. 2b). VAChT KD^{HET} mice were able to perform as well as

(a)

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
$F_{(1,270)}$	2.646	5.109	8.049	4.735	3.323	1.761	0.5487	0.4431
P	0.1143	< 0.05	< 0.01	<0.05	0.0783	0.1946	0.4646	0.5107





WT mice after 5 days of training suggesting that mutant mice are able to learn the task, although they needed more training to reach maximum performance. In agreement with this observation, analysis of performance by averaging the total time that animals spent on the rod during the 10 trials of each day (Fig. 3a) revealed that WT improve their performance (one-way repeated measure ANOVA: $F_{(7,105)} = 31.144$, $P < 0.001$) and that maximum performance is achieved on the third day (Tukey *post hoc* revealed a significant difference between day 8 and day 2 $P < 0.01$, but not between day 8 and day 3 $P = 0.999$). VAChT KD^{HET} mice are also able to improve their performance (one-way repeated measure ANOVA: $F_{(7,105)} = 30.279$, $P < 0.001$), but they achieved their maximum performance only on the fourth day (Tukey *post hoc* analyses revealed significant difference between day 8 and day 3, $P < 0.01$; but not between day 8 and day 4, $P = 0.495$). Exponential fitting of curves generated for each individual mouse for the time spent on the rod in a given day (plotted similar to the average shown on Fig. 2b) shows that maximum performance is identical for two genotypes (Fig. 3b, $t_{(15,15)} = 0.2$; $P = 0.843$). Moreover, time constants (τ) for the learning curve indicated a τ of 2 days for WT mice, whereas VAChT KD^{HET} mice had a τ of 3 days to achieve maximum learning (Fig. 3c). These values are in line with the average performance, but this difference just fails statistical significance (Fig. 3c, Mann–Whitney Rank Sum Test $T = 242$; $P = 0.073$). The fact that both genotypes achieved similar maximum performance suggests that the change in the rate of acquisition for motor learning cannot be explained by alterations in physical performance or coordination. This conclusion is supported by gait analysis (Supplemental Fig. 1), which indicates that VAChT KD^{HET} mice present no alterations in stride length or general gait.

To test whether mutant mice have altered ability to retrieve information necessary to perform in the rotarod, we compared performance of the two genotypes in the last trial of a given day with the first trial of the next day. We found no significant differences between the two genotypes (Fig. 3d, two-way repeated measure ANOVA shows no effect of genotype: $F_{(1,180)} = 0.0119$, $P = 9.17$; significant effect of intersession: $F_{(6,180)} = 8.831$, $P < 0.001$ and no interaction between genotype and intersession: $F_{(6,180)} = 0.882$, $P = 0.509$), revealing that in the day to day basis both genotypes are able to retrieve the necessary information learned in the previous day to perform successfully in the rotarod on the next day. We also tested performance retention 10 days after the eighth trial (day 18) by comparing the last trial of day 8 with the performance in the first trial of day 18 for each genotype (Fig. 3e; $t_{(15,15)} = 0.376$, $P = 0.709$). VAChT KD^{HET} and WT mice showed no significant differences in performance in the first trial on day 18th, suggesting that performance maintenance was equivalent between genotypes (Fig. 2c; $t_{(15,15)} = -1.132$, $P = 0.267$). On day 18, VAChT KD^{HET} and WT mice improved their performance (two-way repeated ANOVA measure shows a significant effect of genotype: $F_{(1,270)} = 4.798$, $P<0.05$; significant effect of trial: $F_{(9,270)} = 9.698$, $P < 0.001$ and interaction between genotype and trial: $F_{(1,270)} = 2.536$, $P < 0.01$) and both genotypes reached similar maximum performance, although KD^{HET} needed more trials to do so (Fig. 2c; Tukey *post hoc* show significant difference between the genotype only on trial 3, 4, 5, respectively, $P < 0.01$, $P < 0.01$, $P < 0.001$). Overall, these data indicate that procedural learning is less efficient in VAChT KD^{HET} mice.

VAChT KD^{HET} mice have specific alterations in object recognition memory

Cognitive processing in VAChT KD^{HET} mice was tested using the temporal order version of the object recognition task. In agreement with published data, during the test phase of this task WT mice preferred the object presented less recently, as shown by increase exploration of object A (Fig. 4a, clear bars) over object B in the test session ($t_{(7)} = 8.056$, $P < 0.0001$). In contrast, mutant mice were unable to distinguish between the more and less recently presented object (Fig. 4a; $t_{(7)} = 0.6705$, $P = 0.524$).

To test if reduction of VAChT expression also affects spatial memory, we utilized the hidden platform version of the MWM. There was no effect of genotype in escape latency during training sessions (Fig. 4b; $t_{(7,12)} = 0.02010$, $P = 0.9842$) and both VAChT KD^{HET} and WT control littermates spent similar amount of time in the target quadrant during a probe trial carried out 24 h after the last training session (Fig. 4c; $t_{(7,12)} = 0.1531$, $P = 0.8800$). We also investigated whether mutant VAChT mice present deficits in spatial working memory. Both genotypes presents alternation scores above chance, 50% (WT: $t_{(17)} = 8.457$, $P < 0.0001$; KD^{HET}: $t_{(13)} = 5.186$, $P < 0.0002$). Spontaneous alternation in the Y maze shows no effect of genotype in the percent alternation scores (Fig. 4d; $t_{(30)} = 0.7383$, $P = 0.4661$) or in the number of arms entered (Fig. 4e; $t_{(30)} = 0.9069$, $P = 0.3717$). Taken together, these results suggest that reduced VAChT levels affect object recognition memory, but not spatial or working memory.

Pharmacological restoration of cholinergic tone improves memory encoding in VAChT KD mice

The present observations suggest that VAChT KD^{HET} mice have a specific deficit in the temporal order task of the object recognition memory. Is this a consequence of an acute decrease in cholinergic tone or a general developmental effect related to the persistent reduction in cholinergic tone? If reduced

Figure 2: Motor skill learning in mice with reduced cholinergic tone. (a) Result of a two-way repeated measures ANOVA for each day reveals a genotype effect on the second, third and forth days during motor skill learning. (b) Cumulative performance on the rod during training. The lines represent mean time on the rod for each genotype in days 1–8 (*) $P < 0.05$; (**) $P < 0.01$. (c) Lines represent the mean ± SEM for performance of WT (open symbols) and VAChT KD^{HET} (closed symbols) on the rotarod for each day. For all the experiments $n = 16$ for both genotypes.

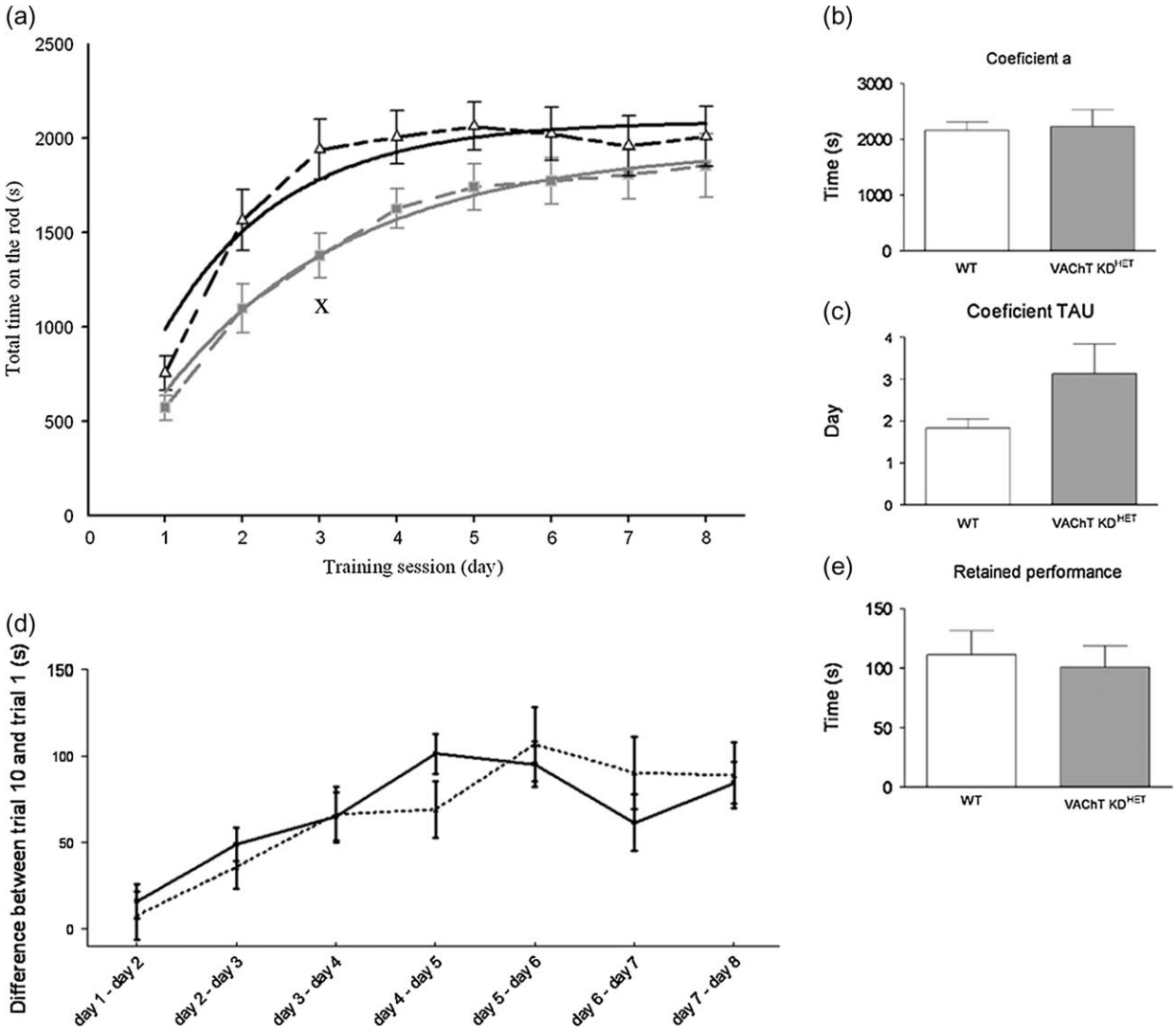


Figure 3: VAChT KD^{HET} mice learn slower than WT mice a procedural task. (a) Dashed lines are the mean ± SEM of the cumulative time mice spent on the rod for each day of training (10 trials per day) for WT (open symbols) and VAChT KD^{HET} mice (closed symbols). A one-way repeated measures ANOVA shows a significant difference between the first day and all the other day in both genotypes ($P < 0.05$) indicating that both genotypes were able to learn. To evaluate in which day the distinct genotypes reached maximum performance on the accelerating rotarod we also compared the eighth day of trial with all the other days. (°) Indicates statistic significant differences for wild-type mice ($P < 0.05$) and (x) indicates statistic difference for VAChT KD^{HET} mice ($P < 0.05$) when compared to the eighth day. The data can be fitted well (solid lines) to the exponential function $y = a(1 - e^{-bx})$. For all analysis $n = 16$ for both genotypes. (b) Identical curves as shown in A were generated to evaluate the performance of each individual animal and the curves were fitted with the exponential equation ($y = a(1 - e^{-bx})$ to obtain the values for a, which indicate the asymptote for both genotypes. The results represent the mean ± SEM values of asymptote performance for WT (white bars) and VAChT KD^{HET} mice (gray bars). (c) In order to evaluate how fast WT and VAChT KD^{HET} learn, we calculated the time constant (τ) for motor skill learning. Results represent the mean ± SEM of the values obtained for WT (white bars) and VAChT KD^{HET} mice (gray bars). (d) Consolidation of learning on the rotarod was evaluated by plotting the difference between the performance in the last trial of each day and the performance in the first trial of the next day. There is no difference in consolidation for the two genotypes. (e) The retention of performance for WT (white bars) and VAChT KD^{HET} mice (gray bars) on the rotarod was evaluated by plotting the difference between the last trial of the eighth day and the first trial of the 18th.

acetylcholine release causes object recognition memory deficits it would be expected that restoring ACh at the synapse with cholinesterase inhibitors could result in reversal of the behavioral deficits. Injection of saline prior to the trial sessions or the test session did not alter the behavior of WT or VAChT KD^{HET} mice (not shown). Furthermore, wild-type mice were able to discriminate against an object presented less recently and treatment with galantamine (0.5, 1.0 and

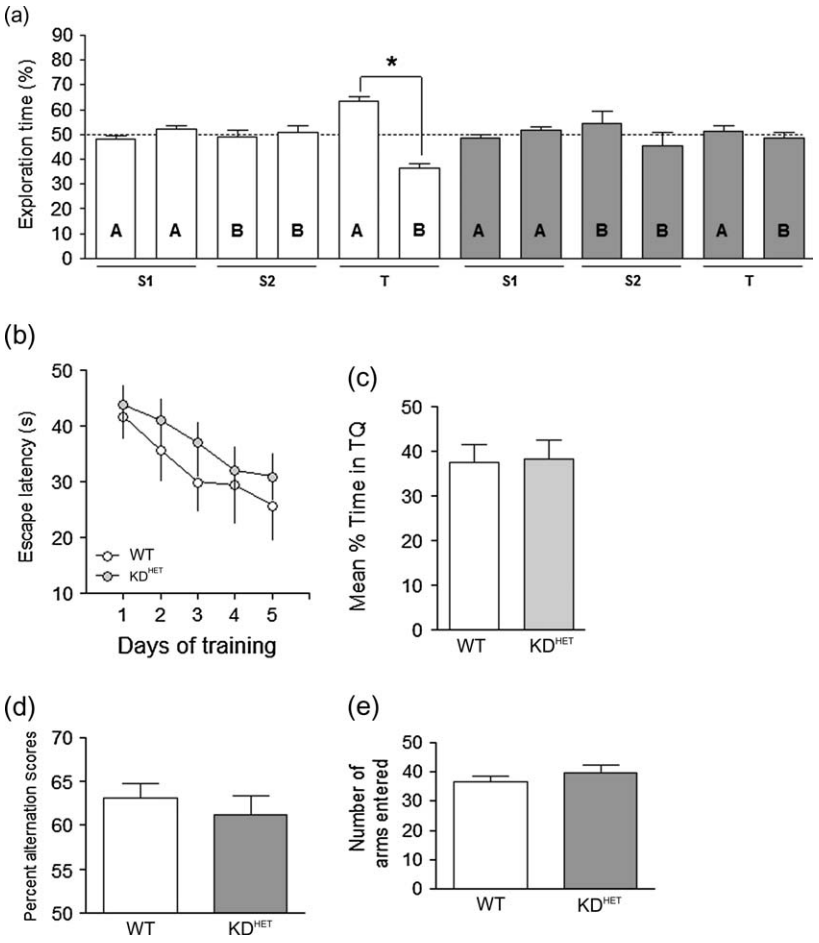


Figure 4: Memory deficits in VAChT KD mice. (a) Data represent mean ± SEM of % of total exploration; letters A and B represent the object; S1, S2 and T represent sample 1, sample 2 and test session, respectively. $n = 8$ for both genotypes. (*) Indicates statistical difference between exploration of object A and B above chance exploration (50%). (b) In the Morris water maze (MWM) task, WT mice and VAChT KD^{HET} are able to learn the location of platform at the end of the 5-day sessions. Data represent mean ± SEM, WT, $n = 8$, VAChT KD^{HET}, $n = 13$. (c) In the probe test of the MWM, we find no difference between WT ($n = 8$) and VAChT KD^{HET} ($n = 13$) performance, which was evaluated by the time spent in the target quadrant. (d) VAChT KD^{HET} mice have intact spatial working memory, measured by percent alternation in Y maze. Data represent mean ± SEM of alternation (WT, $n = 18$; KD^{HET}, $n = 14$). (e) There is no difference in the number of arms entered in the Y maze, between WT and VAChT KD^{HET} mice.

3 mg/kg) injected 30 min prior to the first sample session (Fig. a–c, respectively) did not alter their performance ($t_{(7)} = 3.034$; $P < 0.05$; $t_{(10)} = 7.089$; $P < 0.0001$; $t_{(9)} = 5.638$; $P < 0.001$; respectively, for 0.5, 1 and 3 mg/kg of galantamine).

At 0.5 mg/kg galantamine did not affect VAChT KD^{HET} performance (Fig. 5a; $t_{(6)} = 0.7007$, $P = 0.5097$). However, as can be seen in Fig. 5b,c, treatment with 1 or 3 mg/kg of galantamine significantly improved temporal order memory in VAChT KD^{HET} mice ($t_{(10)} = 6.784$, $P < 0.0001$; $t_{(8)} = 7.434$, $P < 0.0001$, respectively, for 1 and 3 mg/kg of galantamine), making their performance during the test session indistinguishable from that of WT mice.

Besides its well-known action as an acetylcholinesterase inhibitor, galantamine is also able to directly modulate nicotinic receptors (Pereira *et al.* 1994). Therefore, to test whether the reversal of recognition memory deficit in VAChT KD^{HET} can also be elicited by a more selective cholinesterase inhibitor, we investigated the action of donepezil (Cummings 2003). Donepezil did not alter the performance of WT control littermates in the temporal order recognition memory task ($t_{(7)} = 2.500$, $P < 0.05$), but in agreement with the results obtained with galantamine, donepezil was also able to reverse the deficit of mutant VAChT mice (Fig. 5d; $t_{(7)} = 7.196$, $P < 0.001$). Taken together, these results indicate that preserving ACh during memory acquisition can reverse the deficit in the object recognition temporal order task observed in VAChT KD^{HET} mice.

It is possible that increased cholinergic tone in VAChT KD^{HET} mice treated with cholinesterase inhibitors might also facilitate retrieval of information. To investigate this possibility, we analyzed the effect of the pretest administration on memory retention. To do that, we injected galantamine (1 mg/kg) 30 min prior to the test session. WT control mice presented no alteration in performance when injected with galantamine prior to the test session (Fig. 6a; $t_{(7)} = 2.648$, $P < 0.05$). Moreover, VAChT KD^{HET} mice showed no sign of improvement in recognition memory when injected with galantamine prior to the test session; mice were unable to discriminate against the more and less recently presented object (Fig. 6a; $t_{(7)} = 0.6705$, $P = 0.524$).

We further tested whether cholinesterase inhibition was able to alter VAChT KD^{HET} behavior in a more general way. For this test, we analyzed general locomotor activity of wild-type and VAChT KD^{HET} mice. There was no statistic difference in total spontaneous horizontal activity between control littermates (WT) and VAChT KD^{HET} mice [WT (1778.5 ± 246.7 cm in 60 min, all results described bellow are the mean ± SEM), VAChT KD^{HET} (2558.6 ± 305.3 cm in 60 min); $t_{(7,7)} = 1.987$,

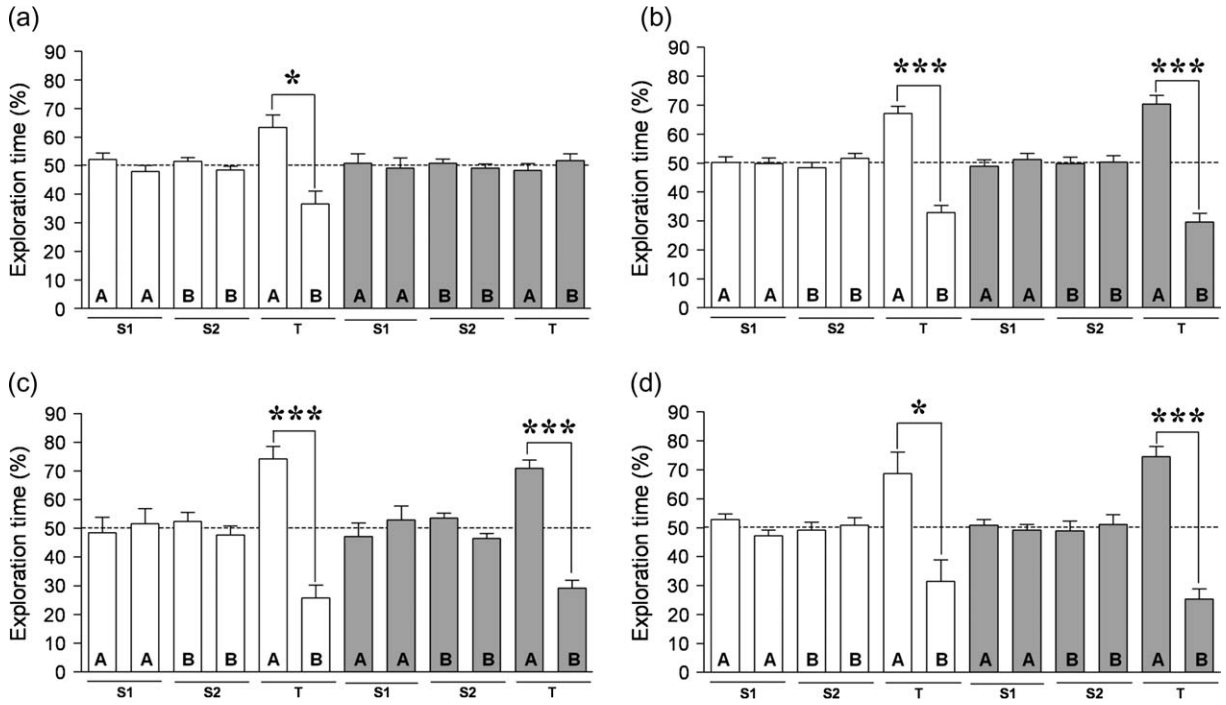


Figure 5: Increased cholinergic tone prior to encoding reverses deficit in recognition memory in VAChT KD mice. (a) At 0.5 mg/kg, galantamine has no effect on exploration performance of WT (white bars, $n = 8$) or VAChT KD^{HET} (gray bars, $n = 7$) as these results are identical to experiments performed after saline injection (not shown). (b) Galantamine at 1 mg/kg did not alter the performance of WT mice (white bars, $n = 11$), but the drug improved the performance VAChT KD^{HET} (gray bars, $n = 11$) on the trial session. (c) At 3 mg/kg, galantamine had a similar effect as with 1 mg/kg and enhanced old object exploration time for VAChT KD^{HET} mice (gray bars, $n = 9$) and did not modify the performance of WT mice (white bars, $n = 10$). (d) Donepezil, at 0.5 mg/kg, improved temporal object order memory of VAChT KD^{HET} mice (gray bars, $n = 6$) but did not alter performance of WT mice (white bars, $n = 6$). Saline injection prior to encoding did not affect recognition memory in both genotypes when compared to noninjected animals (not shown). *Indicates statistical difference between exploration of object A and B above chance exploration (50%) $P < 0.05$ and *** indicates $P < 0.001$.

$P = 0.067$)], although VAChT KD^{HET} mice showed a tendency to higher activity. Rearing was also not statistically different between genotypes [WT (224 ± 43), VAChT KD^{HET} (306 ± 42); $t_{(7,7)} = 1.367$, $P = 0.19$]. These results are paralleled by the horizontal locomotor activity measured at 5 min intervals (Fig. 6b). Galantamine administration did not appear to cause any change in locomotion for both genotypes (Fig. 6b). Total horizontal activity after administration of galantamine was similar for both genotypes [WT (703.6 ± 135.5 cm in 120 min), VAChT KD^{HET} (637.2 ± 143.7 cm in 120 min), Mann–Whitney Rank Sum Test ($T = 61$, $P = 0.505$)], as was rearing [WT (52 ± 9), VAChT KD^{HET} (71 ± 9); $t_{(7,7)} = -1.468$, $P = 0.164$]. Taken together, these experiments indicate that cholinesterase inhibition specifically improves acquisition but not retrieval of object recognition memory in VAChT KD^{HET} mice and it does not change spontaneous locomotor activity in these mice.

Discussion

The present data revealed a key role of VAChT in fine tuning of processes critical for learning and memory that may have important implications for better understanding several human disorders or consequences of drug treatments that affect VAChT expression. These results provide novel insights on the contribution of vesicular ACh in cognitive processing and suggested that the ability to sustain efficient transport of neurotransmitter into synaptic vesicles is of fundamental importance for learning.

VAChT KD mice present reduced capacity to sustain ACh release (Prado *et al.* 2006), a consequence of having reduced expression of this transporter. In fact, immunofluorescence analysis indicates that VAChT levels in synaptic terminals are decreased to the extension previously observed in immunoblot analysis. All the central nervous system areas that are relevant for behaviors studied herein showed the same levels of decrease in VAChT immunoreactivity. In contrast, immunoreactivity for CHT1 appears preserved, supporting the notion that the alteration in VAChT expression does not alter other presynaptic cholinergic proteins.

Reduced levels of VAChT selectively affect motor skill learning

Motor learning depends on improvement of accuracy, speed and general ability to perform a task, and it is accepted that

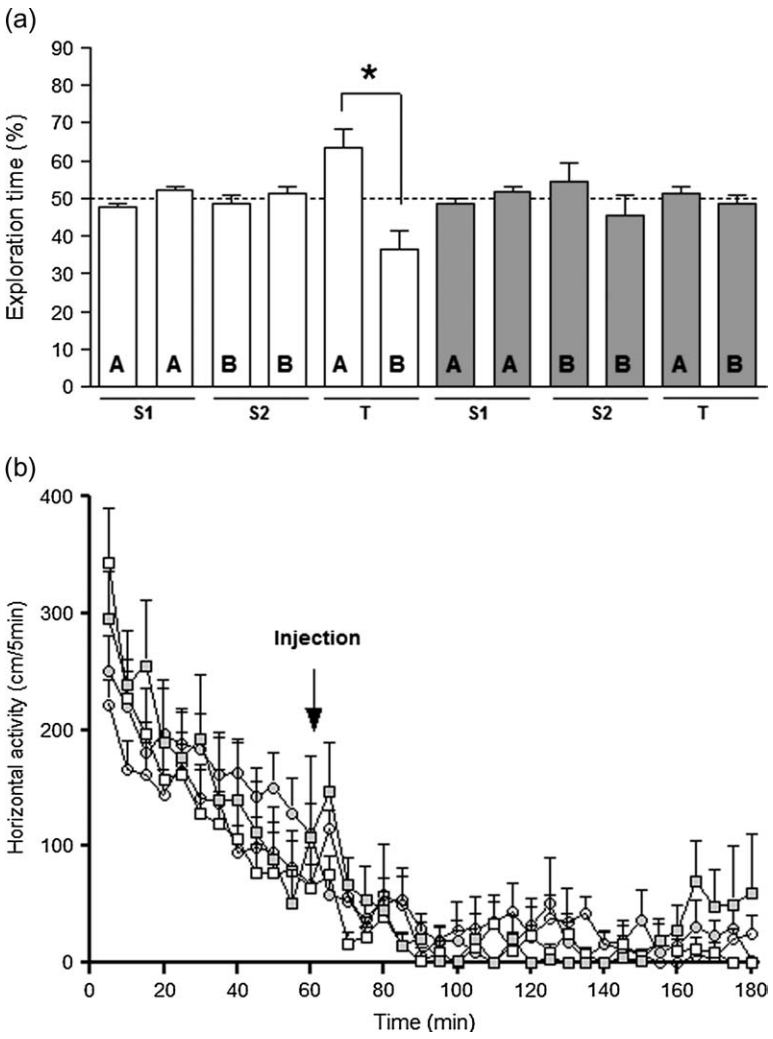


Figure 6: Increased cholinergic tone during retrieval does not facilitate object recognition memory in VAChT KD mice. (a) Galantamine, 1 mg/kg, administered 30 min before test (T), that is, just after retrieval of memory trace has no effect on the recognition memory of WT or VAChT KD^{HET} mice in the temporal object order memory task. Data represent mean ± SEM of % of total exploration; letters A and B represent the object; S1, S2 and T represent sample 1, sample 2 and test session, respectively. WT (white bars, $n = 8$) and KD^{HET} (gray bars, $n = 8$). (b) Locomotor activity of WT (clear symbols) and VAChT KD^{HET} mice (gray symbols) is not different and galantamine (circles) at 1 mg/kg does not alter spontaneous locomotor activity of WT or VAChT KD^{HET} mice compared to saline injection (squares). $n = 8$ for both genotypes.

this is accompanied by substantial plasticity of cortical representations (Nudo *et al.* 1996). Rats treated with IgG-192 saporin, a toxin that targets and eliminates basal forebrain cholinergic neurons, have deficits in learning a forelimb reaching task and exhibit alterations in cortical map representation (Conner *et al.* 2003), pointing to a possible role of distinct cholinergic neuronal groups in motor learning. Interestingly, neither motor coordination and gait, nor maximum performance in rotarod were altered in VAChT KD^{HET} mice, suggesting that motor skill learning is likely to be the only major component affected in these mutants. Our additional experiments evaluating specifically motor learning extended these previous observations and revealed that alterations in vesicular release of ACh modulate the rate of motor skill learning. Interestingly, performance maintenance in the rotarod appears to be minimally affected by reduced VAChT levels. However, although maximum performance could be achieved by VAChT KD^{HET} mice in day 18, the mutants require longer training to achieve that. The reason for this decreased alteration in performance is unknown at the moment.

Cognitive processing in VAChT KD mice

Several studies have addressed neurochemical circuits and anatomical regions involved in novel object recognition memory (Dere *et al.* 2007; Winters & Bussey 2005a). This kind of memory requires judgment of the previous occurrence of stimuli made on the basis of the relative familiarity of individual objects, by integrating information concerning objects and location and by using recency information (Barker *et al.* 2006; reviewed by Dere *et al.* 2007).

The perirhinal cortex is a key player in novel object recognition memory (Barker *et al.* 2006; Winters & Bussey 2005b), whereas the involvement of synaptic transmission in the hippocampus is controversial (Forwood *et al.* 2005; but see also Dere *et al.* 2007). There has been also controversy on the role of cholinergic system in novel object recognition memory based on lesion studies or studies with the p75 neurotrophic receptor selective immunotoxin 192-IgG saporin. Some studies show that the basal forebrain cholinergic system is important for object recognition memory with cholinergic terminals in the perirhinal cortex having a major

role (Warburton *et al.* 2003; Winters & Bussey 2005a). Conversely others failed to find an effect with electrolytic lesions in the medial septum (Ennaceur 1998) or found effects of 192-saporin only long after the lesions were induced (Paban *et al.* 2005), suggesting that the period of testing after the lesion is critical. In contrast, infusion of scopolamine provided strong evidence that cholinergic tone can have profound influence in encoding that is important for object recognition memory (Winters *et al.* 2006, 2007). Our results are consistent with these last observations and suggest that for object recognition recency memory, the ability to sustain cholinergic tone is also critical. Importantly, decreased cholinergic tone does not affect general behavior nor does it have unspecific effects in information processing, as spatial memory appears to be preserved in VAChT KD_{HET} mice (close to 40% decrease in VAChT expression).

The absence of spatial memory deficits in the MWM is unexpected, as some studies investigating the consequences of cholinergic lesions have suggested a role of acetylcholine in spatial memory (Dickinson-Anson *et al.* 1998; LeBlanc *et al.* 1999; Nilsson *et al.* 1987, 1992). In contrast, other studies using more selective lesions of cholinergic neurons failed to detect alterations in performance in the MWM task (Baxter & Gallagher 1996; Baxter *et al.* 1996; Frielingsdorf *et al.* 2006; Leanza *et al.* 1995). These observations are consistent with experiments using mice null for M1 muscarinic receptor that show spared spatial memory in this task (Miyakawa *et al.* 2001). However, because of the limited reduction in VAChT levels in VAChT KD^{HET} mice it is possible that more pronounced reductions in cholinergic tone could still affect spatial memory. To test this possibility directly future studies involving conditional brain specific VAChT knockout mice would be necessary.

The present experiments also addressed the question of whether decreased VAChT levels affect encoding or retrieval of object recognition memory. The ability of cholinesterase inhibitors to reverse the object recognition memory impairment in VAChT KD^{HET} mice was strictly dependent on improving cholinergic tone during encoding of information. Indeed, mice treated with cholinesterase inhibitors before memory retrieval performed as untreated VAChT KD^{HET} mice. The data suggest that cognitive alterations responsible for the failure of VAChT KD^{HET} mice to perform normally in object recognition memory tasks are specifically related to learning deficits. It is important to note that these cholinesterase inhibitors did not increase the exploration time during the sample phase of the task, or change exploration in the open field, suggesting that increased cholinergic tone indeed facilitated encoding information more effectively. These results expand the role of cholinergic tone to facilitate recency memory and are consistent with recent observations that indicate a role for muscarinic receptors in encoding information related to the novel object recognition task (Winters *et al.* 2006, 2007).

Cholinergic tone may assist memory encoding by either facilitating excitatory input to the cortex (Gioanni *et al.* 1999) or by modulating synaptic plasticity (Dringenberg *et al.* 2007). Alternatively, cholinergic activity may decrease excitatory feedback circuitry and this interference can affect information encoding (Hasselmo 2006; Hasselmo & Bower 1992; Hasselmo *et al.* 1992; Winters *et al.* 2007). Finally, cholinergic tone may regulate electrical properties of individual cells facilitating firing (Fransen *et al.* 2002; Klink & Alonso 1997). All of these mechanisms can be envisioned to help recruiting neuronal assemblies to encode information. Targeted VAChT mice therefore provide the means to probe how cholinergic tone regulates cellular mechanisms of learning.

Synaptic vesicles are filled with thousand molecules of ACh, however, the turnover rate for mammalian VAChT to take up ACh into vesicles is slow (approximately 1/s) (Varoqui & Erickson 1996). Estimates of vesicle recycling indicate that reuse of vesicles occurs fast after exocytosis, in the order of 15–30 s (Betz & Bewick 1992; Ryan *et al.* 1993). These observations suggest that synaptic vesicle filling in cholinergic terminals may not reach electrochemical equilibrium during high levels of activity. Central nervous system synapses, with their small pool of synaptic vesicles, might be particularly sensitive to reductions in VAChT levels. This should preferentially affect functions that depend on bursts of presynaptic activity demanding effective recycling of vesicles and ACh to maintain cholinergic tone.

A decrease in VAChT has been reported in Alzheimer's disease (Efange et al., 1997). A reduction of VAChT expression has been found in striatal tissue from patients with Huntingtoń s disease and is also observed in an animal model of this disorder (Smith *et al.* 2006). Moreover, decreased levels of VAChT were reported in rats after sepsis (Semmler *et al.* 2007) and treatment with antipsychotics, which seems to contribute to cognitive deficits observed in this last experimental model (Terry *et al.* 2007). Hence, diminished vesicular storage of ACh because of decreased VAChT expression may contribute to cognitive and neurological symptoms in these disorders. Our data indicate that the ability to sustain ACh release during neuronal activity is important for learning and raise the possibility for a conserved role of cholinergic tone in facilitating explicit and implicit memory encoding. These experiments also suggest that deficits of cholinergic tone because of decreased VAChT levels can be ameliorated by treatments that restore acetylcholine levels in the synapse.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1. Gait analysis of VAChT KD mice. (a) Stride length (mean ± SEM) for WT (white bar), VAChT KD^{HET} (gray bar) was measured between two steps from the same leg. (b) Representative examples of foot prints for WT, VAChT KD^{HET}. $n = 8$ for both genotypes.

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Artigo 2 The Vesicular Acetylcholine Transporter Is Required for Neuromuscular Development and Function

Nesse trabalho testamos se o VAChT seria o único responsável pela armazenagem de ACh em vesículas sinápticas. Minha participação específica foi a observação dos animais antes e após o nascimento para testar a sobrevivência dos mesmos. Fui responsável pela obtenção dos fetos necessários para vários dos experimentos e dissequei os animais para coletar os diferentes tecidos. Realizei também os testes neuromusculares de grip-force e wire-hang dos animais adulto $VAChT^{WT/DEL}$ para a figura 3 e fui responsável pelo estudo da atividade locomotora desses animais.

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The Vesicular Acetylcholine Transporter Is Required for Neuromuscular Development and Function▽

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The vesicular acetylcholine (ACh) transporter (VAChT) mediates ACh storage by synaptic vesicles. However, the VAChT-independent release of ACh is believed to be important during development. Here we generated VAChT knockout mice and tested the physiological relevance of the VAChT-independent release of ACh. Homozygous VAChT knockout mice died shortly after birth, indicating that VAChT-mediated storage of ACh is essential for life. Indeed, synaptosomes obtained from brains of homozygous knockouts were incapable of releasing ACh in response to depolarization. Surprisingly, electrophysiological recordings at the skeletal-neuromuscular junction show that VAChT knockout mice present spontaneous miniature end-plate potentials with reduced amplitude and frequency, which are likely the result of a passive transport of ACh into synaptic vesicles. Interestingly, VAChT knockouts exhibit substantial increases in amounts of choline acetyltransferase, high-affinity choline transporter, and ACh. However, the development of the neuromuscular junction in these mice is severely affected. Mutant VAChT mice show increases in motoneuron and nerve terminal numbers. End plates are large, nerves exhibit abnormal sprouting, and muscle is necrotic. The abnormalities are similar to those of mice that cannot synthesize ACh due to a lack of choline acetyltransferase. Our results indicate that VAChT is essential to the normal development of motor neurons and the release of ACh.

Cholinergic neurotransmission has key functions in life, as it regulates several central and peripheral nervous system outputs. Acetylcholine (ACh) is synthesized in the cytoplasm by the enzyme choline acetyltransferase (ChAT) (16). Choline supplied by the high-affinity choline transporter (CHT1) is required to maintain ACh synthesis (52). A lack of ChAT (4, 35) or the high-affinity choline transporter (21) in genetically modified mice is incompatible with life. ACh plays an important role in wiring the neuromuscular junction (NMJ) during development (38, 43). Embryonic synthesis of ACh is fundamental for the development of proper nerve-muscle patterning at the mammalian NMJ, as ChAT-null mice present aberrant nicotinic ACh receptor (nAChR) localization and increased motoneuron (MN) survival, axonal sprouting, and branching (4, 35).

The vesicular ACh transporter (VAChT) exchanges cytoplasmic ACh for two vesicular protons (37, 41). Previously reported electrophysiological studies showed that quantal size is decreased by vesamicol, an inhibitor of VAChT, but only in nerve terminals that have been electrically stimulated (19, 59, 60, 63). VAChT overexpression in developing *Xenopus* MNs increases both the size and frequency of miniature-end-plate currents (54). In *Caenorhabditis elegans*, mutations in VAChT affect behavior (65). Moreover, a decrease in VAChT expression has functional consequences for mammals, as mutant mice with a 70% reduction in the expression levels of this transporter (VAChT knockdown [KD^{HOM}] mice) are myasthenic and have cognitive deficits (47). Hence, vesicular transport activity is rate limiting for neurotransmission "in vivo" (18, 47).

Exocytosis of synaptic vesicle contents is the predominant mechanism for the regulated secretion of neurotransmitters (55). However, alternative mechanisms of secretion have been proposed (20, 56, 61). Quantal ACh release, comparable to that seen in developing nerve terminals, has been detected in

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myocytes and fibroblasts in culture, which presumably do not express VAChT (14, 24). More recently, it was found that the correct targeting of *Drosophila* photoreceptor axons is disrupted in flies with null mutations in ChAT (64). Remarkably, the inactivation of VAChT did not produce the same result (64). The result suggests that the release of ACh during development is not dependent on VAChT, perhaps because it is nonvesicular or because vesicular storage can occur without VAChT.

To test if the VAChT-independent secretion of ACh has any physiological role in the mammalian nervous system, we generated a mouse line in which the VAChT gene is deleted. These mice lack the stimulated release of ACh from synaptosomes, die after birth, and show several alterations in neuromuscular wiring consistent with a severe decrease in the cholinergic input to muscles during development. These experiments indicate that VAChT has an important role in maintaining activity-dependent ACh release that supports life and the correct patterning of innervation at the NMJ.

MATERIALS AND METHODS

Generation of VAChT knockout mice. The isolation of a VAChT genomic clone was described elsewhere previously (47). This genomic clone was used to construct a gene-targeting vector in which we added LoxP sequences flanking the VAChT open reading frame (ORF) and the TK-Neo cassette. One LoxP sequence was added 260 bp upstream from the VAChT translational initiation codon, and a second LoxP was added approximately 1.5 kb downstream from the stop codon. The TK-Neo cassette was added immediately after the second LoxP and was followed by a third LoxP (S1). Note that this is a vector distinct from what we previously reported for the localization of the TK-Neo cassette (47). The linearized targeting vector was electroporated into J1 embryonic stem cells derived from 129/terSv mice, and selected embryonic stem cell clones harboring homologous recombination (determined by PCR and Southern blotting [not shown]) were injected into C57BL/6J blastocysts to produce chimeric mice. Germ line transmission was achieved, and mice were bred to C57BL/6J mice to produce heterozygote mutant mice ($VAChT^{wt/flox}$). Prior to breeding $VAChT^{flox}$ mice to transgenic mice constitutively expressing Cre, we bred $VAChT^{flox}$ mice with CaMKIIalpha-Cre mice (Cre expression is driven by a fragment of the CaMKIIalpha promoter, kindly donated by Scott Zeitlin [17]) in an attempt to generate brain region-specific conditional knockout mice (these will be reported elsewhere). However, we noted that the progeny of mating between $VAChT^{wt/flox,cre+\text{-}CaMKIIalpha}$ males and $VAChT^{wt/flox}$ females inherited a recombined floxed allele (VAChT-deleted allele, or $VAChT^{del}$). This allele would be identical to that obtained by crossing the $VAChT^{flox}$ mice to Cre mice that constitutively express Cre. This recombination happened because there is ectopic expression of CaMKIIalpha-Cre in the testes, which can be detected by quantitative reverse transcription-PCR (data not shown). The presence of Cre within the testes allows the recombination of the floxed allele, probably during spermatogenesis, and therefore, the $VAChT^{del}$ allele is transmitted to the progeny. The ectopic expression of Cre in the testes was also previously described for other Cre lines (e.g., synapsin-Cre [49]), indicating that this is likely to be a common phenomenon. We backcrossed the progeny ($VAChT^{wt/del}$) to C57BL/6J mice (N4) and confirmed that they were capable of germ line transmission for the $VAChT^{del}$ allele. We then intercrossed $VAChT^{wt/del}$ mice to generate $VAChT^{del/del}$ mice, i.e., a potential homozygous VAChT-null mutant (see below). For comparison purposes, we also obtained ChAT-null mice as a kind gift from Kuo-Fen Lee and Fred H. Gage, Salk Institute (4).

Animals were housed in groups of three to five mice per cage in a temperature-controlled room with 12-h light–12-h dark cycles, and food and water were provided ad libitum. Unless otherwise stated, the experiments were always done using embryonic day 18.5 (E18.5) embryos. All studies were conducted in accordance with NIH guidelines for the care and use of animals and with approved animal protocols from the Institutional Animal Care and Use Committees at the Federal University of Minas Gerais and the University of Western Ontario.

Genotyping, Southern blotting, and sequencing. Genotyping by PCR was performed using tail DNA as a template. The set of three primers used were P1 (5′-TACTTGTCTGTCTGCCTGCCTGTC-3′), P2 (5′-AAGGAGTTGGTTGG CCACAGTAAG-3′), and P4 (5′-TCATAGCCCCAAGTGGAGGG AGA-3′). Oligonucleotides P1 and P2 amplified a 247-bp fragment in the wild-type (wt) allele, while primers P4 and P2 amplified a 329-bp fragment in the *del* allele. The 329-bp fragment amplified by primers P4 and P2 was purified from agarose gel using the QIAquick gel extraction kit (Qiagen) and cloned into the pCR 2.1 vector using the TA cloning kit (Invitrogen). The sequence of the cloned fragment was determined by automated DNA sequencing.

For Southern blot analysis, genomic DNA was digested with the enzymes BamHI and SacI. Digested DNA was subjected to electrophoresis in a 1.5% agarose gel and transferred onto a nylon membrane. After UV cross-linking, DNA on the membrane was hybridized to the NdeI/PmeI VAChT DNA fragment (see Fig. 1 for the position of the probe fragment). Detection was done using the Alkphos direct labeling and detection system kit (GE) according to the manufacturer's instructions.

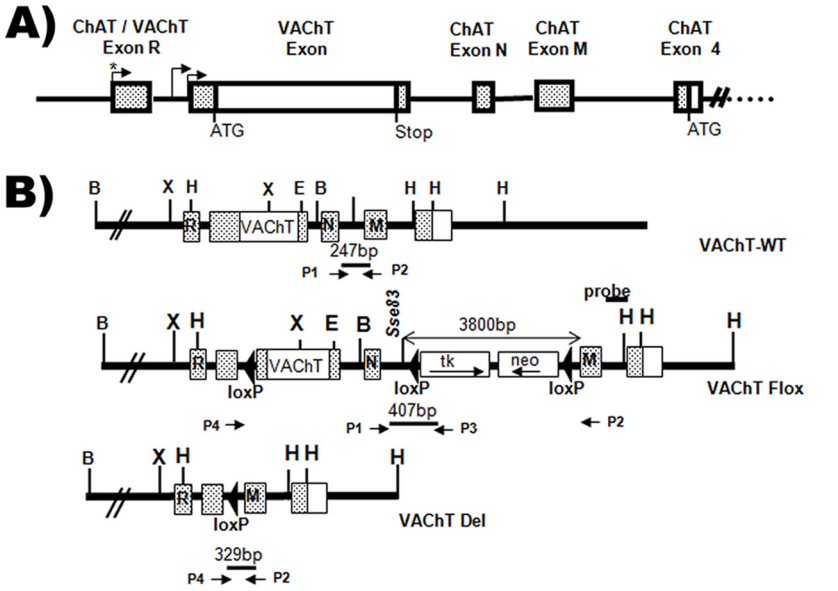
qPCR. For real-time quantitative PCR (qPCR), total RNA was extracted using Trizol (Invitrogen, São Paulo, Brazil) treated with DNase I (Ambion, Austin, TX), and first-strand cDNA was synthesized using a High Capacity cDNA transcription kit (Applied Biosystems, CA) according to the manufacturer's instructions. cDNA was subsequently subjected to qPCR on a 7500 real-time PCR system (Applied Biosystems, CA) using Power SYBR green PCR master mix (Applied Biosystems, CA). For each experiment, a nontemplate reaction was used as a negative control. In addition, the absence of DNA contaminants was assessed in reverse transcription-negative samples and by melting-curve analysis. The specificity of the PCRs was also confirmed by size verification of the amplicons by electrophoresis in acrylamide gels. Relative quantification of gene expression was done with the $2^{-\Delta\Delta CT}$ method using β-actin gene expression to normalize the data. The sequences of the primers used are available upon request.

Western blotting. Immunoblot analysis was carried out as described previously using spinal cord extracts from E18.5 mice (47). Antibodies used were anti-VAChT (Synaptic Systems Gottingen, Germany, and Sigma Chemical Co., São Paulo, Brazil), anti-CHT1 (51), anti-synaptophysin (Sigma Chemical Co.), and anti-actin (Chemicon, CA). Images were acquired and analyzed using ImageQuant TL (GE Healthcare).

Recombinant cDNA construct preparation, cell culture, and transfection. Rat CHT1 subcloned into the expression vector pcDNA3.1(−) and mutated in dileucine-like motif L531A was described previously (51). Human embryonic kidney HEK293 cells were acquired from the Cell Bank, Rio de Janeiro, Brazil. HEK293 cells were grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco). For transient transfections with empty vector (pcDNA3.1) or mutant CHT1 (L531A), HEK293 cells were seeded into 60-mm dishes (Falcon) and transfected using a modified calcium phosphate method (23). Choline and ACh uptake assays were performed at 48 h after transfection.

Choline and ACh uptake assays and ACh release. Choline and ACh uptake assays were performed as described previously (50). Briefly, cells plated into 60-mm dishes were washed twice with Krebs-HEPES medium (124 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, 25 mM HEPES [pH 7.4]) containing 10 mM paraoxon to inhibit acetylcholinesterase. Cells were then incubated with Krebs-HEPES containing 10 μM paraoxon and [^{3}H]choline chloride (1 μM; diluted to 1 mCi/μmol) or [^{3}H]ACh (1 mCi/μmol) for 10 min at 37°C. When hemicholinium-3 (1 μM) was used, the drug was added during this incubation step and maintained during the course of the experiment. Subsequently, cells were washed three times with 1.0 ml of cold Krebs-HEPES with paraoxon (10 μM) and lysed with 500 μl of 5% trichloroacetic acid (TCA) solution. Lysates were centrifuged for 10 min at 10,000 × *g* at 4°C. Pellets were used to measure protein content (3), and radioactivity was measured in the supernatants (100 μl) by liquid scintillation spectrometry to determine choline and ACh uptakes. In the competition assay, choline uptake was performed in the presence of crescent amounts of ACh (3 mM, 10 mM, or 30 mM).

The TCA supernatants obtained as described above were used to determine the [^{3}H]ACh content (45). Briefly, TCA was removed with ether, and quaternary amines were extracted using sodium tetraphenylboron in butyronitrile (10 mg/ml), the organic phase separated by centrifugation was reserved, and tetraphenylboron was precipitated with $AgNO_3$ in water. The suspension was homogenized and centrifuged. The organic phase was transferred into a new plastic tube containing $MgCl_2$ in water to precipitate excess Ag^+. After centrifugation, the solution containing quaternary amines was taken to dryness under a vacuum. The [^{3}H]choline present in dried samples was resuspended and oxidized using choline oxidase (Sigma-Aldrich) in glycylglycine buffer (pH 8). [^{3}H]ACh was extracted using tetraphenylboron in butyronitrile similarly to the procedure described above. Tritium in the organic (predominantly ACh) and aqueous (corresponding predominantly to choline) phases was measured by liquid scintillation spectrom-



C)

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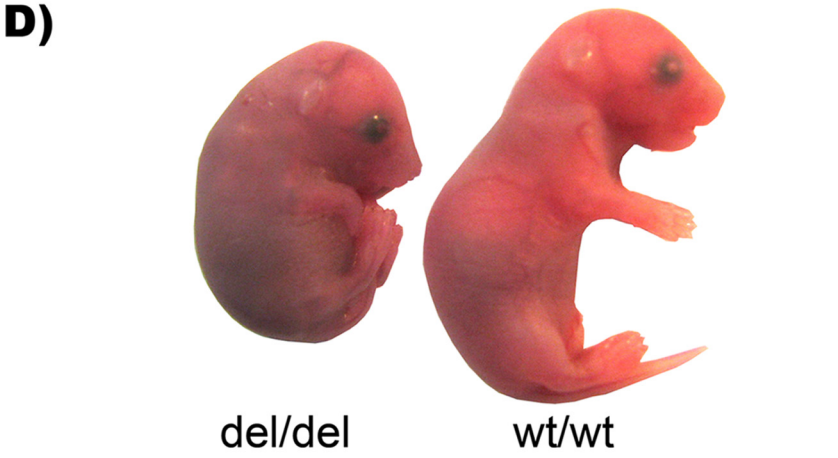
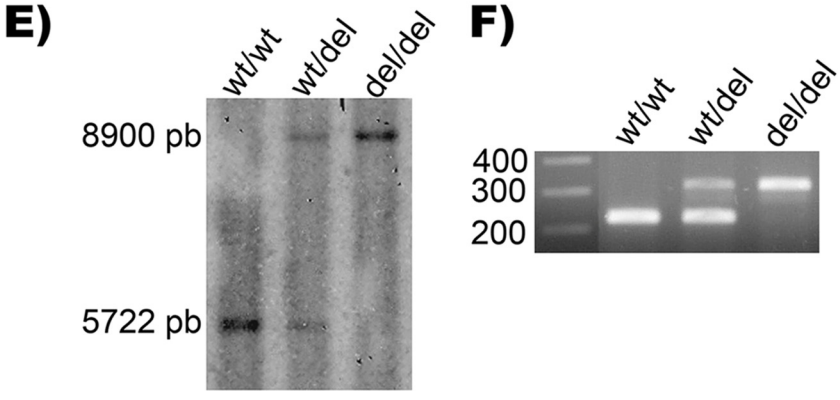
1   TCATAGCCCC AAGTGGAGGG AGAAAAAAAA AAAAAAAGAG AGGCGGCGGT GGAGGAAGAG
                          P4
61  GCAAGAGCGG ACGCGCGGGG AGGGCTGGAG AGACGGCGGG CGGCGGCAGC ATGCCCCTGG

121 GCGGGTGCAC ACGGCCTCTC TGCGCGGCAG GGGCGGGCAG GGGCTGCTCC TGCTCTCTCT

181 GGGCACCGCG CGTCCAGTCT CCCGCCTCAG CCCCTCGGCT TGCCGGCCTA CTAGCATAAC
                                                 NheI/SpeI
241 TTCGTATAAT GTATGCTATA CGAAGTTATG GCGCGCCCCT GCAGGAATTG GATGGTAAAA
        loxP                    AscI
301 GAAAACAAGG GAGGTCTTAC TGTGGCCAAC CAACTCCTT
                          P2
```





etry. Choline and ACh standards (0.1 to 0.5 μCi/ml) were processed in parallel with the samples to assess yield and cross-contamination. The later values were used to correct results of sample analyses. Protein content determined by the method of Bradford was used to normalize the data (3). Choline or ACh uptake into cells that was dependent on CHT1 was measured as a percentage of transport in cells transfected with empty vector. Each *n* value represents the average of data for triplicate samples.

KCl-induced release of [^{3}H]ACh in brain synaptosomes. Crude synaptosomes from whole brains of individual mice were homogenized in ice-cold buffer (0.32 M sucrose, 10 mM EDTA, Tris-HCl [pH 7.4]), and P2 pellets were obtained as described previously (2), washed, and then incubated in a depolarizing solution (90 mM NaCl, 50 mM KCl, 5 mM $NaHCO_3$, 1 mM $MgCl_2$, 1.2 mM Na_2HPO_4, 10 mM glucose, 20 mM HEPES, 2 mM $CaCl_2$, 0.02 mM paraoxon [pH 7.4]) for 5 min at 30°C. Subsequently, samples were centrifuged at 5,500 × *g* for 5 min at 4°C, and pellets were incubated in Krebs-HEPES medium (140 mM NaCl, 5 mM KCl) containing 100 nM of [^{3}H]choline, 5 mM $NaHCO_3$, 1 mM $MgCl_2$, 1.2 mM Na_2HPO_4, 10 mM glucose, 20 mM HEPES, 2 mM $CaCl_2$, and 0.02 mM paraoxon (pH 7.4) for 15 min at 30°C for choline uptake. After centrifugation, synaptosomes were washed three times with choline in ice-cold buffer (50 μM), and pellets were resuspended in ice-cold buffer. Each sample was separated into four aliquots. Two aliquots were incubated in Krebs-HEPES medium, and the other two aliquots were incubated in depolarizing solution containing hemicholinium-3 (1 μM) for 5 min at 30°C. The [^{3}H]ACh released was collected after centrifugation, pellets were digested with 5% TCA, and the radioactivity of both samples was measured using liquid scintillation counting. Total radioactivity (supernatant and pellet) was calculated and then normalized by protein content. For each sample, the average values obtained under depolarizing or nondepolarizing conditions was divided for the total radioactivity. The release of newly synthesized [^{3}H]ACh is predominant under this condition (2); the results are shown as fractional release above baseline release obtained under nondepolarizing conditions.

Tissue ACh measurements. Brains were dissected rapidly, homogenized in 5% TCA, and centrifuged (10,000 × *g* for 10 min) at 4°C. Supernatants were frozen at −80°C until use. For ACh determinations, TCA was removed with ether, and a chemiluminescent assay was done with choline oxidase as described previously (44). The data are presented as means and standard errors of the means (SEM). One-way analysis of variance (ANOVA), followed by Bonferroni's test, was used to analyze the differences in tissue ACh concentrations in VAChT$^{wt/wt}$, VAChT$^{wt/del}$, and VAChT$^{del/del}$ mice; a *P* value of <0.05 was considered to be statistically significant.

Electrophysiology. Electrophysiology experiments were performed similarly to methods described elsewhere previously (47). Hemidiaphragms were isolated from E18.5 embryos, and the muscle with attached nerve was pinned to a Sylgard pad in a 5-ml acrylic chamber continuously perfused at a rate of 1 ml/min with Tyrodes solution containing 137 mM NaCl, 26 mM $NaHCO_3$, 5 mM KCl, 1.2 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 2.4 mM $CaCl_2$, and 10 mM glucose equilibrated with 95% O_2–5% CO_2 at pH 7.4. During recording, tetrodotoxin (3 μM) was included to avoid contractions. Microelectrodes were fabricated from borosilicate glass and had resistances of 8 to 15 MΩ when filled with 3 M KCl. Standard intracellular recording techniques were used to record miniature end plate potentials with an Axoclamp-2A amplifier. Recordings were band-pass filtered (0.1 Hz to 10 KHz) and amplified 200 times prior to digitization and acquisition on an IBM computer running WinEDR (John Dempster, University of Strathclyde). The membrane potential was recorded and used to correct MEPP amplitudes and areas to a standard resting potential of −60 mV. At the end of experiments, 5 μM *d*-tubocurarine was applied to verify that the observed events were due to nicotinic receptors.

FM1-43 imaging. FM1-43 imaging experiments were performed as described previously (47) except that a fixable FM1-43 analog was used. Briefly, diaphragms from E18.5 mice were dissected and mounted onto a Sylgard-lined chamber containing mouse Ringer solution with the following composition: 135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 1 mM Na_2HPO_4, and 11 mM D-glucose. Solutions were aerated with 95% CO_2–5% O_2, and the pH was adjusted to 7.4. FM1-43fx (8 μM) was used to stain recycling synaptic vesicles during stimulus with a high-K^+ solution (60 mM KCl) for 10 min with 16 μM *d*-tubocurarine to prevent contractions. After stimulation, the preparation was maintained in normal K^+ solution for 10 min to guarantee maximal FM1-43fx uptake. Excess FM1-43fx adhering to the muscle cell plasma membrane was removed during a washing period in mouse Ringer solution not containing FM1-43fx for at least 40 min; 16 μM *d*-tubocurarine was present to prevent muscle contraction. Advasep-7 (1 mM) was added during the washing period after FM1-43fx staining to reduce background fluorescence. Diaphragms stained with FM1-43fx were fixed with 4% paraformaldehyde for 40 min and then mounted onto slides and examined by fluorescence microscopy on either an Axiovert 200 M microscope equipped with a 40× water immersion objective using the Apotome system or a Leica SP5 confocal microscope using a 63× water immersion objective and an argon laser (488 nm) for excitation. The spectrum emission was set from 510 to 620 nm. During image acquisition, whole hemidiaphragms were scanned, and the images were obtained from muscle areas with stained NMJs. The total number of junctions per hemidiaphragm was defined by the sum of junctions observed in each image after scanning the entire muscle. The density of junctions was determined by the ratio of the number of junctions/total area (mm^2). The nerve terminal area was measured using Image J, and the size of each terminal was expressed in $pixels^2$. Data were analyzed using an unpaired *t* test. A *P* value of <0.05 was considered to be statistically significant.

Immunofluorescence. Immunofluorescence was performed as described previously (4). Briefly, whole-mount diaphragms from embryos were rapidly dissected and fixed in 4% paraformaldehyde–phosphate-buffered saline (PBS) (pH 7.4) for approximately 3 days. Tissues were cryoprotected with 10% sucrose–4% paraformaldehyde, frozen with isopentane over dry ice, and kept at −80°C until use. Muscles were rinsed two times in PBS, incubated with a 0.1 M glycine–PBS solution for 1 h, and blocked in incubation buffer (150 mM NaCl, 0.01 M phosphate buffer, 3% bovine serum albumin, 5% goat serum, and 0.01% thimerosal) overnight at 4°C. Tissues were immunostained with anti-VAChT (rabbit polyclonal; Sigma Chemical Co.), anti-CHT1 (51), or anti-neurofilament 150 (rabbit polyclonal; Chemicon) in incubation buffer overnight at 4°C. Following three washes of 1 h each with PBS–0.5% Triton X-100, muscles were incubated with Alexa Fluor 546-conjugated goat anti-rabbit antibody (Molecular Probes) and Alexa Fluor 488-conjugated α-bungarotoxin (Molecular Probes) in the buffer described above overnight at 4°C, and the washing step was repeated. Diaphragms were flat mounted in Hydromount medium, and images were collected with an Axiovert 200 M microscope using the Apotome system or a Leica SP5 confocal microscope to acquire optical sections of the tissues. Quantitative analyses of nAChR or CHT1 fluorescence were carried out with Metamorph software (Molecular Devices, Downingtown, PA). For each set of experiments, a threshold was calculated by using a background area of the image. This threshold value determined for VAChT$^{wt/wt}$ was applied to images obtained with other genotypes, and the total fluorescence intensity (integrated intensity) for bungarotoxin-labeled nAChR or terminals labeled by CHT1 antibody was then detected automatically. To count the number of positive terminals or junctions, adjacent sections of the entire muscle were obtained, and the numbers of positive labeled structures were counted with Metamorph similarly to the above-described experiments with FM1-43.

MN quantification. Adult pregnant females were anesthetized with ketamine-xylazine (70 mg/kg and 10 mg/kg, respectively) intraperitoneally and sacrificed by cervical dislocation. Embryos were removed quickly, and spinal cords were removed and immersed in Bouins fixative for 24 h prior to being processed for paraffin embedding. Paraffin blocks were serially sectioned, with sections placed onto microscope slides and stained with thionin. MNs were counted as described previously (12).

In vivo analysis of muscle function. Locomotor activity, grip force, and wire hang tests were performed essentially as described previously (15, 47).

FIG. 1. Generation of VAChT$^{del/del}$ mice. (A) Generation of VAChT *del* mice using the Cre-LoxP system. Boxes represent the different exons of ChAT or VAChT. Open boxes represent the ORF of VAChT and ChAT. Note that the VAChT gene is within the first intron of ChAT. (B) Schematic representation of the VAChT gene locus, the *floxed* allele, and the *del* allele. P1, P2, P3, and P4 indicate the positions of PCR primers used for genotyping. (C) Sequence analysis of the 329-bp fragment amplified with primers P2 and P4. Restriction sites and LoxP sequences are indicated. (D) VAChT mutant mice (VAChT$^{del/del}$) died rapidly after birth in cyanosis (not shown). Embryos from E18.5 exhibited flaccid limbs and kyphosis (hunchback). (E) Southern blot analysis confirmed the presence of the *del* allele in VAChT mutant mice. (F) Genotype of VAChT mutant mice by PCR.

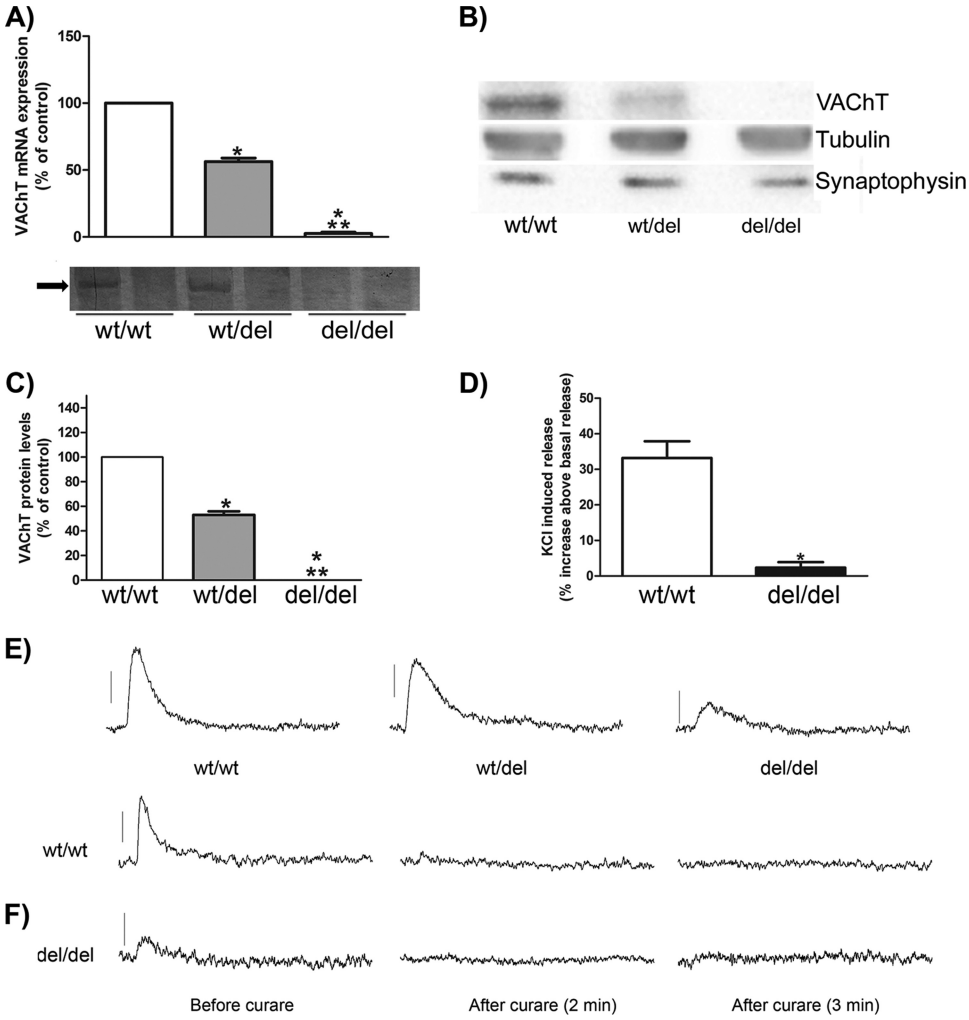


FIG. 2. VAChT expression and ACh release in VAChT$^{del/del}$ E18.5 mice. (A) Quantitative analysis of VAChT mRNA levels by qPCR. PCR products were run in a polyacrylamide gel. *, statistically different from wt; **, statistically different from wt/del {one-way ANOVA with Bonferroni post hoc [$F(2,6 = 920)$]; $P < 0.0001$; $n = 4$}. Lanes labeled with a − show the respective negative control without the sample. (B) Western blot analysis of VAChT, synaptophysin, and tubulin in spinal cord extracts. (C) Average values for the amount of VAChT from densitometric analyses of several Western blots. Tubulin immunoreactivity was used to normalize protein loading. Data are presented as percentages of VAChT$^{wt/wt}$ levels. *, statistically different from wt; **, statistically different from wt/del {one-way ANOVA with Bonferroni post hoc [$F(2,9 = 927.9)$]; $P < 0.0001$; $n = 4$}. (D) Effects of KCl-induced depolarization on [^{3}H]ACh release from synaptosomes. *, statistically different from wt/wt ($P < 0.05$ by t test). (E) MEPPs from the NMJ. MEPPs recorded in VAChT$^{wt/del}$ muscle show no significant change in amplitude compared to VAChT$^{wt/wt}$ mice. VAChT-null mice showed the existence of scarce MEPPs with decreased amplitude compared to VAChT$^{wt/wt}$ and VAChT$^{wt/del}$ mice. (F) *d*-Tubocurarine abrogated MEPPs in both VAChT$^{wt/wt}$ and VAChT$^{del/del}$ mice.

RESULTS

Generation of mice null for VAChT. We have generated mice in which the VAChT ORF was deleted using Cre-Lox technology (Fig. 1A and B). The deletion of the VAChT ORF was confirmed by DNA sequencing (Fig. 1C), and we named the VAChT-deleted allele VAChT^{del}. The posture of VAChT$^{del/del}$ mice at E18.5 resembles that of ChAT-null mice, with flaccid limbs and signs of slight kyphosis (Fig. 1D). VAChT$^{del/del}$ mice die rapidly in cyanosis within 2 to 5 min after birth. Southern analysis (Fig. 1E) and PCR genotyping (Fig. 1F) confirmed the presence of the *del* allele in heterozygous and homozygous VAChT mutant mice. These mice are a novel mutant line distinct from the one that we have previously described and that presents close to 70% and 45% reductions in VAChT expression (VAChT KD^{HOM} and VAChT KD^{HET}, respectively). Contrary to the mouse line reported here (VAChT$^{del/del}$), the VAChT KD lines survive to adulthood (47).

To confirm that the genetic manipulation that putatively deleted the VAChT ORF indeed suppresses VAChT mRNA expression, we used qPCR and E18.5 embryos (Fig. 2A). VAChT$^{wt/del}$ mouse brain presented a 50% decrease in the VAChT mRNA level compared to VAChT$^{wt/wt}$ littermate controls. No VAChT mRNA was detected in VAChT$^{del/del}$ mouse brain. The reverse transcription-PCR amplicons were also separated by electrophoresis in a polyacrylamide gel. VAChT$^{del/del}$ mice generated no DNA fragment corresponding to VAChT (Fig. 2A, inset gel). VAChT$^{wt/del}$ mice exhibited

a 50% decrease and VAChT$^{del/del}$ mice exhibited a 100% decrease in VAChT protein levels assayed by Western blotting of spinal cord extracts (Fig. 2B and C). The amount of synaptophysin, a protein present in synaptic vesicles, was unchanged in the spinal cord of VAChT$^{del/del}$ mice (Fig. 2B).

To investigate the importance of VAChT for the evoked secretion of ACh, we prepared crude synaptosomes from the forebrain of E18.5 wt and homozygous mutant mice. We labeled ACh in these synaptosomes with the precursor [^{3}H]choline and monitored the release of labeled neurotransmitter as previously described (2, 26, 27, 32). VAChT$^{del/del}$ mice are capable of producing ACh (see Fig. 5). KCl depolarization was able to increase the release of [^{3}H]ACh in synaptosomes obtained from wt mice but not from VAChT$^{del/del}$ mice (Fig. 2D). Therefore, this experiment indicates that in the absence of VAChT, depolarization-evoked ACh release is hindered.

In order to analyze ACh secretion under nondepolarizing conditions, we performed electrophysiological analysis of the nerve-muscle diaphragm preparation. Figure 2E shows MEPPs recorded from NMJs of VAChT$^{wt/wt}$, VAChT$^{wt/del}$, and VAChT$^{del/del}$ E18.5 mice. VAChT$^{wt/del}$ mice presented no change in the amplitude of MEPPs compared to control mice (0.99 ± 0.09 mV for wt/wt and 0.92 ± 0.09 for wt/del for 31 MEPPs in three and four mice, respectively). Surprisingly, we could detect small-amplitude MEPPs in the NMJ from E18.5 embryos of VAChT$^{del/del}$ mice (Fig. 2E). These experiments were difficult to perform, as the frequency of MEPPs in VAChT$^{del/del}$ mice was noticeably low compared to that of VAChT$^{wt/wt}$ embryos (del/del = 0.0072 ± 0.0009 Hz [11 MEPPs obtained from four fibers from two mice]; wt/wt = 0.0308 ± 0.002 Hz [three fibers from three mice]; wt/del = 0.0364 ± 0.008 Hz [six fibers from four mice]). MEPPs from VAChT$^{del/del}$ mice were of smaller amplitude (0.54 ± 0.07 mV). However, given the overt morphological changes at the NMJs from E18.5 VAChT$^{del/del}$ mice (see Fig. 6, 7, and 8), both pre- and postsynaptic contributions to these changes are likely. Treatment of NMJs from control littermates and homozygous VAChT mutants with *d*-tubocurarine (5 μM) abolished miniature detection, indicating that the MEPPs were likely recorded due to the activation of nAChR (Fig. 2F). In agreement with the fact that VAChT$^{wt/del}$ mice presented no alteration in MEPPs at the NMJ, adult VAChT$^{wt/del}$ mice presented no overt neuromuscular dysfunction that could be detected in a test of fatigue or grip force (Fig. 3A and B). In addition, spontaneous locomotor activity was unchanged in VAChT$^{wt/del}$ mice (Fig. 3C).

We considered whether ACh uses another type of transporter to load synaptic vesicles in the absence of VAChT. One candidate is CHT1, which has been found to reside predominantly in synaptic vesicles by us (15, 50–52) and others (22, 36). Like other secondary active transporters for aqueous solutes, CHT1 probably functions bidirectionally (33). "Reverse transport" by CHT1 would be required to mediate ACh uptake by synaptic vesicles. We tested the possibility that CHT1 transports ACh in addition to choline by using a cell line expressing recombinant CHT1. We were not able to do the test in nerve terminals per se, as the pharmacological blockade of CHT1 would decrease ACh synthesis and potentially produce effects on small MEPPs not due to the inhibition of vesicular CHT1. To untangle the multiple possible roles of CHT1, we used a

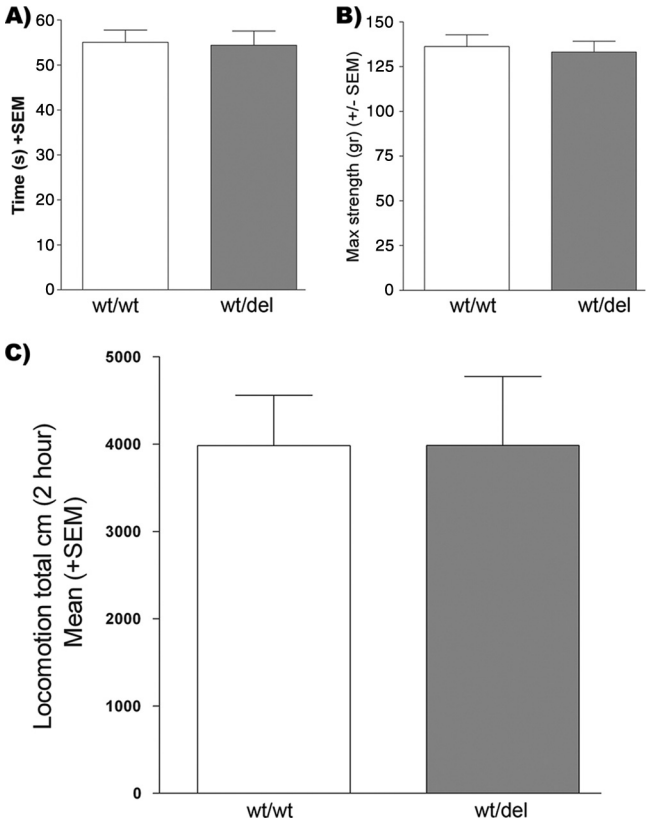


FIG. 3. Neuromuscular function in VAChT$^{wt/del}$ mice. (A) Grip force measured for VAChT$^{wt/wt}$ and VAChT$^{wt/del}$ mice. There is no significant difference between the two genotypes. (B) Time spent hanging upside down from a wire netting for VAChT$^{wt/wt}$ and VAChT$^{wt/del}$ mice. No significant difference was observed. (C) Spontaneous locomotor activity is not changed between the genotypes.

mutant form of CHT1 (L531A) that does not undergo endocytosis, and which remains predominantly on the cell surface, to transfect HEK293 cells. The strategy is expected to maximize ACh uptake by transfected cells should CHT1 be able to transport ACh (51). As expected, transfected cells took up fourfold-more choline than did nontransfected cells (Fig. 4A) (51). ACh in concentrations similar to those found in the cytoplasm of cholinergic terminals (41) inhibited choline uptake, indicating a good likelihood that ACh competes with choline for binding to CHT1 (Fig. 4B). However, the transfected cells took up no more ACh than did nontransfected cells (Fig. 4C). The results demonstrate that CHT1 does not significantly transport ACh, and thus, they do not support the possibility that CHT1 mediates the uptake of ACh by synaptic vesicles.

The results leave open the possibility that a passive transport system similar to that described previously for isolated cholinergic vesicles of *Torpedo* is present in mammalian synaptic vesicles (8). In the right circumstances, even the passive uptake of ACh by synaptic vesicles might be sufficient to generate the small MEPPs observed here. Indeed, recent experiments by Parsons and collaborators using synapse-like microvesicles from rat PC12 cells found that intact vesicles loaded with ACh lose their neurotransmitter content even when a high-affinity analog of vesamicol completely blocks VAChT. The result demonstrates an ACh leakage

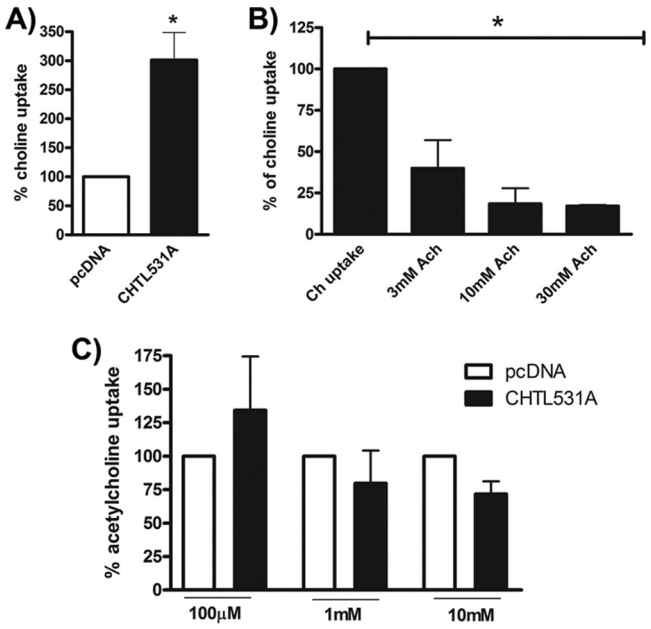


FIG. 4. (A) Choline uptake in HEK293 cells transiently transfected with empty vector (pcDNA3.1) or L531A CHT1 cDNAs. The data represent the means ± SEM of data from five independent experiments (in duplicates) and are normalized to data for cells expressing empty vector (pcDNA3.1). *, significant difference ($P < 0.05$ by t test). (B) ACh competition assay using HEK293 cells transiently transfected with empty vector (pcDNA3.1) or L531A CHT1 cDNAs. The data represent the means ± SEM of data from four independent experiments and are normalized to data for cells expressing the empty vector (pcDNA3.1). *, significantly different from control uptake. (C) ACh uptake in HEK293 cells transiently transfected with empty vector (pcDNA3.1) or L531A CHT1 cDNAs. The data represent the means ± SEM of data from three independent experiments and are normalized to data for cells expressing the empty vector (pcDNA3.1).

mechanism in synapse-like microvesicles that might be bidirectional (S. M. Parsons, personal communication).

However, in order for the passive uptake of ACh by synaptic vesicles to be possible in VAChT$^{del/del}$ mice, cytoplasmic stores of ACh must be maintained. In the absence of vesicular storage, many neurotransmitters are degraded (62), but in *C. elegans*, the mutational inactivation of VAChT leads to an increase in the ACh content of the worm (29). Therefore, we measured the amount of intracellular ACh in the brains of mutant mice. In E18.5 embryos, the amount in VAChT$^{del/del}$ mice was more than fivefold greater than that in VAChT$^{wt/wt}$ mice (Fig. 5A). There was also a clear tendency for the level of ACh in the brain of VAChT$^{wt/del}$ embryos to be increased compared to that of control wt mice (Fig. 5A). In adult VAChT$^{wt/del}$ mice, the ACh content was significantly increased compared to that of control wt mice (Fig. 5B). Because vesicles in VAChT$^{del/del}$ mice are likely depleted of ACh, the concentration increase for ACh in the cytoplasm of cholinergic terminals is probably greater than what the bulk analysis indicates. Hence, an increased concentration of cytoplasmic ACh in VAChT$^{del/del}$ mice might support the passive uptake of ACh into synaptic vesicles and produce small MEPPs.

Why is there so much more ACh in VAChT$^{del/del}$ mice? One possible explanation is increases in the amounts of either ChAT or CHT1, which are involved in the synthesis of ACh. To test for this possibility, we performed qPCR analysis of E18.5 embryos. Transcript levels for ChAT were increased in a gene dosage-dependent way (Fig. 5C). VAChT$^{wt/del}$ mice had nearly twofold-more ChAT mRNA than their control littermates, whereas VAChT$^{del/del}$ mice had nearly fourfold more (Fig. 5C). In addition, we found that VAChT$^{del/del}$ mice had nearly twofold-more CHT1 mRNA than control littermates, whereas VAChT$^{wt/del}$ mice had no significant change (Fig. 5D). At the protein level, we also detected an increase in the amounts of ChAT and CHT1 in homozygous mutant animals (Fig. 5E and F). These observations suggest that increases in ChAT and CHT1 expression levels likely underlie the increase in the amount of ACh in VAChT$^{del/del}$ mice.

Abnormal neuromuscular patterning is a major feature of NMJ developed in the absence of ACh (4, 35). In VAChT$^{del/del}$ mice, nerve terminals have fivefold-more ACh but lack the protein responsible for the active storage of the transmitter in vesicles. Does a lack of VAChT affect NMJ development? Can the lack of VAChT be compensated by the excess intraterminal ACh in VAChT mutants? In order to answer these questions, we evaluated nerve branching, nAChR localization, and the genesis of nerve terminals by labeling the NMJ of wt, VAChT$^{wt/del}$, and VAChT$^{del/del}$ mice with distinct markers. To begin, we tested whether NMJs of VAChT$^{del/del}$ mice showed immunoreactivity for VAChT. We found no VAChT immunoreactivity, as expected (Fig. 6A); in comparison, CHT1 immunolabeling was easily detected (Fig. 6B). Interestingly, analysis of nAChR labeling using fluorescent α-bungarotoxin–Alexa Fluor 543 (BTX-543) (Fig. 6, red) suggested an altered nAChR distribution (Fig. 6A and B and higher magnification in C). Indeed, clusters of nAChR labeled with BTX-543 showed stronger labeling and a larger area in VAChT$^{del/del}$ mice than the corresponding labeling in control and VAChT$^{wt/del}$ mice (Fig. 6C and D) (the increase in labeling was close to 70%).

The rescue of MNs from physiologically programmed cell death that follows the blockade of neuromuscular activity during development is a well-known phenomenon (38, 39). The disturbance of ACh synthesis also affects MN apoptosis (4). In order to test if in the absence of VAChT MNs went through the normal wave of apoptosis, we counted lumbar MNs from wt and VAChT$^{del/del}$ E18.5 embryos (Fig. 6E). Of note, there was a significant increase in the number of MNs in VAChT mutant mice compared to wt controls (36%), suggesting that VAChT-independent ACh secretion did not generate the muscle activity necessary for the programmed cell death of MNs during development. The increase in MN survival was similar to but somewhat less severe than that observed for ChAT-null mice (Fig. 6E) (51% increase in the number of neurons compared to wt controls).

To examine if the enhanced nAChR labeling and enhanced MN numbers are accompanied by an increase in the number of nerve terminals in VAChT$^{del/del}$ mice, we quantified the number of CHT1-positive nerve terminals. Immunoreactivity for CHT1 (Fig. 7A) was increased at the NMJ, confirming the biochemical data shown in Fig. 5D and F. We also quantified the number of CHT1-positive nerve terminals, and we detected a significant increase in the number of CHT1-positive nerve

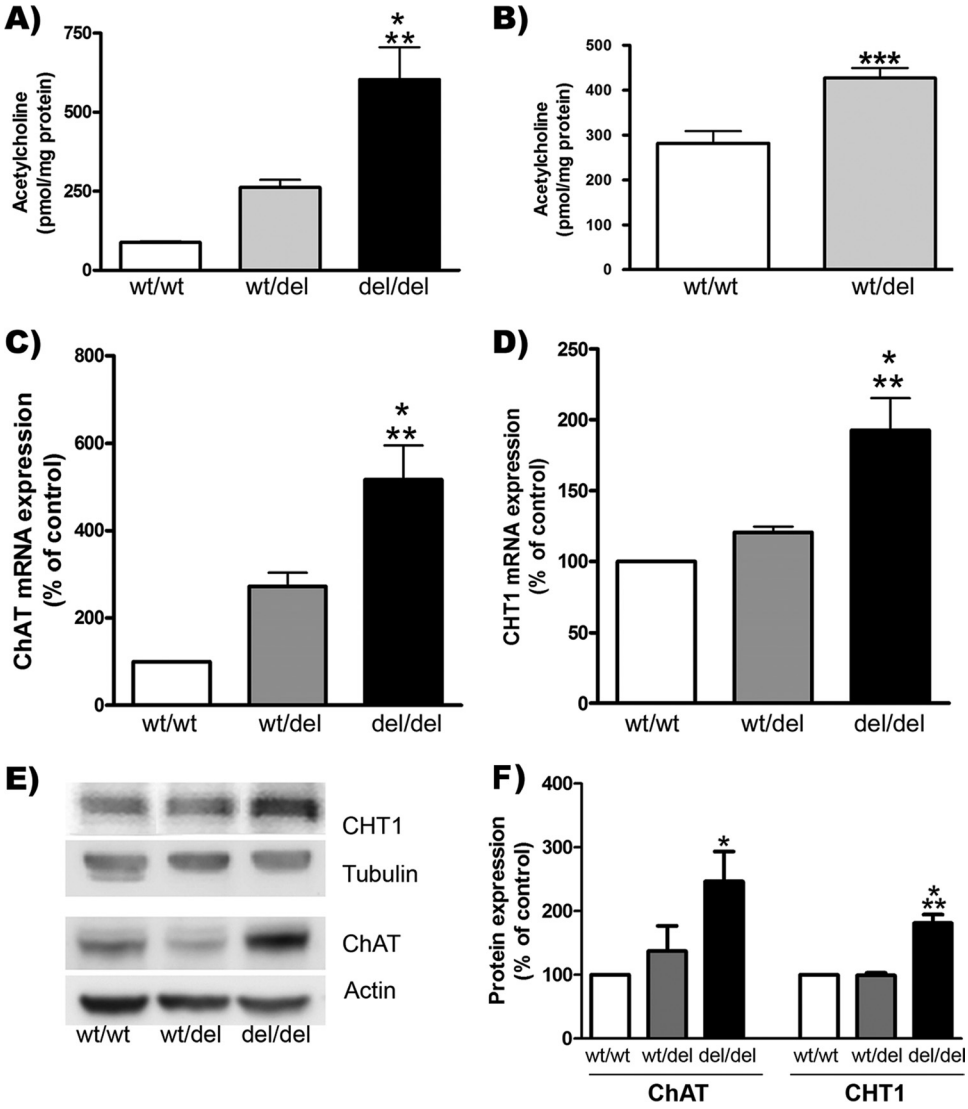


FIG. 5. Neurochemical alterations in VAChT$^{del/del}$ mice. (A) Intracellular ACh contents in brains of VAChT mutant mouse embryos. Data are means ± SEM (three to five mice). An asterisk indicates a statistically significant difference by one-way ANOVA with Bonferroni post hoc test [$F(2,10 = 12.72)$]. (B) Intracellular ACh content in brains from adult VAChT$^{wt/wt}$ and VAChT$^{wt/del}$ mice ($n = 4$ to 6 brains) (***, $P < 0.001$). (C) ChAT mRNA levels detected by qPCR from E18.5 mouse brains [$F(2,9 = 18.28)$] ($n = 4$). (D) CHT1 mRNA levels detected by qPCR from E18.5 mouse brains. *, statistically different from VAChT$^{wt/wt}$ mice; **, statistically different from VAChT$^{wt/del}$ mice [$F(2,11 = 12.52)$] ($n = 5$). (E) ChAT and CHT1 protein expression in E18.5 spinal cords. (F) Quantification of protein expression (three to four animals) ($P < 0.05$) [CHT1 $F(2,6) = 35.21$; ChAT $F(2,15) = 4.599$]. *, statistically different from wt/wt; **, statistically different from wt/del.

endings in VAChT-null mutants (Fig. 7B). To further test if the nerve endings in the diaphragm of VAChT$^{del/del}$ mice were able to undergo exocytosis-endocytosis, we used a form of the activity-dependent dye FM1-43, FM1-43fx, that can be used for protocols requiring tissues to undergo fixation. Preparations to be stained with FMI-43fx underwent KCl-mediated depolarization as described previously (47, 53) and were then washed and fixed prior to the quantification of fluorescently labeled nerve terminals. The results show that synaptic vesicles in VAChT$^{del/del}$ mice undergo exocytosis-endocytosis. Moreover, muscles from homozygous mutants have an increased density of stained nerve terminals compared to control wt mice (40% increase) (Fig. 7C and D). Figure 7E shows an example of terminals labeled with FM dye, and Fig. 7F indicates that in addition to an increase in the number of terminals, the area of the individual terminals labeled with FM1-43 from VAChT$^{del/del}$ mice is also increased compared to that from VAChT$^{wt/wt}$ mice ($P = 0.0018$). The increase in the number of nerve terminals appears to be a consequence of the complete loss of VAChT, as VAChT KD^{HOM} mice that preserve 30% of normal levels of the transporter show no such increase (48; data not shown). VAChT KD^{HOM} mice also did not show an increase in the number of MNs, suggesting that reducing VAChT levels by up to 70% can still support enough release of neurotransmitter during development to maintain the program of MN cell death (data not shown).

To further examine axonal targeting at the NMJ in the absence of VAChT, we labeled diaphragms from wt,

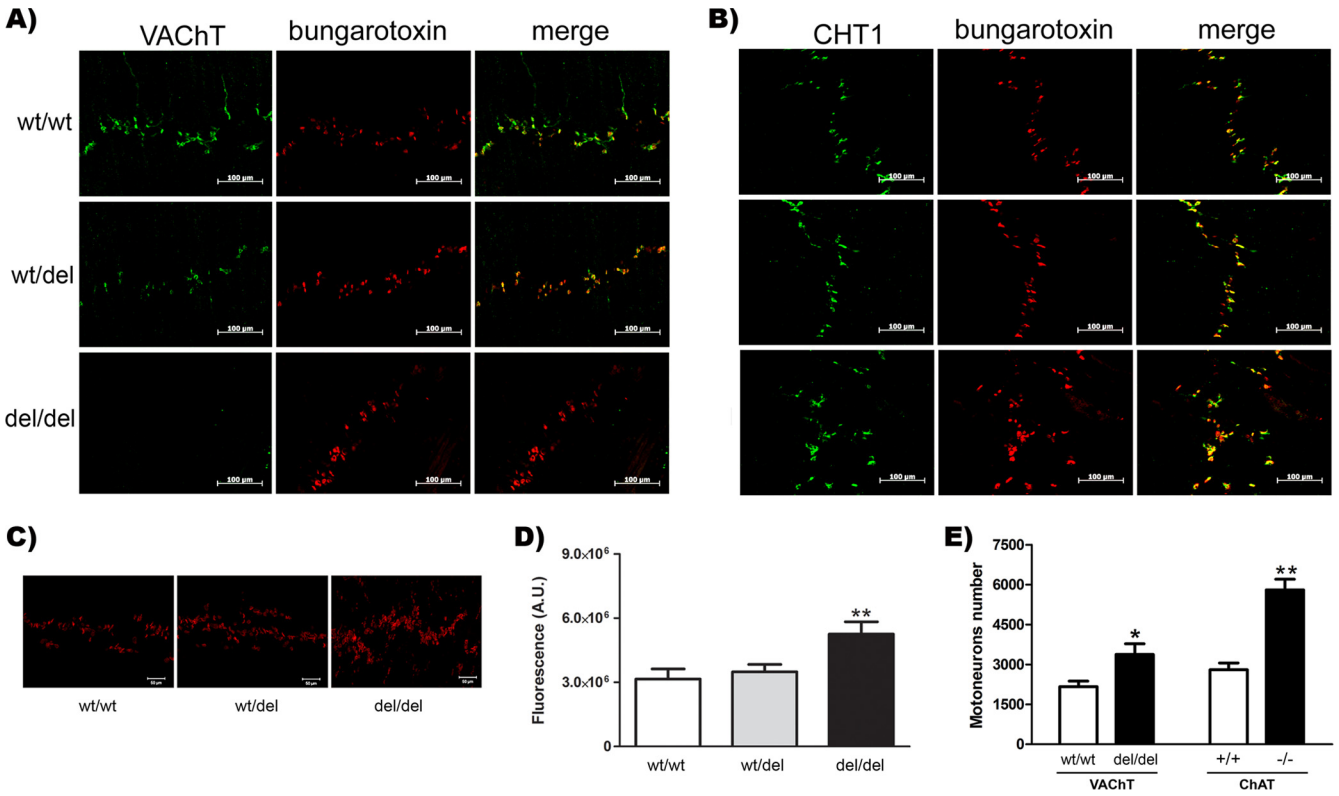


FIG. 6. Alterations in MN number and in NMJ morphology in VAChT$^{del/del}$ E18.5 mice. (A) VAChT immunoreactivity was easily detected in presynaptic termini of VAChT$^{wt/wt}$ and VAChT$^{wt/del}$ diaphragms but was absent in VAChT$^{del/del}$ mice. (B) CHT1 immunoreactivity was detected in all genotypes, although VAChT$^{del/del}$ mice showed an altered distribution of nerve endings (see below and Fig. 5). (C) Abnormal distribution of nAChR in VAChT$^{del/del}$ NMJs. Images show that nAChRs from VAChT$^{del/del}$ mice present stronger labeling and are distributed over a broader region than those from wt and VAChT$^{wt/del}$ mice. (D) Quantification of nAChR fluorescence. Four independent experiments were performed, and the results are expressed as means ± SEM. An asterisk indicates a statistically significant difference (one-way ANOVA with Bonferroni post hoc) [$F(2,20 = 5.632)$]. A.U., arbitrary units. (E) The number of lumbar MNs was significantly increased in VAChT and ChAT-null mice. Clear bars, wt mice; dark bars, homozygous mutant mice.

VAChT$^{wt/del}$, and VAChT$^{del/del}$ mice with an anti-neurofilament antibody (Fig. 8, red) and nAChR with BTX-Alexa Fluor 488 (Fig. 8, green). These experiments show that there is no difference in axonal branching between wt and VAChT$^{wt/del}$ mice. Axon branches from the nerves labeled with the anti-neurofilament antibody were of the characteristic size and generally contacted a cluster of nAChR (Fig. 8). In contrast, VAChT$^{del/del}$ mice had an increase in axonal sprouting and branching that contacted improperly arranged nAChR clusters (Fig. 8). The morphology of the NMJ from VAChT$^{del/del}$ mice was remarkably similar to that reported for ChAT-deficient mice. In fact, in hematoxylin- and eosin-stained muscles, we note that sheets of condensed parallel fusiform nuclei with abundant myofibrillar tissue could be easily discerned in VAChT$^{wt/wt}$ and VAChT$^{wt/del}$ mice (Fig. 9A and B). In contrast, myofibrilar tissue was replaced with fragmented myofibrils in VAChT$^{del/del}$ mice (Fig. 9C). In some cases, there was a complete loss of normal architecture in mutant muscles, and degenerated myofibrils were replaced with fibrotic and fatty tissue (Fig. 6C). Relative to the controls, skeletal muscles from VAChT$^{del/del}$ mice showed marked atrophy. These findings suggest that in the absence of VAChT, despite the nerve terminals having increased ACh contents, the outcome for NMJ development was as severe as the lack of ACh synthesis.

DISCUSSION

The present work addresses the role of VAChT in sustaining the release of ACh. We found that VAChT is fundamental for ACh release in the brain and the NMJ. Moreover, in the absence of the vesicular transport of ACh, there are profound effects on axons, terminal numbers, and synaptic and muscle morphology at the NMJ. Indeed, VAChT-null mice, despite presenting fivefold-more ACh than control mice, recapitulate the NMJ phenotype found in mice that cannot synthesize ACh due to a lack of ChAT. These observations bear important consequences for understanding how developing synapses function and the mechanisms by which transmitter secretion during development regulates synaptic targeting.

VAChT knockout mice do not survive postnatally. The pharmacological inhibition of VAChT causes paralysis and death compatible with an NMJ blockade (5), indicating that interference with VAChT might be lethal. Given the observations that ChAT-null mice have abnormal NMJ development (4, 35), the question arises of whether it is just the presence of ACh that is required or if the VAChT-mediated storage of ACh during development is also important. Previous experiments with munc18-1 null mice, which have no regulated secretion of a neurotransmitter, also suggested that NMJ development is reg-

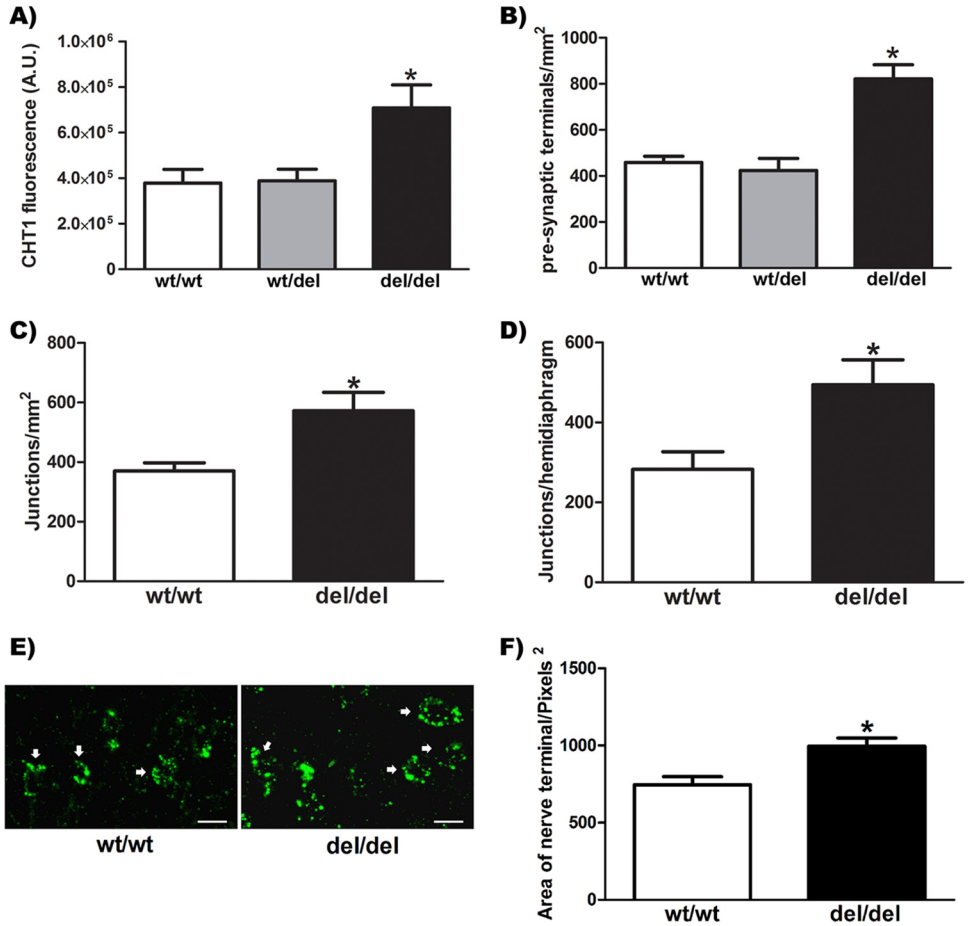


FIG. 7. Synaptic alteration in VAChT$^{del/del}$ E18.5 mice. (A) Quantification of CHT1 fluorescence (arbitrary units [A.U.]) in nerve terminals. An asterisk indicates a statistically significant difference (one-way ANOVA with Bonferroni post hoc test) [$F(2,20 = 5.632)$]. (B) Density of nerve terminals immunolabeled for CHT1 in hemidiaphragms from VAChT$^{wt/wt}$, VAChT$^{wt/del}$, and VAChT$^{del/del}$ mice. *, statistically different by ANOVA with Bonferroni post hoc test [$F(2,17) = 18.43$]. (C) Density of nerve terminals stained with FM1-43fx in hemidiaphragms of VAChT$^{wt/wt}$ and VAChT$^{del/del}$ mice (*, $P = 0.0218$ for VAChT$^{wt/wt}$ versus VAChT$^{del/del}$ mice by unpaired t test; $n = 6$). (D) Number of nerve terminals stained with FM1-43fx per hemidiaphragm (*, $P = 0.0260$ for VAChT$^{wt/wt}$ versus VAChT$^{del/del}$ mice by unpaired t test; $n = 6$). (E) Representative images of NMJs stained with FM1-43fx in hemidiaphragms of VAChT$^{wt/wt}$ and VAChT$^{del/del}$ mice (scale bar, 10 μm). (F) Average area of single nerve terminals in mouse hemidiaphragms stained with FM1-43fx (*, $P = 0.0018$ for VAChT$^{wt/wt}$ versus VAChT$^{del/del}$ mice by unpaired t test). At least 30 end plates were analyzed for each genotype.

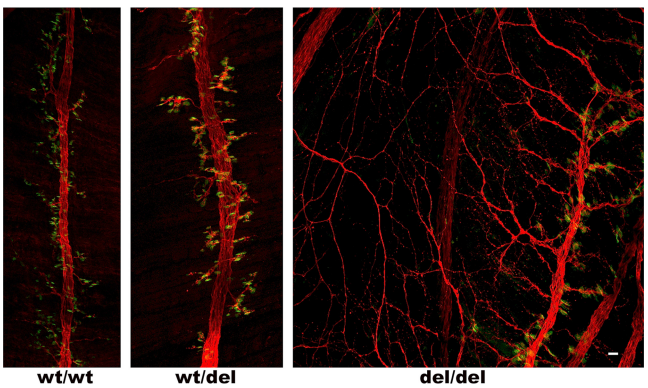


FIG. 8. Altered morphology at the NMJ of VAChT$^{del/del}$ E18.5 mice. Whole diaphragms were stained with anti-neurofilament antibody (red), and nAChRs were labeled with α-bungarotoxin (green). Confocal stacks were obtained, and maximal projections are shown in the images. The image is representative of three experiments. Note the large increase in axonal sprouting in VAChT-null mice.

ulated by synaptic vesicle exocytosis, although for these mutants, it has not been established whether ACh synthesis and storage are affected (28). A number of previously reported studies suggested that distinct pathways of ACh secretion might exist at cholinergic synapses (56, 60, 61, 64). Moreover, vesamicol-independent ACh release, presumably from synaptic vesicles, can be detected in response to pharmacological treatments (2, 7, 10, 11, 46). Hence, if VAChT-independent mechanisms of ACh release have functional significance, they might partially compensate for the lack of the transporter in at least some of its physiological roles.

Interestingly, experiments with an independent mouse line, VAChT KD^{HET} mice, that have close to a 40% reduction in VAChT expression levels showed that a moderate reduction in the level of VAChT causes no neuromuscular phenotype and only small changes in neuromuscular neurotransmission (47). Similar results were obtained with VAChT$^{wt/del}$ mice in the present report, suggesting an important safety mechanism at

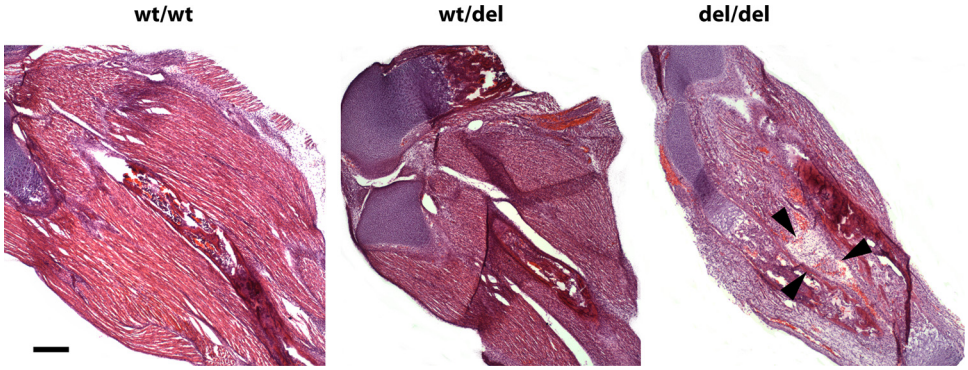


FIG. 9. Muscle morphology of E18.5 embryos from VAChT$^{wt/wt}$, VAChT$^{wt/del}$, and VAChT$^{del/del}$ genotypes. Skeletal muscles (gastrocnemius) were stained with hematoxylin and eosin. Black arrows indicate a loss of normal myofibrillar architecture. Bar, 250 μm.

the NMJ that allows decreased VAChT expression to be compensated. However, VAChT KD^{HOM} mice, with close to a 70% decrease in VAChT protein levels, do show alterations in neuromuscular neurotransmission and motor function (47). It should be noted, however, that synapses in the central nervous system are more sensitive to reductions in VAChT expression, and both VAChT KD^{HET} mice (47) and VAChT$^{wt/del}$ mice (our unpublished observations) present selective cognitive deficits in object recognition memory.

Despite this substantial compensation, the rapid postnatal death of VAChT$^{del/del}$ mice argues that the active storage of ACh by this transporter is critical at the NMJ, as the mice succumb to respiratory failure. In agreement with this conclusion, synaptosomes from VAChT$^{del/del}$ mice do not release newly synthesized ACh in response to depolarization. Lethal mutants of VAChT have also been generated in *Drosophila melanogaster*. These mutants are apparently expressed as well as wt alleles, but they have affected VAChT transport activity (31). Lethal alleles of *unc-17* in *C. elegans* have also been identified, indicating that VAChT is critical for survival in several organisms (1).

VAChT regulates cholinergic synaptic development. Whereas it is clear that ACh storage by VAChT is important for motor function, it is possible that during development, other mechanisms of ACh release, which are independent of this transporter, might become relevant. In nerve-muscle cocultures, a nonquantal release of ACh can be detected in developing growth cones (56). Moreover, compelling genetic evidence from *Drosophila* suggests that the correct axonal targeting of photoreceptors depends on ACh synthesis but not on the expression of VAChT or on synaptic vesicle exocytosis (64). Hence, at least in *Drosophila*, a VAChT-independent mechanism of secretion appears to be important during development. In the light of these findings, we examined whether neuromuscular development in mouse embryos depends on the VAChT-mediated storage of ACh.

Surprisingly, recordings from the diaphragm of VAChT$^{del/del}$ mice revealed small MEPPs, raising the possibility that they arise from small quanta of ACh. Experiments with curare confirmed that these MEPPs were due to the activation of nAChR. We tested the possibility that CHT1 can transport ACh, which might have explained the transport of ACh in vesicles lacking VAChT. A functional mutant of CHT1 retained on the cytoplasmic membrane was expressed in HEK293 cells, and the transfected cells were tested for an enhanced uptake of ACh. None was detected. Moreover, at the low internal pH of synaptic vesicles, CHT1 probably cannot transport substrates (30). We cannot completely eliminate the possibility that ATP or another vesicular constituent is released and activates nicotinic receptors, although the blockade of the small MEPP by curare indicates that ACh itself is the agent.

Early work on vesicles isolated from *Torpedo* electric organs identified a passive accumulation of ACh that could account for up to one-third of the total transport (8). More recent unpublished data indicate that isolated synapse-like microvesicles loaded with radiolabeled ACh lose their neurotransmitter by a VAChT-independent pathway (S. M. Parsons, personal communication). The experiments suggest that ACh can permeate vesicular membranes in the absence of active transport. Given that levels of intracellular ACh are increased fivefold in VAChT$^{del/del}$ mice, creating a very large gradient between the cytoplasm and the lumen of synaptic vesicles, we favor the possibility that the small MEPPs detected in VAChT$^{del/del}$ NMJs result from the passive entry of ACh into vesicles. In fact, vesicles from VAChT$^{del/del}$ NMJs can be loaded with FM1-43, confirming that "empty" vesicles undergo exocytosis-endocytosis (9, 40). However, a stimulated release of newly synthesized ACh from brain synaptosomes obtained from VAChT$^{del/del}$ mice did not occur, indicating that this putative passive transport is much less efficient and may require much more time than the active VAChT-mediated transport.

It seems unlikely that the VAChT-independent secretion of ACh, as recently detected for *Drosophila* (64), has a major role during the development of the mammalian NMJ based on our assessment of muscle morphology, axonal patterning, MN survival, and synaptogenesis in VAChT$^{del/del}$ mice. It is well established that the survival of MN, as well as proper axonal and synaptic targeting, depends on effective competition for neurotrophic support that can be modulated by muscle activity during embryogenesis. ACh has been recognized to act as a signal that induces proper axonal branching, nerve terminal size, and number and maturation of synapses. It likely generates muscle activity leading to the secretion of neurotrophic factors during embryonic development (4, 35). The programmed cell death of MNs is also regulated by ACh, and

ChAT-null mice have an increased number of MNs (4, 6, 35). These results are consistent with the well-known phenomenon of increased survival of MNs after a pharmacological blockade of muscle nAChR during development (38, 39, 42). Our observation that VAChT$^{del/del}$ mice, despite having a fivefold increase in tissue ACh levels, have alterations in neuromuscular development similar to that seen for ChAT-deficient mice strongly suggests that passive uptake by vesicles or even a leakage of ACh from nerve terminals cannot compensate for the lack of VAChT. In fact, the increase in ChAT, CHT1, and ACh contents should conspire to facilitate ACh leakage in VAChT$^{del/del}$ mice but apparently to no avail. It is curious, however, that the VAChT-independent ACh secretion described previously for *Drosophila* (64) does not operate in the mammalian NMJ. The difference between mice and flies may relate to the fact that ACh in *Drosophila* photoreceptors is not the chemical transmitter of these synapses as it is for the mammalian NMJ (64); rather, ACh in the fly photoreceptor seems to function as a developmental cue, whereas histamine is the actual neurotransmitter.

Cytoplasmic ACh in the absence of VAChT. We also find that the removal of the VAChT gene, which is embedded in the first intron of the ChAT gene, causes several neurochemical alterations in cholinergic synapses. The large increase in the ACh content is opposite from what happens in mice null for vesicular monoamine transporter 2, as they have a decrease in intracellular monoamine contents (62). *C. elegans* carrying a blocking mutation of VAChT also has an increase in ACh contents (29, 65).

The increase in the ACh content in mutant mice is likely due to increased levels of expression of ChAT and CHT1. The change in ChAT expression may be related to a compensatory mechanism due to the lack of ACh release, but it might also arise from the physical removal of a large fragment of the ChAT gene, which includes a large part of the first intron (after the R exon), the N exon, and part of the second intron. This deletion physically places the M promoter of ChAT close to the promoter for VAChT and potentially could increase the level of expression of ChAT mRNAs. However, given the fact that the CHT1 expression level is also increased, it is possible that the lack of evoked ACh release triggers signals that upregulate the ACh synthesis machinery. Moreover, the increase in the number of MNs can also contribute to the increased levels of CHT1 and ChAT in the spinal cord. Given that individual synaptic terminals at the NMJ show increased CHT1 expression levels by immunofluorescence, we favor the possibility that both increased expression levels and the increased number of neurons contribute to the higher levels of CHT1 and ChAT. It remains to be determined if cholinergic neurons in the brain present similar changes in morphology and sprouting.

Our data suggest that changes in levels of expression of ChAT and CHT1 "in vivo" can effectively increase the ACh content in cholinergic terminals. Moreover, it seems that the excess ACh in the cytoplasm can be accumulated without degradation, suggesting that "in vivo," this excessive amount of ACh does not impair ACh synthesis. These results agree with data from previously reported experiments performed in the presence of the VAChT inhibitor vesamicol, which, in the superior cervical ganglion, impairs ACh release and allows an accumulation of cytoplasmic ACh (13). These data suggest that ACh synthesis is not regulated by mass action, as previously proposed by a number of investigators (25, 57, 58), because in the absence of ACh release, the transmitter continues to accumulate.

Previous results indicated that both the exocytosis-endocytosis of synaptic vesicles and the quantal release of the neurotransmitter occur in developing axons (24, 34). Our experiments indicate that VAChT regulates a key step for physiologically relevant neurotransmission during the development of the NMJ.

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Artigo 3 Cholinergic impairment affects long-term object recognition memory retention, but not remote memory.

Nesse artigo nós estudamos a importância do tônus colinérgico para a memória declarativa. Nós estudamos particularmente a importância da liberação de ACh na consolidação da memória. Nesse trabalho fiz todos os experimentos.

Cholinergic impairment affects long-term object recognition memory retention, but not remote memory.

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Abstract.

The release of the neurotransmitter acetylcholine (ACh) depends on the vesicular loading by the vesicular acetylcholine transporter (VAChT). ACh is important for different cognitive functions such as learning, memory and attention. To address the role of acetylcholine in object recognition memory we used heterozygous VAChT KD mice (VAChT KD^{HET}), which have 40% decrease of VAChT as well as pharmacological manipulations of the cholinergic system. We provide evidence that the reduced cholinergic tone affects consolidation of object recognition long-term memory without affecting remote memory.

Key words: acetylcholine, consolidation, remote memory, atropine, memory, learning

Introduction

It is well known that memories may last days, months, or even a lifetime. In fact, the mechanisms that underlie the formation of new hippocampus dependent memories have been broadly explored (Morris 1990; Izquierdo and Medina 1997; Scoville and Milner 2000). However, the neurochemical basis underlying the persistent changes in the strength of synaptic connections necessary for memories to persist is still not totally understood.

Most studies about long-term memory (LTM) persistence in rodents are done using memory tasks that are fear-related. For this kind of memory to persist, protein synthesis and BDNF expression in the hippocampus (Bekinschtein et al. 2007), as well as the activation of ventral tegmental area/hippocampus dopaminergic connections seem necessary at specific post-learning times (Rossato et al. 2009). Interestingly, the late molecular changes, as well as memory persistence, seem to be dependent on the training protocols. Strong, but not weak, inhibitory avoidance (IA) training results in increased c-Fos expression in the dorsal hippocampus 24 h post-training (Katche et al. 2010) and memory persistence is also observed up to 14 days after training (Rossato et al. 2009). Taken together, these results indicate that fear memory persistence involves delayed molecular alterations in the hippocampus and is highly dependent on the training intensity.

On the contrary, much less is known about declarative memory persistence in rodents. Declarative memories correspond to our ability to recollect everyday events and factual knowledge and, although considered genuinely human, this type of memory can be investigated in animals based on their abilities to recognize, for example, a new object in a familiar context (Dere et al. 2005; Dere et al. 2007). There is a consensus that the hippocampus is necessary for acquisition, retention and retrieval of both explicit and declarative memories, that deal with conscious recollection of facts and episodes (review by Winters et al. 2010). Even minimal damage to medial temporal lobes (MTL) results in significant impairment to autobiographical episodic memory (Viskontas et al. 2000) and, recently, it was demonstrated that MTL subfields contribute uniquely to the formation of declarative memories that endure over time (Carr et al. 2010). Regarding studies with rodents, the consolidation of long-term novel object recognition memory (NOR) requires hippocampal mRNA translation (Myskiw et al. 2008), protein synthesis (Rossato et al. 2007) and LTP expression (Clarke et al. 2010) However, there are controversies about hippocampus

participation on NOR memory processing (Forwood et al. 2005; Dere et al. 2007). Few studies have analyzed ORM after periods longer than 48h (Dornelles et al. 2007; Stefanko et al. 2009) or the mechanisms involved in the persistence of ORM over time (Stefanko et al. 2009).

Some studies show that the basal forebrain cholinergic system is important for object recognition with cholinergic terminals in the perirhinal cortex having a major role (Warburton et al. 2003; Winters and Bussey 2005). Conversely, others failed to find an effect with electrolytic lesions in the medial septum (Ennaceur 1998) or found effects of 192-saporin only long after the lesions were induced (Paban et al. 2005), suggesting that the period of testing after the lesion is critical. In contrast, infusion of scopolamine provided evidence that cholinergic tone can have profound influence on encoding for object recognition memory (Winters et al. 2006; Winters et al. 2007).

We have recently described a novel animal model of cholinergic deficiency, the vesicular acetylcholine transporter (VAChT) knockdown mice (VAChT KD, Prado et al. 2006). Heterozygous VAChT KD mice (VAChT KD^{HET}) have 40% decrease in the VAChT, the protein responsible to take up cytosolic ACh into synaptic vesicles and thereby a key regulator of ACh availability for release (Prado et al. 2002; Ribeiro et al. 2006; Lima Rde et al. 2010). The VAChT KD^{HET} mice show temporal OR memory deficits that can be reversed by administration of acetylcholinesterase inhibitors, which increase the availability of ACh in the synaptic cleft (Prado et al. 2006; de Castro et al. 2009b). Here we used this mice to further explore the role of ACh release for OR memory. We find surprisingly that cholinergic deficits caused 24h-LTM deficiency, but did not affect memory persistence at 96 hours. These results provide new insight on the role of cholinergic neurotransmission for memory consolidation.

Materials and methods

Animals

For this work we used VAChT KD^{HET} and VAChT$^{WT/DEL}$, the later an heterozygous VAChT knockout mouse line with 50% decrease in VAChT expression described in (Prado et al. 2006) and (de Castro et al. 2009a), respectively. Reduction of 40-50% in VAChT expression causes similar decrease in ACh release in the brain (Prado et al. 2006). Animals were housed in groups of three to five per cage in a temperature-controlled room with 12:12 light-dark cycles, and food and water were provided ad libitum. Every possible effort was

made to minimize animal suffering and all procedures were conducted in accordance with NIH guidelines for the care and use of animals. All animal protocols are approved by the Institutional Animal Care and Use Committees at the Federal University of Minas Gerais..

Object recognition

The object recognition test is based on differential spontaneous exploration of novel and familiar objects (Ennaceur and Delacour 1988) The apparatus used was an open box made of PVC 50 x 35 x 25 cm (height) surmounted by a video camera and a light. Two objects made of glass or plastic were used. Their weight was such that they could not be displaced by mice or they were fixed with tape under the object. As far as we could ascertain, they had no natural significance for mice and they had never been associated with reinforcement. Initial tests showed that mice did not have any preference for the objects used.

The general procedure consisted of three different phases: a familiarization phase, a training phase and a test phase. On the 1st day, mice were individually submitted to a single familiarization session of 10 min, during which they were introduced to the empty arena. On the second day (24 h later), animals were submitted to a single 10 min training session during which two identical objects (A1 and A2) were placed in symmetrical positions from the centre of the arena and each object was 15 cm from the side walls. After a delay during which mice returned to their home-cage, they were reintroduced into the arena (test phase) and exposed to two objects, a familiar object and a novel object (A and B), placed at the same locations as during the training phase. The role (sample or new object) as well as the position of the two objects during the test session was interchanged between mice and across sessions. To control odor cues, the apparatus was cleaned with 90% ethanol and ventilated between each session and animal. If a mouse had a total exploration time of less than 10 seconds (sum of exploration of both objects), they were discarded. All session were performed during the first part of the light cycle and mice were acclimated to the room for at least 15 minutes before the beginning of each session. All the injections were performed after the training session to avoid any effect of injections during the training and the effect was observed only on consolidation.

Experiment 1: Long-term memory

To examine long term memory of mice with reduced expression of VAChT, they were tested 24h after training. In addition, WT mice were treated with atropine (1mg/kg, i.p.) or with NaCl (9%) just after training to confirm the importance of ACh in memory consolidation (see figure 1 a). We also used galantamine (1mg/kg) to reverse the deficit of memory in mutant mice.

Experiment 2: Effect of increased training sessions

Previously experiments have shown that VAChT KD^{HET} mice can perform like WT mice after numerous days of training in accelerated rotarod (de Castro et al. 2009b). To see if mutant mice are able to form memory trace after several training sessions, the mice received one training (10min) per day for four days with the same object and were tested 24h after the four training sessions (fig 2a). We also tested whether atropine injections in WT mice after each training could block memory formation.

Experiment 3: Effect of training duration

In the previous experiment, several different parameters were changed in comparison to the LTM experiment. One of these parameters was the total time that mice were exposed to the object during training, as this time was changed from 10 minutes to 40 minutes. In order to test if the duration of the training session could influence memory formation, the mice were trained during a unique 40 min training session and tested 24h after training for 10 min (fig 3 a).

Experiment 4: Effect of Object

To determine object importance in the 4 trial experiment, mice underwent one trial (the second day) and, during Day 3, they were placed in an open field for 10 min without an object and were tested on the 6^{th} day for 10 min (fig 3 c).

Experiment 5: Effect of delay between trial and test sessions

In this experiment, we tested whether the delay between training and test sessions was important for the formation of memory in mice which had reduced Ach release. Animals were trained for 10 min on the second day, returned to their home cage for the next 4 days, and were tested on the 6^{th} day. When WT mice were tested, they were also injected with atropine (1mg/kg) or with NaCl (9%) just after training (fig 4 a).

Statistics and data collection

The basic measure was the total time spent by mice exploring an object during the session. Exploration of an object was defined as follows: directing the nóse at a distance < 1 cm to the object and/or touching it with the nóse (Ennaceur and Delacour 1988). Result was expressed as mean ± SEM and we used Sigmastat 3.1 software for statistical analysis. Statistical analysis was performed using the two-way analysis of variance (ANOVA) with repeated measures and when appropriate, a Tukey post-hoc comparison test was used. If some data were not normal, we assumed the ANOVA was sufficiently robust to perform this test.

Results

VAChTKD^{HET} mice have impairment in long-term memory formation (Fig1 b). The two factors ANOVA revealed no genotype effect ($F_{(1,14)}$ = 1.038 , P = 0.326), a significant effect of object ($F_{(1,14)}$ = 25.824, P<0.001) and interaction between the two factors ($F_{(1,14)}$ = 15.262, P<0.01). WT mice explored the new object for a longer period during the second exposure (P<0.001), whereas VAChT KD^{HET} mouse explored the new object and the familiar object similarly (P = 0.420). The impairment in LTM due to decreased cholinergic neurotransmission was confirmed with an independent line of VAChT-modified mice, VAChT$^{wt/del}$ mice (fig 1 c). VAChT$^{WT/DEL}$ mice also showed a deficit of LTM and the two factors ANOVA revealed no effect of genotype ($F_{(1,18)}$ = 0.291, P = 0.596), a significant effect of object ($F_{(1,18)}$ = 34.629, P<0.001) and interaction between the genotype x object ($F_{(1,18)}$ = 25.550, P<0.001). A Tukey post-hoc test showed a difference between exploration for the two objects by WT mice (P<0.001) whereas no difference was observed for VAChT$^{WT/DEL}$ mice (P = 0.556).

To further test a role of cholinergic activity we injected WT mice with atropine (1mg/kg, i.p.) just after training. WT mice injected with atropine were not able to remember the familiar object (Fig1 d). The injection procedure did not affect general exploration, as no effect of treatment was detected ($F_{(1,12)}$ = 0.499, P = 0.493). There was a significant effect of the object ($F_{(1,12)}$ = 21.162, P<0.001) and interaction between the two factors was observed ($F_{(1,12)}$ = 8.919, P<0.05). The saline injected mice explored the new object for longer (P<0.001), whereas atropine injected mice explored the two objects similarly (P = 0.307). These results suggest that LTM can be blocked with injection of muscarinic receptor antagonist or by the general reduction of ACh release.

The importance of ACh during the consolidation for the VAChT KD^{HET} mice was confirmed by injection of galantamine just after training, which reversed the impairment in LTM (fig1 e). The two factors ANOVA showed no effect of genotype on exploration ($F_{(1,13)}$ = 1.201, P = 0.293), a significant effect of object ($F_{(1,13)}$ = 8.517, P<0.05) and interaction between object and treatment ($F_{(1,13)}$ = 5.939, P<0.05). VAChT KD^{HET} mice injected with galantamine injection explore the new object for longer period (P<0.01), while VAChT KD^{HET} mice injected with saline did not recognize familiar object (P = 0.747). We repeat the experiment with VAChT$^{WT/DEL}$ injected with saline or galantamine (Fig1 f). The two factors ANOVA revealed no effect of genotype ($F_{(1,9)}$ = 0.0583 , P = 0.815), a significant effect of object ($F_{(1,9)}$ = 9.094, P<0.05) and interaction between object x treatment ($F_{(1,9)}$ = 9.217,

P<0.05). VAChT$^{wt/del}$ mice injected with galantamine explored the new object for a longer period (P<0.001) ande VAChT$^{WT/DEL}$ mice injected with saline showed similar exploration for both objects (P = 0.889). These experiments suggest that increasing cholinergic tone with cholinesterase inhibitors reverses LTM deficit.

Re-exposition to an object in several consecutive training increases ACh release (Degroot et al. 2005). Moreover, we find that increased training improves the response of VAChT KD^{HET} mice in the rotarod, suggesting that perhaps the deficit in LTM could be reversed in mutant mice that received more training (de Castro et al. 2009b). In order to test if OR memory can be rescued by increasing the exposure of mutant mice to the object, we trained mice with the same objects for 4 consecutive days and tested them on the 5th day for OR memory (Fig2a). The results are presented in figures 2b and c and show that, after 4 training sessions, VAChT KD^{HET} mice behave the same way as WT mice and spend more time exploring the new object [The two-factors ANOVA revealed no effect of genotype ($F_{(1,19)}$ = 0.989 , P = 0.333), a significant effect of object ($F_{(1,19)}$ = 40.470, P<0.001) and no interaction between the two factors ($F_{(1,19)}$ = 1.572, P = 0.225)]. Importantly, pharmacological manipulation of the muscarinic system with atropine showed the same result. We injected atropine after each training session. Figure 2d and 2e show that atropine injection in WT mice did not disturb memory [Two way ANOVA: no effect of genotype ($F_{(1,16)}$ = 0.743 , P = 0.402), a significant effect of object ($F_{(1,16)}$ = 55.103, P<0.001) and no interaction between these two factors ($F_{(1,19)}$ = 1.445, P = 0.247)]. These results suggest that by increasing the number of training sessions, mutant mice or WT mice injected with atropine were able to form intact OR memory

It is possible that 10 min exposure is not sufficient to encode the memory trace in the absence of cholinergic input, but in 4 days, 40 min exposure to the object did. In order to test if increasing the period of learning is sufficient to reverse the deficits in LTM during cholinergic deficiency, we submitted the mice to a single but longer period of training (figure 3a). After 40min training exploring the object, VAChT KD^{HET} mice still presented a deficit in OR memory LTM (fig 3 b). In this experiment, we observed an unexpected difference in the exploration ($F_{(1,18)}$ = 5.535, P<0.05), and we could not explain this through a previous difference in exploration during the training ($t_{(18)}$ = 0.723 P = 0.479). We also observed a significant effect of object ($F_{(1,18)}$ = 39.329, P<0.001) and an interaction between the two factors ($F_{(1,18)}$ = 32.988, P<0.001). The post-hoc Tukey showed an increase in exploration of the novel object compared to the familiar object in WT mice (P<0.001), whereas the VAChT

KD^{HET} mice exhibited similar exploration for both objects (P = 0.714), which suggested that the time the mice were exposed to the object did not influence memory trace encoding.

To test the importance of reintroduction to the box (context) for memory encoding in VAChT KD^{HET} after 4 training sessions, we introduced the mice to the box for 3 consecutive days after the initial training. The box was empty so the mice were not re-exposed to the object (fig 3c). Surprisingly, mutant mice did not need to be re-exposed to the object to encode the memory trace and remember the familiar object (fig 3d). The two way ANOVA yielded no effect of genotype ($F_{(1,18)} = 0.732$, P = 0.403), a significant effect of object ($F_{(1,18)} = 49.001$, P<0.001) and no interaction between genotype and object ($F_{(1,18)} = 0.313$, P = 0.583).

The experiments above suggest that cholinergic deficits impair LTM at 24 hours; however memory was preserved at 96 hours if mice were re-exposed to the context. In order to text the possibility that mice with cholinergic deficiency did not present OR memory at 24 h, but did at 96 h we tested the mice with a delay between the training and the test session (fig4a). When the interval between the trial and the test was extended to 4 days instead of 24h, VAChT KD^{HET} mice showed intact OR memory (fig 4b). In fact, the two factors ANOVA revealed no effect of genotype ($F_{(1,23)} = 2.184$, P = 0.153), a significant effect of object ($F_{(1,23)} = 25.837$, P<0.001) and no interaction between these two factors ($F_{(1,23)} = 1.261$, P = 0.273). This indicates that both, mutant and WT mice, present the same behavior and explore the unfamiliar object longer. To further test the involvement of the cholinergic system, we repeated this experiment in WT mice that received atropine or saline injection after the training. Confirming the data obtained in VAChT KD^{HET} mice, we did not find a memory deficit in atropine-injected mice when compared to saline injected mice (Fig 4c) [Two factors ANOVA revealed no effect of genotype ($F_{(1,13)} = 0.812$, P = 0.384), a significant effect of object ($F_{(1,13)} = 37.923$, P<0.001) and no interaction object x drug interaction($F_{(1,13)} = 0.983$, P = 0.339)]. These results suggest that increasing the delay between training and the test was sufficient to detect normal memory in mice with cholinergic disturbance.

Discussion

We use here both, pharmacological approaches and genetic-modified mice with different protocols for object recognition memory, to demonstrate that intact cholinergic function plays a role for the consolidation of LTM, but not for remote memory.

Increasing the strength of the training, by exposing mice to the objects for 40 min, did not allow the VAChT KD^{HET} mice to form LTM memory. This is surprising because the strength of the training has been shown to be important for memory formation. In the inhibitory avoidance test, the persistence of memory depends on the strength of training; in fact, a strong foot sock induced persistent LTM lasting over 14 days (Rossato et al. 2009). Additionally, the exploration of objects promotes an increase in ACh release in the hippocampus (Degroot et al. 2005). Therefore, a strong training and a longer time exploring the object may increase the ACh release and promote LTM. A longer training, however, was not sufficient to reverse the LTM deficit observes in VAChT KD^{HET} mice, perhaps because synaptic vesicles filling by VAChT could not cope with the demand.

The role of ACh for OR LTM has been demonstrated in several studies (Ennaceur and Meliani 1992; Bartolini et al. 1996; Warburton et al. 2003; Winters and Bussey 2005; Rutten et al. 2006; de Castro et al. 2009b). Using post-training injections, we provide support for an important contribution of ACh for LTM consolidation. Experiments with galantamine treatment rescue the deficit of LTM in two independent strains of VAChT-mutant mice, suggesting that this deficit is linked to decreased release of ACh during the post-training period. The effect of this cholinesterase inhibitor seem to be important during the consolidation period, as in previous experiments we have shown that injection 30 min prior to the test session does not rescue OR LTM deficits in VAChT KD^{HET} mice (de Castro et al. 2009b). This result is also in agreement with recent data showing that administration of scopolamine just after the training session blocks memory 24h after training (Barbosa et al. 2010). These results, however, are different from those described by Warburton and collaborators, as the authors did not observe an effect of scopolamine injection (0.05 mg/kg, i.p) when rats were injected just after the training (Warburton et al. 2003). This may be due to the fact that they used a low dose of scopolamine (0.05mg/kg), since the doses used in other experiments were between 0.1 and 1 mg/kg (more detail in Dere et al. 2007).

Remote memory has been characterized as memory trace that becomes independent of hippocampus and likely involves cortical activation (Bontempi et al. 1999; Teng and Squire 1999). According to the systemic consolidation theory, the hippocampus stores experiences

for a short period of time before the information is transferred to the cortex for durable storage (reviewed in Wiltgen et al. 2004; Frankland and Bontempi 2005). Recent experiments have suggested that the hippocampus is important for object recognition memory consolidation (Balderas et al. 2008; Broadbent et al. 2010; Clarke et al. 2010) and ACh influences LTM in the hippocampus (Izquierdo et al. 1992). Interestingly, the time necessary for a memory to become independent of the hippocampus is highly dependent on the type of test used. In contextual fear memory, for example, 14 to 28 days are necessary while for socially-acquired food preference tasks, the delay is 10 days (Frankland and Bontempi 2005). According with the object recognition memory trace model elaborated by Romero-Granados and collaborator, the cortical system consolidation occurred during the 48h after the training and they observed that after 9 days the memory is distributed trough cortical area (Romero-Granados et al. 2010). If the system consolidation is similar in our experiments, thus, the memory observed 4 days after the training may be considered as a remote memory. The activation of the hippocampus for LTM as well as the implication of the cortical region for remote memory are both well established (For reviewWiltgen et al. 2004; Frankland and Bontempi 2005). Therefore it seems that encoding of remote memory is not a continuum with LTM, and mice may develop a remote memory without expressing LTM. Our data indicate that ACh seems to be dispensable for remote memory formation in this task, whereas it is essential for consolidation of LTM.

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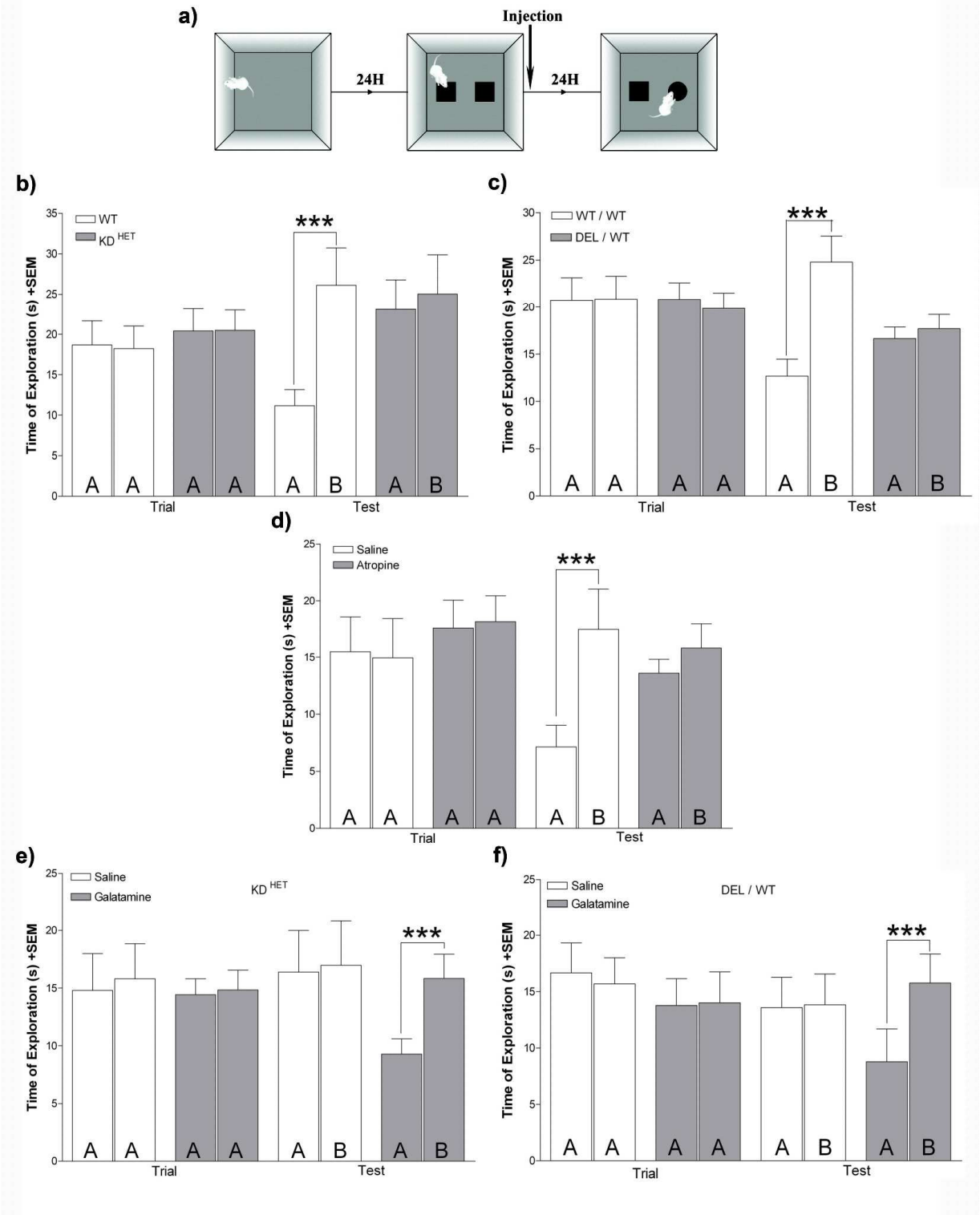

Figure 1 **Pharmacological and genetic down-regulation of cholinergic neurotransmission affect consolidation of OR LTM**

a) Scheme of the different sessions for investigation of LTM. b) Time of exploration for the object during the training and the test session. VAChT KD^{HET} mice present a deficit in long term memory. c) Object recognition LTM for VAChT$^{WT/DEL}$ mice. d) Object recognition LTM

test in WT mice injected with atropine (1mg/kg, i.p.) just after training. e) Injection of galantamine (1mg/kg, i.p.) after the training in VAChT KD^{HET} reverse LTM deficit. f) Injection of galantamine (1mg/kg, i.p.) after training in $VAChT^{WT/DEL}$. All the Results are expressed as mean + SEM of total exploration time. The letters A and B represent, respectively, the familiar and new object. *** represent a significant difference between the new object (A) and the familiar object (B) $p<0.001$ with a two way repeated measure ANOVA.

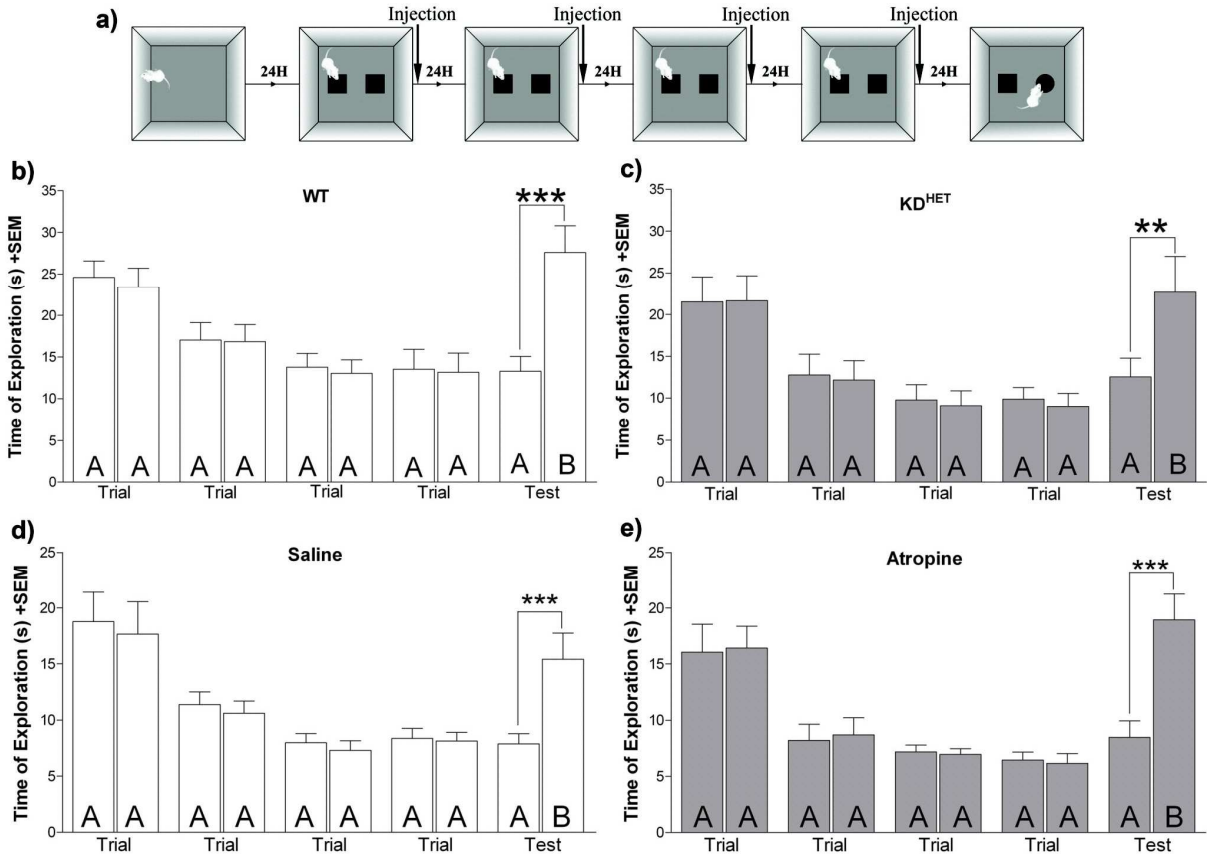

Figure 2 **Object recognition memory after 4 periods of training**

a) Scheme of the experiment. b) Exploration time for WT mice. c) Exploration time for VAChT KD^{HET} mice d) Exploration time for WT mice injected with saline e) Exploration time for WT mice injected with atropine. Results are expressed as mean + SEM of total exploration time. The letters A and B represent the object. ** represent a significant difference between the new object (A) and the familiar (B) $p<0.01$ and *** $p<0.001$ with a two way repeated measure ANOVA.

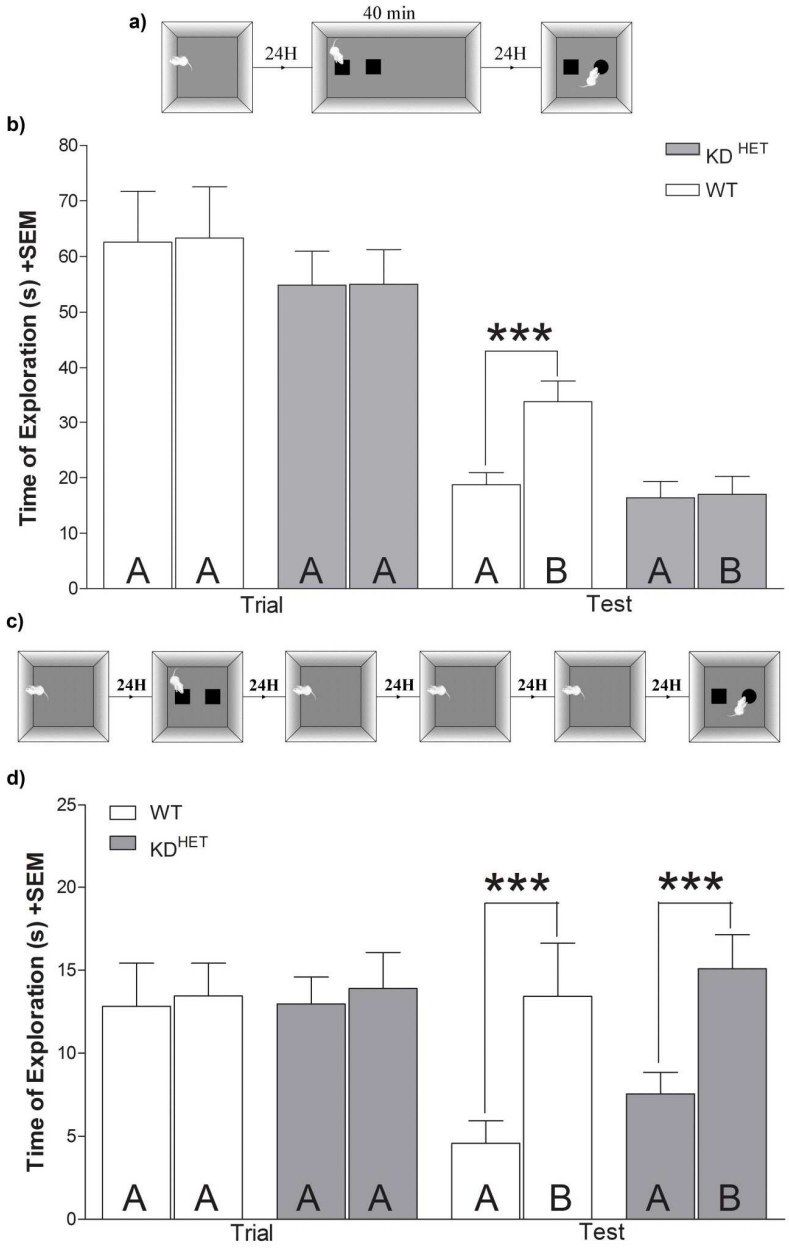

Figure 3. **Effect of the strength of training and reintroduction to the open field.** a) Scheme of the protocol used for the 40 minute training. b) Exploration time for WT and VAChT KD^{HET} mice that were trained during 40 min and tested 24h after. c) Scheme of the protocol used for testing the effect of the object during the 4 trial test. d). Exploration time for WT and VAChT KD^{HET} mice trained as described in c. All the Results are expressed as mean + SEM of total exploration time. The letters A and B represent the object. *** represent a significant difference between the new object (A) and the familiar (B) $p<0.001$ with a two way repeated measure ANOVA.

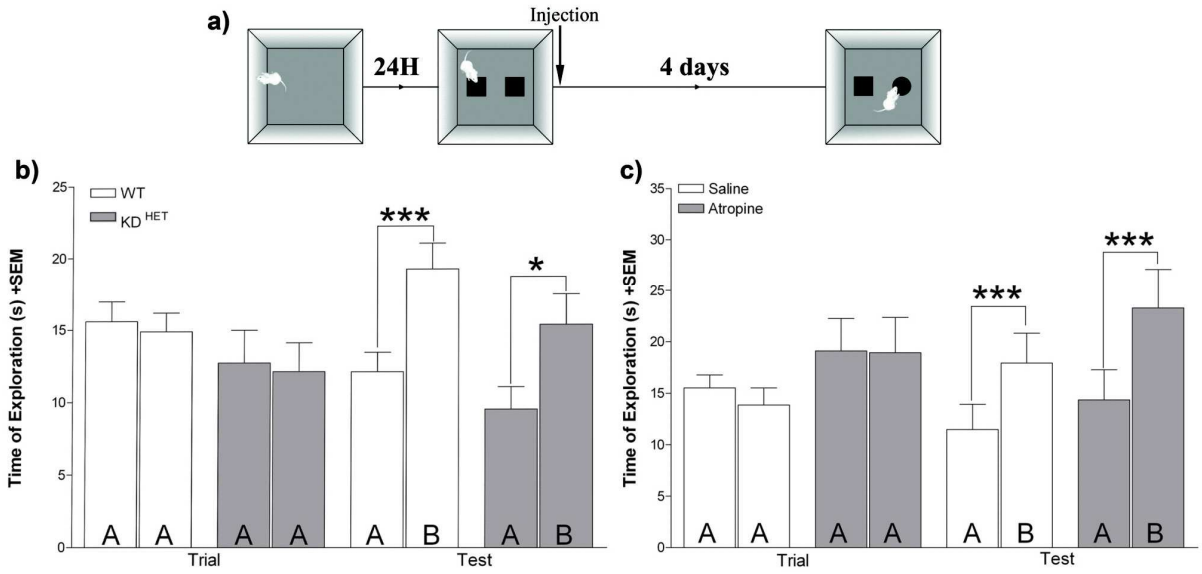

Figure 4 **Object recognition LTM at 96 h.**

a) Scheme for the protocol to test memory after 4 days. b) Exploration time for WT and VAChT KD^{HET} tested 4 days after training. c) Exploration time for WT mice injected with atropine or saline after the training and tested 4 days later. The letters A and B represent respectively the familiar and new object. * $p<0.05$ and ** $p<0.01$ represent a significant difference between objects with a two way repeated measure ANOVA.

Artigo 4 Novel strains of Mice Deficient for the Vesicular Acetylcholine Transporter: insights on transcriptional regulation and control of locomotor behaviour.

Nesse trabalho testamos o efeito de uma deleção de VAChT sobre a locomoção. Realizei os testes neuromusculares de grip-force, wire-hang e cat walk dos animais adultos $VAChT^{FloxNeo/FloxNeo}$ e $VAChT^{FloxNeo/Del}$ para a figura 6. E fui responsável pelos estudos da atividade locomotora e do teste da cruz elevada (Fig 8 e 9). Eu testei os $VAChT^{Flox/Flox}$ nós mesmo testes para ver se a remoção da cassete neo poderia restaurar uma atividade normal nesse animal (Fig 11).

Novel strains of Mice Deficient for the Vesicular Acetylcholine Transporter: insights on transcriptional regulation and control of locomotor behaviour.

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Abstract

Acetylcholine (ACh) is thought to play numerous roles in the CNS, including the control of locomotor activity. However, defining the actual contribution of brain ACh to specific behaviours has been challenging, mainly because of the difficulty in generating suitable animal models of cholinergic dysfunction. The vesicular acetylcholine transporter (VAChT) provides the last cholinergic specific step for acetylcholine release and its gene is embedded within the first intron of the choline acetyltransferase (ChAT) gene, forming the cholinergic gene locus. The importance of acetylcholine for breathing and the exquisite genomic organization of the VAChT gene make it a challenge for genetic manipulation. We have approached this problem by interfering with no-coding regions of the VAChT gene to generate knockdown mouse lines to probe for cholinergic function. We used homologous recombination to generate a VAChT allele that was flanked by loxP sequences, but still kept the Tk-Neo resistance cassette, which was inserted approximately 1.5 kb downstream from the VAChT stop codon, in a ChAT intronic region (FloxNeo allele). We show that mice with the FloxNeo allele show differential VAChT and ChAT expression in distinct neuronal populations. These mice present relatively intact VAChT expression in the somatomotor subset of cholinergic neurons, but have pronounced decrease in VAChT expression in other cholinergic neurons in the brain. As a consequence, the new mutant mice present preserved neuromuscular function, but altered brain cholinergic activity. We show that these new mutants are hyperactive when exposed to the open-field, a result consistently observed with pharmacological and genetic alteration of cholinergic receptors. Genetic removal of the Neomycin resistance cassette rescues VAChT expression and the hyperactivity phenotype. These results suggest that release of ACh is normally required to “turn down” neuronal circuits controlling locomotion in the brain.

Key words: Acetylcholine, hyperactivity, transcriptional regulation, VAChT-mutant mice.

Introduction

Acetylcholine (ACh) is the major peripheral neurotransmitter controlling the parasympathetic and the sympathetic autonomic nervous system as well as the somatic motor system. Moreover, the cholinergic system is thought to play key roles in many functions in the CNS, including the control of locomotor activity, emotional behavior, and higher cognitive processes such as learning and memory (Everitt and Robbins 1997;Wess *et al.* 2007;Taly *et al.* 2009).Changes in cholinergic neurotransmission are associated with a variety of important neurological disorders including Alzheimer's disease, schizophrenia, Parkinson's disease, epilepsy and attention-deficit hyperactivity disorder (Scarr 2009).

ACh changes cellular activity of target cells through metabotropic muscarinic receptors (Wess *et al.* 2007;Wess *et al.* 2003) and ionotropic nicotinic receptors (Grutter *et al.* 2005;Taly *et al.* 2009). The brain expresses five different types of muscarinic receptors (M1-M5). The nicotinic receptors, which are formed by five identical or homologous subunits, are generated from twelve different subunits (nine α-subunits and three β-subunits) (Taly *et al.* 2009). The various pentameric nAChR subunit combinations have different pharmacological and kinetic properties, and are widely distributed in the brain. Similar complexity is observed for the different G-coupled muscarinic receptors. Because of this complexity, defining the actual contribution of brain ACh to specific behaviours has been challenging.

There have been several attempts to generate animal models of cholinergic dysfunction by elimination of cholinergic neurons using electrolytic or excitotoxic methods, as well by the more selective strategy of cholinergic immunolesion (Everitt and Robbins 1997). Although these studies have provided important information regarding the cholinergic system, they also have raised a number of inconsistent results concerning behavioural processes that are affected by altering cholinergic transmission (Everitt and Robbins 1997). The fact that in many cases these techniques may not consistently eliminate cholinergic neurons could explain some of the differences. In addition, other signalling molecules, such as neuropeptides, growth factors and co-transmitters, can be co-released by cholinergic neurons, further confounding the interpretation of neuronal degeneration-induced cholinergic deficiency. Furthermore, neuronal death causes inflammation which can also complicate interpretation of the experiments. Therefore it is important to develop alternative, more consistent and targeted approaches to complement these previous studies to investigate specific roles of ACh in brain functions.

Using genetics to generate mouse models of cholinergic deficiency is equally challenging. ChAT KO mice do not survive post-labour and adult heterozygous ChAT KO

mice have compensations in choline uptake and show no behavioural phenotype (Misgeld *et al.* 2002;Misgeld *et al.* 2002). In order to attempt to generate models of cholinergic deficiency, we have recently generated novel mouse lines by targeting the vesicular acetylcholine transporter (VAChT knockdown - VAChT KD and VAChT knockout - VAChT $^{del/del}$). VAChT is essential for ACh release as mice null for VAChT expression do not survice (de Castro *et al.* 2009a). In contrast, reduction of VAChT expression by 40% (VAChT KD^{HET}) and 70% (VAChT KD^{HOM}) allows survival (Prado *et al.* 2006). Analysis of ACh release in VAChT$^{del/del}$ and VAChT KD mice indicate that decreased expression of VAChT perturbs storage of ACh in vesicles. During stimulation, impaired ACh storage becomes more pronounced leading to significant decrease in ACh release, particularly in mice with 70% reduction of VAChT (Prado *et al.* 2006;Lima *et al.* 2010). VAChT KD^{HOM} mice are myasthenic and present social and object recognition memory deficits (Prado *et al.* 2006) and cardiac dysfunction (Lara *et al.* 2010), indicating that perturbation of ACh storage affects several physiological functions (Prado *et al.* 2006;Guidine *et al.* 2008;de Castro *et al.* 2009a;de Castro *et al.* 2009b;Lara *et al.* 2010). All these phenotypes can be rescued by inhibition of cholinesterase, indicating that they are the result of decreased ACh release due to the exocytosis of partially-filled synaptic vesicles and are not the result of developmental changes (Prado *et al.* 2006;Lima *et al.* 2010;Lara *et al.* 2010).

The VAChT gene locus is complex. The entire VAChT open reading frame is encoded by one single exon that is contained inside the first intron of the ChAT gene (Eiden 1998). Control of expression of VAChT and ChAT is poorly understood, and distinct cholinergic neurons show different requirements for regulatory regions within the cholinergic gene locus (Naciff *et al.* 1999;Misawa *et al.* 2003;Schutz *et al.* 2000;Schutz *et al.* 2003). In order to further investigate the roles of the cholinergic system we developed novel strains of VAChT targeted-mice. We have changed the initial strategy used to generate VAChT KD mice. Instead of adding the Tk-Neo resistance cassette to the 5'-untranslated region of the VAChT gene, we added the cassette approximately 1.5 kb downstream from the VAChT stop codon, in a ChAT intronic region. We show that interrupting the intron between ChAT exons N and M with a Tk-Neo cassette keeps VAChT expression in the somatomotor subset of cholinergic neurons relatively intact, but causes pronounced decrease in VAChT expression in other groups of cholinergic neurons in the CNS. As a consequence, these mice present preserved neuromuscular function, but altered brain cholinergic activity. We show that these new mutant mice are hyperactive when exposed to a new environment, a result consistently observed with pharmacological and genetic alteration of cholinergic receptors (Molinengo *et*

al. 1989;Shannon and Peters 1990;Ukai *et al.* 1994;Gerber *et al.* 2001;Miyakawa *et al.* 2001;Gomeza *et al.* 2001;Granon *et al.* 2003;Granon and Changeux 2006). Genetic removal of the Neomycin resistance cassette rescues VAChT expression and the hyperactivity phenotype. These results suggest that release of ACh is normally required to "turn down" neuronal circuits controlling locomotion.

Materials and Methods

Generation of VAChT mutant mice. Construction of the gene-targeting vector was described previously (de Castro *et al.* 2009a). In short, one LoxP sequence was added 260 bp upstream from the VAChT translational initiation codon, and a second LoxP was added approximately 1.5 kb downstream from the stop codon. The Neomicin-resistance gene (TK-Neo cassette) was added immediately after the second LoxP and was followed by a third LoxP (Figure 1). The linearized targeting vector was electroporated into J1 embryonic stem cells derived from 129/terSv mice, and selected embryonic stem cell clones harbouring homologous recombination (determined by PCR and Southern blotting [not shown]) were injected into C57BL/6J blastocysts to produce chimeric mice. Germ line transmission was achieved, and mice were bred to C57BL/6J mice to produce heterozygous mutant mice ($VAChT^{WT/FloxNeo}$). Heterozygous mice were intercrossed to generate the homozygous ($VAChT^{FloxNeo/FloxNeo}$) and wild-type controls ($VAChT^{WT/WT}$) used in these experiments. $VAChT^{FloxNeo/del}$ and $VAChT^{WT/del}$ mice were generated by intercrossing $VAChT^{WT/FloxNeo}$ to heterozygous VAChT KO mice ($VAChT^{wt/del}$;(de Castro *et al.* 2009a). Only male mice were used in this study. Animals were housed in groups of three to five per cage in a temperature-controlled room with a 12:12 light-dark cycles in microisolator cages. Food and water were provided ad libitum. Mouse colonies were maintained at the University of Western Ontario, Canada, in accordance with Canadian Council of Animal Care (CCAC) guidelines for the care and use of animals. All animal procedures were approved by the University of Western Ontario Institutional Animal Care and Use Committee.

Genotyping, Southern blotting. Genotyping by PCR was performed using tail DNA as a template. The set of three primers used were P1 (5-GAGAGTACTTTGCCTGGGAG GA -3), P2 (5- GGCCACAGTAAGACCTCCCTTG -3), P3 (5- GCAAAGCTGCTATTGGC CGCTG -3) and P4 (5-TCATAGCCCCAAGTGGAGGG AGA-3). For Southern blot analysis, genomic DNA was digested with the enzymes *Bam*HI and *Sac*I. Digested DNA was subjected to electrophoresis in a 1.5% agarose gel and transferred onto a nylon membrane.

After UV cross-linking, DNA on the membrane was hybridized to the NdeI/PmeI VAChT DNA fragment (see Fig. 1 for the position of the probe fragment). Detection was done using the Alkphos direct labelling and detection system kit (GE Healthcare) according to the manufacturer's instructions.

qPCR. For real-time quantitative PCR (qPCR), total RNA was extracted using the Aurum Total RNA for fatty and fibrous tissue kit from Biorad. Quantification and quality analysis of RNA in the extracted samples was done by microfluidic analysis (Agilent Technologies' Bioanalyzer). First-strand cDNA was synthesized using the iSCRIPT cDNA SYNTHESIS KIT from Biorad. cDNA was subsequently subjected to qPCR on a CFX-96 Real Time System (Biorad) using the iQ SYBR GREEN SUPERMIX (Biorad). For each experiment, a non-template reaction was

used as a negative control. In addition, the absence of DNA contaminants was assessed in reverse transcription-negative samples and by melting-curve analysis. Relative quantification of gene expression was done with the $\Delta\Delta CT$ method using β-actin gene expression to normalize the data.

Western blotting. Immunoblot analysis was carried out as described previously (de Castro *et al.* 2009a). Antibodies used were anti-VAChT (rabbit polyclonal 1:100, Synaptic System, Germany), anti-CHT1 (rabbit polyclonal 1:500, kindly provided by R. Jane Rylett, University of Western Ontario, London, Canada), anti-CHAT (rabbit polyclonal 1:1000, Chemicon) and anti-actin (Chemicon, CA). Images were acquired using the FluorChem Q System from Alpha Innotech and analysed using the AlphaVie software.

Immunofluorescence analysis of brain slices were performed as described previously (de Castro *et al.* 2009a). Images were acquired using an Axiovert 200M using the ApoTome system or a LEICA SP5 confocal microscope as previously described (de Castro *et al.* 2009b).

Tissue ACh measurements. Brains were dissected rapidly, homogenized in 5% TCA, and centrifuged (10,000 X *g* for 10 min) at 4°C. Supernatants were frozen at -80°C until use. For ACh determinations, TCA was removed with ether, and a chemiluminescent assay was done with choline oxidase as described previously (44). The data are presented as means and standard errors of the means (SEM). One-way analysis of variance (ANOVA), followed by Bonferroni's test, was used to analyze the differences in tissue ACh concentrations in $VAChT^{FloxNeo/del}$, $VAChT^{FloxNeo\ /FloxNeo}$,$VAChT^{WT/del}$ and wild-type controls ($VAChT^{WT/WT}$); a $P < 0.05$ was considered to be statistically significant.

Electrophysiology. Recordings were performed on hemidiaphragm. Animals were sacrificed and the diaphragm with attached rib bone was rapidly dissected and placed into Tyrode's solution containing (in mM) NaCl (124), KCl (5), $NaHCO_3$ (26), NaH_2PO_4 (1.2), $MgCl_2$ (1.3), $CaCl_2$ (2.4), glucose (10). This solution was gassed with a mixture of 5%CO_2/95%O_2 and in this condition had a pH of 7.4. The diaphragm was bisected, and one half was transferred into a custom recording chamber in which the muscle was held in place with metal pins that passed through the surrounding tissue and were inserted into a Sylguard bed. During recording, the muscle was continuously perfused with gassed Tyrode solution containing 0.0003 mM tetrodotoxin to avoid spontaneous action potentials. Borosilicate (WPI) microelectrodes were fabricated on a Narashige PN-30 puller, and had resistances of 5-15 MOhm when filled with 3 M KCl. Fine branches of the motor nerve were visually identified under 100X magnification and the muscle fiber was impaled using a fine micromanipulator (WPI). Membrane potential and synaptic potentials were amplified 10X with an Axon Instruments Axoclamp 2A, and membrane potential was monitored throughout the experiment. To digitalize the miniature endplate potentials (MEPPs), the signal was high-pass filtered at 0.1 Hz to subtract the resting potential and amplified a further 200-1000X using an Ectron 750 amplifier. This signal was fed to a Lab Master A-D conversion board controlled by Strathclyde Electrophysiology Software (University of Strathclyde, Glasgow, Scotland). To measure quantal size, a software event detector was used to record 25 ms of data on either side of the MEPP. The threshold of the event detector was set just below the peak of the noise so as not to miss any small MEPPs. Under these conditions, approximately 15% of detected "MEPPs" were false positives and were manually detected and removed. To measure MEPP frequency, membrane potential was recorded without selection, and MEPPs were manually identified and counted.

Grip force and wire-hang.

Mice were brought to the testing room and allowed to acclimatize for 10 minutes before initiating tests. A Grip Strength Meter from Columbus Instruments (Columbus, OH) was used to measure forelimb grip strength as an indicator of neuromuscular function as described previously (Prado *et al.* 2006;de Castro *et al.* 2009a). Briefly, the grip strength meter was positioned horizontally and mice were held by the tail and lowered toward the apparatus. Mice were allowed to grasp the smooth, metal, triangular pull bar (forelimbs only) and were then pulled backward in the horizontal plane. The force applied to the bar at the

moment the grasp was released was recorded as the peak tension (kg). The test was repeated 10 consecutive times within the same session and the highest value from the 10 trials was recorded as the grip strength for that animal. Mice were not trained prior to testing and each mouse was tested once (10 trials equal one test session).

For wire-hanging experiments the laterals of a cage top were covered with tape to prevent the mice to reach the borders (Sango *et al.* 1996) . The mouse was gently put on the cage top, which was then briefly shaken to induce the mouse to grasp the wire in the top. The cage top was then inverted and suspended approximately 40cm above an empty cage. Time spent hanging upside down was determined with a cut-off time of 60 sec.

Gait analysis. Mice were subjected to gait assessment (Neumann *et al.* 2009) using a CatWalk automated gait analysis system (Noldus Information Technology). The apparatus is made of a 1.3m long glass plate with dim fluorescent light beamed into the glass from the side. The reflexion of the paw in contact to the glass was recorded by a video camera. Mice were placed in the walkway and allowed free exploration for 1 min before recording the first run. A minimum of 3 correct runs (the mouse cross the walkway with no interruption or hesitation) for each mouse was recorded. Runs were analysed using the Noldus software and only the runs where it was possible to discern all steps were used for the analysis. We only used the mean stride length of hind paw as data, stride length is the distance between two successive prints of the same paw.

Locomotor activity and habituation. Locomotor activity was automatically recorded (Accuscam Instrument, Inc. Columbus, OH). The open field arena was a 20 cm×20 cm platform surrounded by 30 cm high walls. Mice were acclimated to the testing room for 20 minutes prior to beginning the test, and had not experienced a cage change for at least 24 hours. Mice were placed in the center of the apparatus and allowed to freely explore the arena for 5 min. Horizontal locomotion and rearings were recorded and used as measures of locomotion and exploration, respectively (Vianna *et al.* 2001). Locomotor activity was measured at 5 min intervals and cumulative counts (120 min) were taken for data analysis as described elsewhere (Gainetdinov *et al.* 2003). For the intersession habituation, mice were exposed for 120 min to the same open field during 3 consecutive days. Measurements of total activity were obtained and one-way ANOVA and Tukey's Multiple Comparison Test was used to test for statistical significance. Activity was measured by the Versamax software.

Elevated Plus-maze. Animals were placed in the center of the elevated plus maze (Med Associate Inc.) and activity was recorded for five minutes with a webcam connected to a computer. Total amount of time spent in the open and in the open sections of the maze was calculated with the Anymaze software; an animal was considered to be completely within a section of the maze when its center of gravity was in this section. The result was expressed as the percentage of time spent in the open arm.

Statistical Analysis. Data were statistically analyzed by a two-tailed Student's *t* test or by two-way or repeated measure ANOVA. If data were not normal, we used the adequate non-parametric test. The specific statistical analyses used are noted in the text and legends.

Results

Generation of VAChT-deficient mice.

We generated a new VAChT targeted mouse line by inserting a lox-P flanked TK-neo cassette in the 3' region of the VAChT gene, in the intron between exons N and M of the ChAT gene, and a third lox-P sequence 260 bp upstream from the VAChT translational initiation codon (Figure 1). Successful recombination of the mutated VAChT allele was confirmed by Southern-blot and PCR analyses (Fig. 1C and 1D).

We initially characterized this novel mouse line by evaluating VAChT expression expecting that the TK-neo cassette would not alter VAChT gene expression. However, we found that $VAChT^{FloxNeo/FloxNeo}$ mice showed a large decrease in VAChT expression in the striatum (76% decrease in VAChT mRNA- Figure 2A), but VAChT expression in the spinal cord was decreased only by 46% (Figure 2B). We have found in previous experiments that decreased expression of VAChT in up to 50% in the spinal cord does not alter neuromuscular function (Prado *et al.* 2006;de Castro *et al.* 2009a). Because of the distinct levels of expression of VAChT in the brain compared to the spinal cord with the $VAChT^{FloxNeo}$ allele, we decided to investigate further this allele owing to the possibility to knock-down VAChT expression in the brain, but preserve peripheral cholinergic function. To examine this possibility, we crossed $VAChT^{FloxNeo/FloxNeo}$ mice to heterozygous VAChT-null mice in order to generated $VAChT^{FloxNeo/del}$ mice anticipating that this novel mouse line might present even more significant knockdown of VAChT in the brain, but relatively preserved peripheral function. Genotyping of these mice was obtained by PCR (Fig. 1E).

We examined VAChT expression in $VAChT^{FloxNeo/del}$ mice compared to $VAChT^{wt/wt}$ mice. The levels of mRNA for VAChT were decreased in the striatum of $VAChT^{FloxNeo/del}$

mice even further (89% decrease - Fig. 2C). Similarly to VAChT$^{FloxNeo/FloxNeo}$ mice, VAChT expression in the spinal cord of VAChT$^{FloxNeo/del}$ mice was relatively preserved (57% decrease, Figure 2D). Confirming results obtained previously, levels of mRNA for VAChT$^{wt/del}$ mice decreased 50% when compared to VAChT$^{wt/wt}$ mice (Figure 2C and (de Castro *et al.* 2009a). We also examined other components of cholinergic nerve terminals that can impact cholinergic tone. Of significant interest both VAChT$^{FloxNeo/del}$ and VAChT$^{wt/del}$ presented an increase in ChAT mRNA expression in the spinal cord, but not in the striatum (Fig. 2C and 2D) and this was compatible with previous findings for the del allele (de Castro *et al.* 2009a). This increase in ChAT expression is likely related to the removal of the VAChT gene with a decrease in the distance between two ChAT promoters (see Fig. 1). In contrast, ChAT mRNA expression was not changed in VAChT$^{FloxNeo/FloxNeo}$ mice (Fig, 2A and 2B). Also, CHT1 and AChase mRNA expression were not changed in any of the VAChT mutants in either the striatum or spinal cord (Fig 2A-D). In order to investigate the expression of VAChT in distinct brain regions we used immunofluorescence. In brain sections VAChT expression was drastically reduced in the striatum, cortex and hippocampus of VAChT$^{FloxNeo/del}$ and VAChT$^{FloxNeo/FloxNeo}$ mice compared to VAChT$^{wt/del}$ or VAChT$^{wt/wt}$ mice (Fig. 3 and 4). In contrast CHT1 labeling was preserved. We also examined expression of VAChT in the facial motor nuclei (Fig. 4). Staining in the cell bodies was similar in all genotypes, although both VAChT$^{FloxNeo/del}$ and VAChT$^{FloxNeo/FloxNeo}$ mice had a decreased labelling in the punctuated fluorescence for nerve terminals that contact these neurons. To further investigate if indeed VAChT expression was reduced in the brain, we used striatum tissues. Western-blot analysis showed a decrease of 75 to 85% in the expression of VAChT in the striatum of VAChT mutants (supplementary Fig. 1).

To explore VAChT expression in the periphery we stained VAChT in the NMJ of diaphragms. In contrast to the decreased VAChT expression in distinct brain regions, we found negligible differences for VAChT expression in nerve-endings at the diaphragm of VAChT$^{FloxNeo/del}$ and VAChT$^{FloxNeo/FloxNeo}$ mice when compared to the two control genotypes (Fig. 5A). Furthermore, analysis of nicotinic ACh receptor labelling using fluorescent bungarotoxin suggested normal nAChR distribution (Fig. 5A).

To test if neuromuscular transmission was preserved in VAChT$^{FloxNeo/del}$ and VAChT$^{FloxNeo/FloxNeo}$ mice we recorded from the NMJ of the diaphragm. Both the amplitude and the frequency of miniature end-plate potentials (MEPPs) were increased for VAChT$^{FloxNeo/del}$,VAChT$^{FloxNeo/FloxNeo}$ and VAChT$^{wt/del}$ mice when compared to VAChT$^{wt/wt}$ (Fig. 5B and C). These results are compatible with our previous observations that close to

50% reduction of VAChT at neuromuscular junctions affects quantal release of ACh only mildly (Prado *et al.* 2006). These results suggest that quantal release in the two mutant mice with decreased expression of VAChT in the brain was well preserved, if not improved at the NMJ.

To examine whether VAChT$^{FloxNeo/del}$ and VAChT$^{FloxNeo/FloxNeo}$ mice had preserved muscular function we performed a series of neuromuscular tests. These mutant mice showed no difference in grip-force and wire-hang tasks suggesting preserved neuromuscular function (Fig. 6A-D). Moreover, because previous observations showed that VAChT KD^{HOM} mice had gait problems (Prado *et al.* 2006), we also tested if VAChT$^{FloxNeo/del}$,VAChT$^{FloxNeo/FloxNeo}$ and VAChT$^{wt/del}$ mice presented any gait abnormality. In agreement with the lack of neuromuscular phenotype, we found no gait deficiency in these mutant mice (Fig. 6 E-F). These results indicate that VAChT$^{FloxNeo/del}$ and VAChT$^{FloxNeo/FloxNeo}$ mice do not present the neuromuscular phenotype of VAChT KD^{HOM}.

We have previously demonstrated that decreased VAChT expression leads to proportional increase in the amount of total ACh in the brains of mutant mice (Prado *et al.* 2006;de Castro *et al.* 2009a). Because we did not detect any alteration in either ChAT or CHT1 in the striatum, we measured the amount of ACh in the brains of mutant mice as an indirect assessment of ACh output. We determined the ACh content in the striatum of VAChT$^{FloxNeo/del}$ mice (the line with largest decrease in VAChT expression) and VAChT$^{wt/del}$ mice. VAChT$^{FloxNeo/del}$ mice presented several-fold more ACh in the striatum than wild-type control mice, whereas the increase in VAChT$^{wt/del}$ mice with around two-fold (Fig. 7). These data show a gene-dosage effect in these VAChT mutant mice and corroborate the mRNA and protein findings that the decrease in VAChT expression is more accentuated in VAChT$^{FloxNeo/del}$ when compared to VAChT$^{WT/del}$ mice.

VAChT$^{FloxNeo/FloxNeo}$ and VAChT$^{FloxNeo/del}$ mice are hyperactive

To start to assess the consequences of decreased VAChT expression for central functions we examined locomotor activity in the open field, which has been shown to be altered by antagonists of muscarinic receptors as well as by genetic elimination of some nicotinic and muscarinic receptors (Gerber *et al.* 2001;Miyakawa *et al.* 2001;Molinengo *et al.* 1989;Shannon and Peters 1990;Bymaster *et al.* 1993;Ukai *et al.* 1994;Granon *et al.* 2003;Granon and Changeux 2006;Maubourguet *et al.* 2008). Figure 8 indicates that VAChT$^{FloxNeo/del}$ mice showed increased locomotion throughout the 2 hour monitoring period when compared to wild-type controls (Kruskal-Wallis test show difference between the

genotypes ($H_{(3)}$ =31.680, p<0.001) . The average total distance traveled by $VAChT^{FloxNeo/del}$ mice in 2 h was 2.1-fold higher than that of WT controls. An intermediate increase (1.4-fold) in locomotor activity was observed in $VAChT^{FloxNeo/FloxNeol}$ mice when compared to wild-type controls (Fig 8A and B). Activity levels of $VAChT^{wt/del}$ mice showed a tendency to increase however it did not meet statistical significance. These results suggest that decreased VAChT expression to the levels found in $VAChT^{FloxNeo/del}$ mice causes abnormal motor activity and that this effect is gene-dosage dependent. In addition, vertical exploration in the open field was increased in $VAChT^{FloxNeo/del}$ mice as shown by the number of rearings (Figure 8C and D; Kruskal-Wallis test; ($H_{(3)}$ =13.764, p<0.05; post-hoc Dunn reveal a significant higher rearing number of $VAChT^{FloxNeo/del}$ compared to WT controls).

Lack of habituation does not seem to be the cause of the hyperactivity as all three mutants showed decreased motor activity across the 2-hour test session (Figure 8A). $VAChT^{FloxNeo/FloxNeo}$ and $VAChT^{FloxNeo/del}$ mice were retested after 24h and 48h under the same conditions and both genotypes in the second and third days showed significant decrease in locomotor activity [*between-sessions habituation* in the open field; Figure 9A, two-way repeated measures ANOVA- main effect of genotype,$F_{(2, 86)} = 15.825$, $p < 0.001$, day $F_{(2, 86)} = 35.318$, $p < 0.001$ and interaction genotype x day $F_{(4, 86)} = 2.505$, $p < 0.05$], further suggesting no impairment in habituation in the novel environment. It is important to note that even after the third day, both $VAChT^{FloxNeo/FloxNeo}$ and $VAChT^{FloxNeo/del}$ mice remained hyperactive when compared to WT (Tukey test respectively P<0.01 and P<0.001).

We also tested for change in anxiety level. The time spent in the center vs. the periphery of the open field was evaluated in the same open field trials used to quantify locomotor movement. As shown in Figure 9B $VAChT^{FloxNeo/del}$ mice spent significant more time in the center of the open field apparatus (Kruskal-Wallis test, $H_{(3)}$ =11.537, p<0.05; post-hoc Dunn's method, $p < 0.05$) which could be an indication of reduced anxiety (Crawley 1999). However, when we assessed the willingness of VAChT mutant mice to explore a novel unprotected environment (open arms) of the elevated plus maze, the time spent in the open arms (Fig. 9C; One Way Analysis of Variance $F_{(2,58)}$ = 1,603, NS), and the number of entries in the open arms in the elevated plus maze test (Fig. 9D; One Way Analysis of Variance $F_{(2,58)}$ = 1,845, NS) were not significantly affected in $VAChT^{FloxNeo/del}$ mice. These data indicate that $VAChT^{FloxNeo/del}$ mice do not show consistent changes in anxiety-related behaviors.

Genetic rescue of VAChT-mutant mice hyperactive behavior

If decreased VAChT expression causes hyperactivity, it would be expected that correcting VAChT levels should allow for rescue of this phenotype. The VAChT^{FloxNeo} allele carries a TK-Neo cassette 3' from the ORF of VAChT and this is likely the cause of decreased VAChT expression (Fig.1). Cre excision of loxP flanked DNA sequences is a stochastic event (Rajewsky *et al.* 1996), we therefore crossed VAChT$^{FloxNeo/wt}$ mice to distinct Cre mice (see Methods) to obtain an allele in which the TK-Neo cassette was deleted (Fig. 1B; VAChT^{Flox} allele). We screened the offspring from this cross by PCR to identify founder mice carrying only the floxed VAChT gene, with removal of the TK-Neo cassette. VAChT floxed founders (VAChT^{Flox}) were crossed to C57BL/6J mice to confirm germ-line transmission and the progeny obtained were intercrossed to obtain VAChT$^{Flox/Flox}$ mice and WT controls (PCR in Fig. 1F).

We investigated VAChT expression at the mRNA and protein levels and found that VAChT$^{Flox/Flox}$ mice have essentially the same level of expression for this transporter as VAChT$^{wt/wt}$ mice in the striatum, cortex, spinal cord and hippocampus (Fig. 10). Moreover, ChAT and CHT1 expression were not changed in VAChT$^{flox/flox}$ mice (Fig. 10). Accordingly VAChT$^{flox/flox}$ mice showed no deficits in neuromuscular function in the grip-force (Fig. 11A) or wire-hang (not shown). Measures of anxiety in the elevated plus maze were identical to measures of WT controls (Fig. 11B and C). When we tested VAChT$^{Flox/Flox}$ mice in the open-field we also found that locomotor activity was identical to that of VAChT$^{wt/wt}$ mice and no habituation deficits were observed (Fig. 11D-F). These results strongly suggest the recovery of VAChT expression by removal of the TK-Neo cassette rescued the hyperactivity phenotype of VAChT^{FloxNeo}.

Discussion

The VAChT^{FloxNeo} allele shows differential regulation of VAChT expression.

The present experiments explore some of the remarkable features of the cholinergic gene locus to target VAChT and generate mice with decreased cholinergic function. We show that interference with the VAChT-ChAT locus, by insertion of a TK-Neo cassette, in the intron between exons N and M, differently affected the expression of VAChT in the brain and the spinal cord. Owing to the relative preservation of cholinergic function in the spinal cord and NMJ, we were able to show that one of the consequences of reduction of VAChT expression in the forebrain, and consequent reduction of ACh release, is hyperactivity. This

phenotype shows a gene-dose effect with lesser expression of VAChT causing a more pronounced hyperactivity.

VAChT has a unique genomic organization; its open reading frame is encoded within the first intron of the ChAT gene. This arrangement, frequently called the cholinergic gene locus (CGL) (Eiden 1998), is conserved in nematode (Alfonso *et al.* 1994a;Alfonso *et al.* 1994b), Drosophila (Kitamoto *et al.* 1998) and mammals (Erickson *et al.* 1994).Transcriptional control of the CGL is rather complex as multiple promoters and alternative splicing is used to generate different mRNA species from both VAChT and ChAT genes (Cervini *et al.* 1995). Transgenic mice containing different DNA segments of the CGL locus fused to reporter genes have been used to identify regulatory regions that are important for the expression of VAChT and ChAT *in vivo* (Kitamoto *et al.* 1995;Kitamoto *et al.* 1998;Naciff *et al.* 1999;Schutz *et al.* 2000;Schutz *et al.* 2003;Salvaterra *et al.* 1993;Yasuyama *et al.* 1995). These studies indicate that multiple regulatory elements are necessary to control expression in the CGL and suggest that regulation of the CGL is different in different types of cholinergic neurons. Moreover, this regulatory strategy seems to be conserved in insects and vertebrates (Lee and Salvaterra 2002;Schutz *et al.* 2003). A core promoter containing regulatory elements necessary to activate the CGL in cholinergic cells and to repress its activity in non-neuronal cells is present in the sequence spanning approximately 4 kb upstream of the R exon (Lonnerberg *et al.* 1995;Lonnerberg *et al.* 1996). Other regulatory elements have been described in the genomic region between exon-M and the first ChAT coding exon (Schutz *et al.* 2003), however the complete set of regulatory sequences controlling the CGL remains to be determined. A cholinergic group-specific transcriptional activator has been identified in *Drosophila*. Mutant flies that lack expression of the transcription factor abnormal chemosensory jump6 (acj6) showed decreased ChAT in primary olfactory neurons, whereas expression in mechanósensory neurons was unaffected (Lee and Salvaterra 2002).

Our results give further support to the subset-specific regulation of the CGL. Because the Tk-Neo cassette used to generate the $VAChT^{FloxNeo}$ allele was introduced 450bp upstream from the beginning of M-exon, it is reasonable to suggest that its presence interfered with the function of additional regulatory elements. As sensorymotor cholinergic neurons rely mainly on the core promoter, VAChT expression in these neurons may be relatively preserved while all the other groups of cholinergic neurons in the brain had pronounced decrease in VAChT expression. Interestingly, ChAT expression was not altered in the $VAChT^{FloxNeo}$ allele. This might suggest that VAChT and ChAT rely on different regulatory elements. In contrast, mice

that present the VAChT^{del} allele showed an increased expression of ChAT in the spinal cord but not in the striatum. These results agree with our previous experiments showing increased ChAT expression in the spinal cord of VAChT^{del} mice (de Castro *et al.* 2009a); see also Fig 2 and 3). This occurs likely due to the proximity of the M-promoter of ChAT to VAChT promoters after excision of the intervening DNA sequences flanked by loxP (see Fig. 1). Our experiments suggest that whereas ChAT expression may be regulated by elements that were modified by the del allele in the spinal cord the missing genomic fragment does not seem to be necessary for regulation of ChAT expression in the striatum (Fig 2C and D). Overall, our experiments examining VAChT and ChAT expression point to differential gene regulation between the striatum, and likely other brain forebrain regions, and the spinal cord. The significance for this differential regulation for normal cholinergic physiology is poorly understood, but likely play important roles to maintain the expression of critical genes for cholinergic function between these distinct sets of neurons. The fact that VAChT expression was rescued by the removal of the Tk-Neo cassette shows unequivocally that the two loxP sequences that flank the VAChT gene do not alter transcription.

VAChT$^{FloxNeo/FloxNeo}$ and VAChT$^{FloxNeo/del}$ mice are hyperactive.

As decreased VAChT expression leads to proportional decrease in ACh release in the brain (Prado *et al.* 2006;de Castro *et al.* 2009a) the availability of mutant mouse lines displaying different levels of VAChT expression (VAChT$^{WT/del}$ mice: 50% decrease; VAChT$^{FloxNeo/FloxNeo}$ mice: 75% decrease; VAChT$^{FloxNeo/del}$ mice: 85% decrease) provided us with unique tools to evaluate the consequences of reduced VAChT levels for brain functions. Also, understanding the consequences of decreased VAChT expression is made easier now by the new mouse lines that do not show confounding peripheral phenotypes.

Because ACh is known to play a major role in the regulation of locomotor control (Di *et al.* 1994), we used these mutants to investigate the role of VAChT in locomotor activity. We found that up to 50% decrease in VAChT expression in the brain does not change locomotor activity in mice, similar to previous experiments with VAChT KD^{HET} mice and VAChT$^{wt/del}$ mice. However, our data show clearly thata more pronounced decrease in VAChT expression causes a gene-dose dependent hyperactivity in a new environment. These results suggest that release of ACh is normally required to regulate neuronal circuits controlling locomotion.

Injection of muscarinic antagonists in distinct brain regions cause pronounced augmentation in locomotor activity levels (Molinengo *et al.* 1989;Shannon and Peters

1990;Ukai *et al.* 1994) and that a hyperactivity phenotype was observed in mouse strains lacking M1 and M4 muscarinic receptors (Gerber *et al.* 2001;Miyakawa *et al.* 2001;Gomeza *et al.* 2001) as well as mouse strains null for the nicotinic β2 receptor (Granon *et al.* 2003;Granon and Changeux 2006;Maubourguet *et al.* 2008). Paradoxically, systemic injections of nicotinic agonists can cause increase in locomotor activity (Reavill and Stolerman 1990;Museo and Wise 1990b;Museo and Wise 1990a;Panagis *et al.* 1996). However, this effect should be considered with caution, as the hyperactivity most probably results from desensitization of specific types of nicotinic receptors due to prolonged activation. Therefore, ACh may regulate locomotor circuitry in multiple and redundant ways. Our data provide additional support to the notion that insults that cause cholinergic presynaptic deficiency can also increase activity.

Locomotor hyperactivity is a symptom present in many disorders including Attention Deficit Hyperactivity Disorder (ADHD), schizophrenia, Alzheimer's diseases and some forms of autism (Scarr 2009). Interestingly, all these disorders have in common some degree of cholinergic deficit. VAChT$^{FloxNeo/del}$ and VAChT$^{FloxNeo/FloxNeo}$ mice are novel complementary models to understand the specific consequences of decreased cholinergic activity in the brain and should be useful to further investigate the role of ACh in distinct brain functions. Importantly, as mice VAChT$^{Flox/Flox}$ mice have preserved VAChT expression and do not show any phenotype, we will be able to generate mice with total suppression of ACh release in specific brain regions. These conditional mutants will be valuable to investigate the role of specific groups of cholinergic neurons in brain function.

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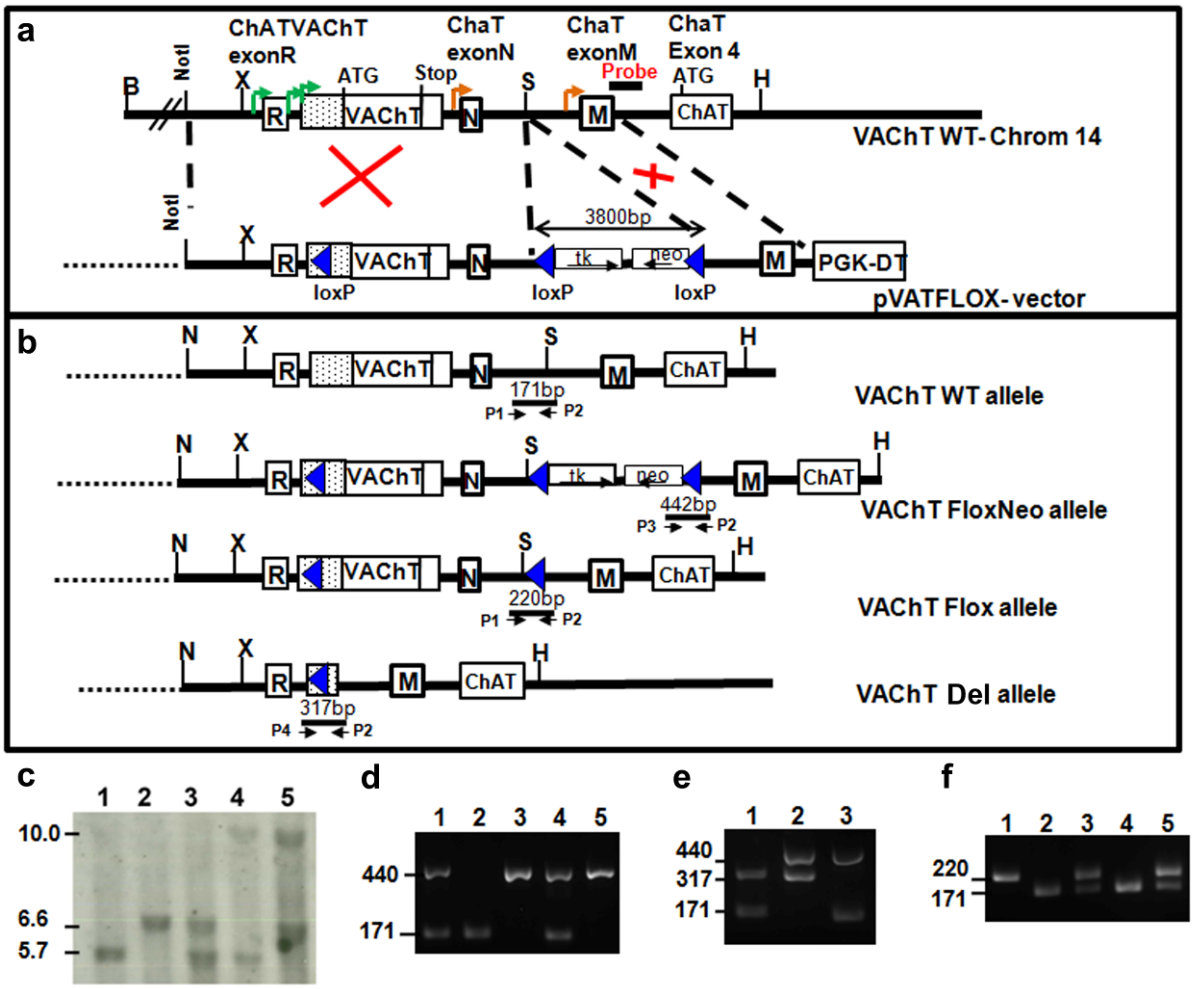


Figure 1: Schematic Drawing of the Cholinergic Gene Locus and Generation of VAChT Deficient Mice. a) Boxes represent the different exons of ChAT or VAChT. The position of the initiation codon (ATG) for VAChT and ChAT and the stop codon (Stop) of VAChT are indicated. Potential transcription initiation sites are indicated for VAChT (green arrowheads) and ChAT (orange arrowheads). Note that the VAChT gene is within the first intron of ChAT. b) Different VAChT alleles generated. P1, P2, P3 and P4 indicate the primers used for PCR genotyping and the fragment sizes generated. LoxP sequence, some restriction enzymatic sites and probe annealing are represented. c) Southern blot analysis of WT (lane 1), $VAChT^{FloxNeo/FloxNeo}$(lane 2), $VAChT^{WT/FloxNeo}$ (lane 3), $VAChT^{WT/Del}$(lane 4) and $VAChT^{FloxNeo/Del}$(lane 5). d) PCR analysis of $VAChT^{WT/FloxNeo}$ (lanes 1 and 4), $VAChT^{WT/WT}$ (lane 2), and $VAChT^{FloxNeo/FloxNeo}$ (lanes 3 and 5). e) PCR analysis of $VAChT^{WT/del}$ mice (lane 1), $VAChT^{FloxNeo/Del}$ (lane 2), and $VAChT^{WT/FloxNeo}$ mice (lane 3). f) PCR analysis of $VAChT^{Flox/Flox}$ mice (lane1), $VAChT^{WTWT}$ mice (lane2 and 4), and $VAChT^{WT/Flox}$ (lane 3 and 5).

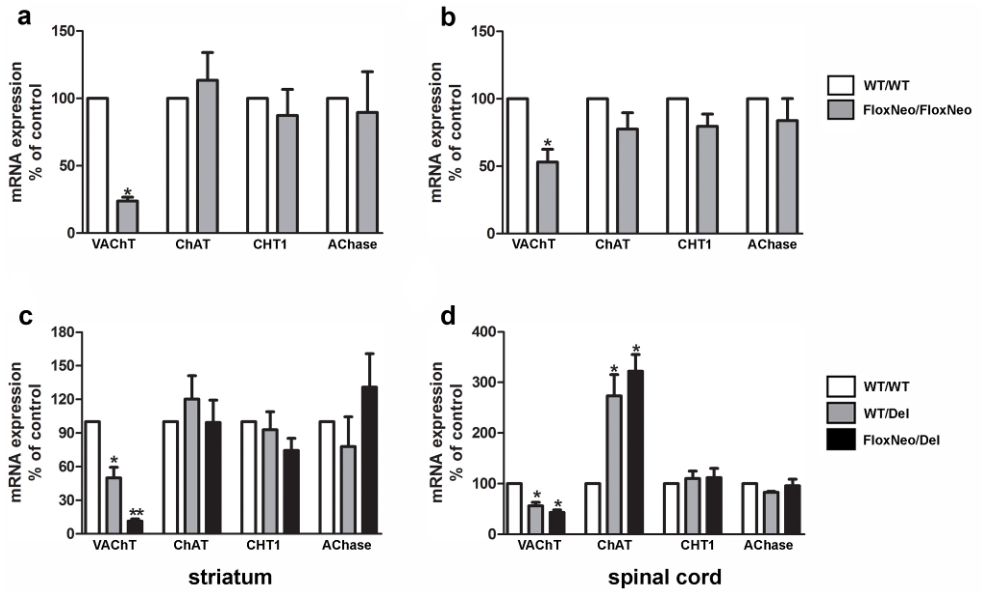


Figure 2: **VAChT mRNA expression is changed in VAChT mutant mice**. a) VAChT, ChAT, CHT1 and AChase mRNA levels in striatum and b) spinal cord of WT and $VAChT^{FloxNeo/FloxNeo}$ mice. c) VAChT, ChAT, CHT1 and AChase mRNA levels in striatum and d) spinal cord of $VAChT^{WT/WT}$, $VAChT^{FloxNeo/Del}$ and $VAChT^{WT/Del}$ mice. mRNA expression levels were quantified by qPCR using actin to normalize the data. Graphs represent average of 4-6 different mice. (*) and (**) indicate $p<0.01$ and $p<0.001$ respectively.

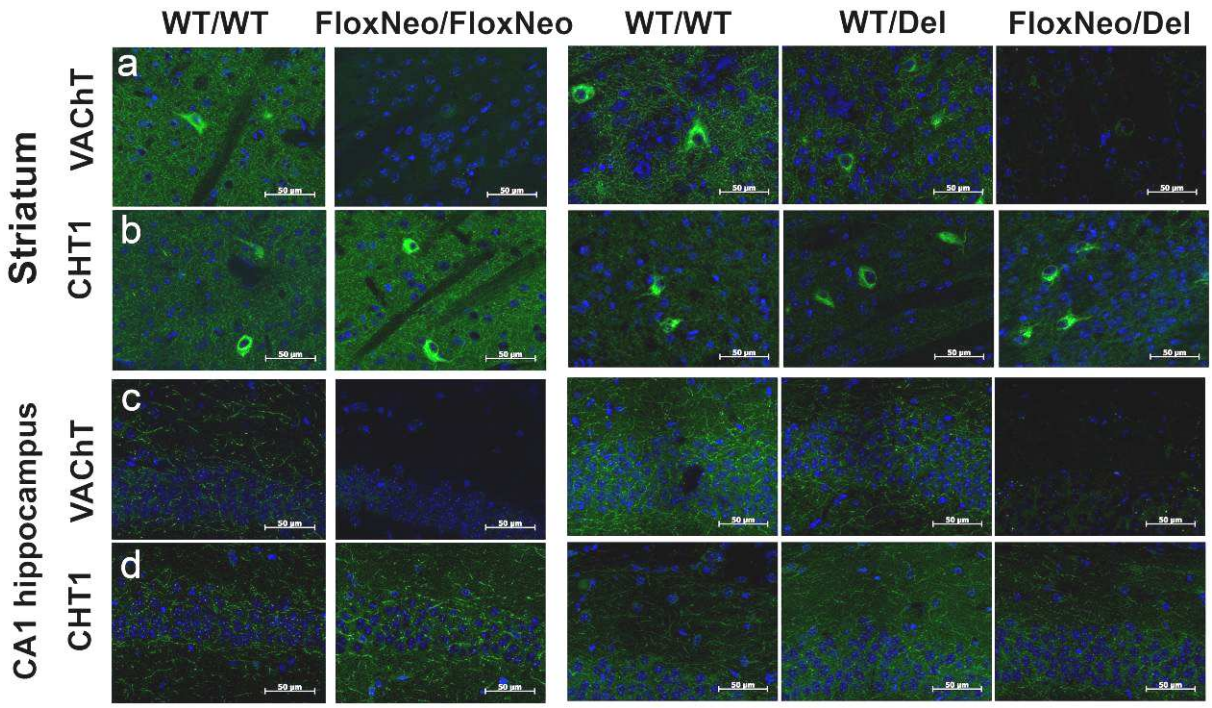


Figure 3: **VAChT immunorreactivity is altered in VAChT mutant mice**. a) Representative optical sections from striatum stained with a VAChT antibody (green) or b) stained with CHT1 antibody (green). c) Representative optical sections from hippocampus stained with a VAChT antibody or d) CHT1 antibody (green). Dapi labelling (blue) was used to stain nuclei. Scale bar 50 µm.

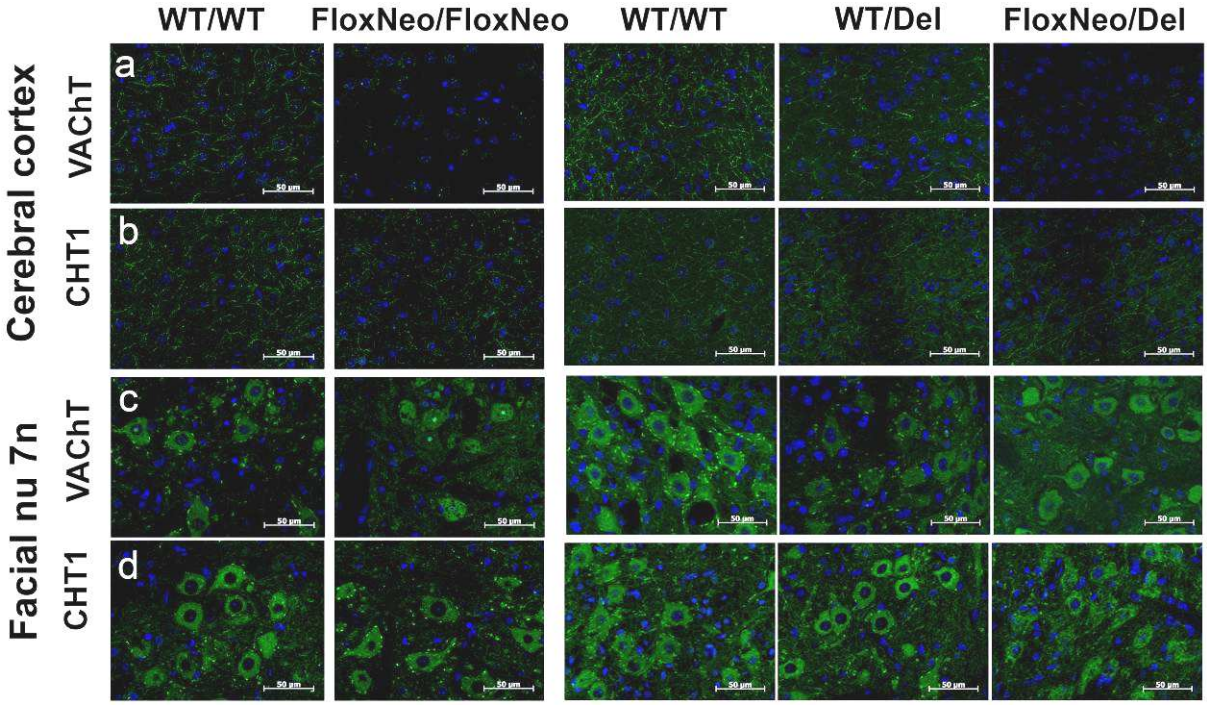


Figure 4: **VAChT immunorreactivity is altered in VAChT mutant mice**. a) Representative optical sections from cortex stained with a VAChT antibody (green) or b) CHT1 antibody (green). c) Representative optical sections from facial motor nuclei stained with a VAChT antibody or d) CHT1 antibody (green). Dapi labelling (blue) was used to stain nuclei. Scale bar 50 µm.

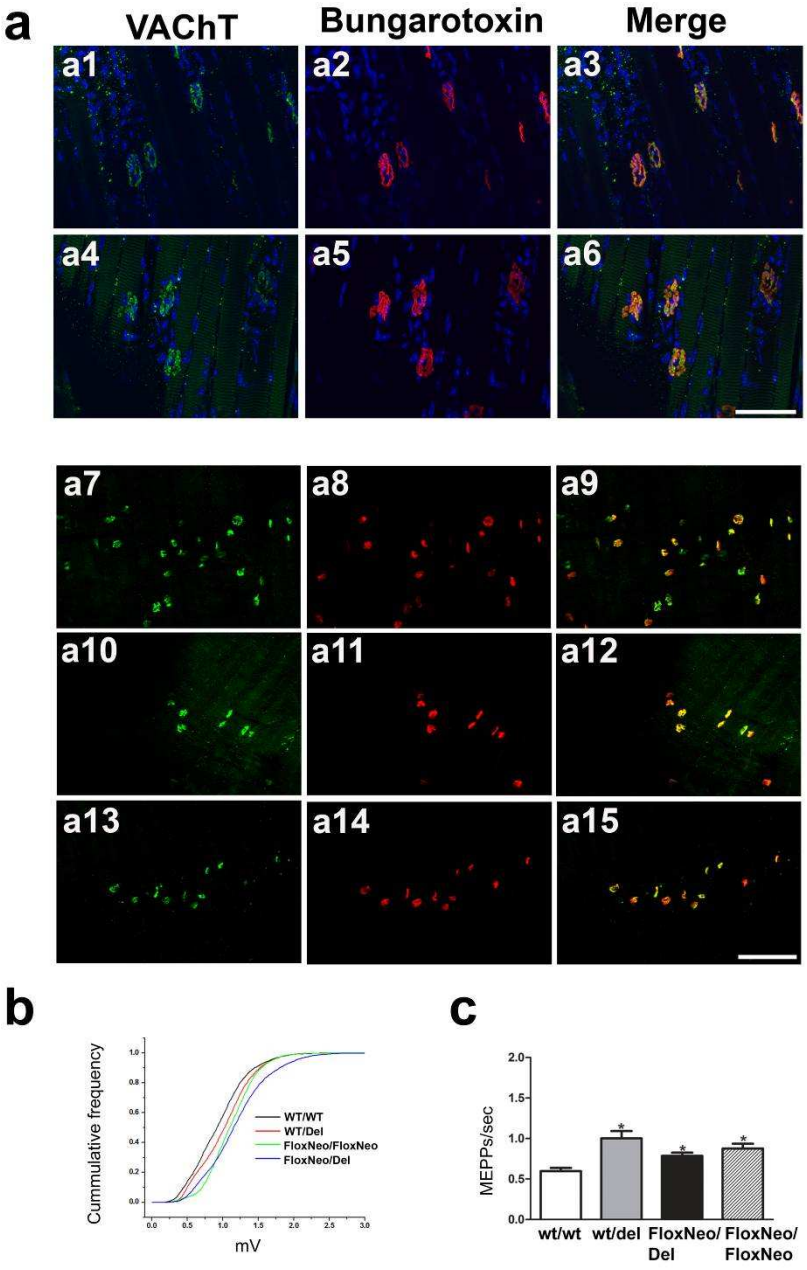


Figure 5: NMJ morphology and transmission in VAChT mutant mice. a) Diaphragms were immunolabelled with VAChT antibody (green) and α-bungarotoxin (red) to label nicotinic receptors. Right columns show overlay pictures. Dapi (blue) was used to stain nuclei. Images are representative of 3 independent experiments. WT control mice (a.1-3), VAChT$^{FloxNeo/FloxNeo}$ mice (a.4-6), WT control mice (a.7-9), VAChT$^{WT/Del}$ mice (a.10-12), and VAChT$^{FloxNeo/Del}$ mice (a.13-15). No alterations were observed between the genotypes. Scale bar 50µm. b) Quantal size of the four genotypes quantified by plotting the cumulative frequency of MEPP amplitudes. WT control (black line), VAChT$^{WT/Del}$ mice (red line), VAChT$^{FloxNeo/FloxNeo}$ mice (blue line) and VAChT$^{FloxNeo/Del}$ mice (green line). c) Frequency of MEPPs at synapses for the four genotypes. (*) indicates statistically significant difference from control wild-type mice (two-way ANOVA followed by Bonferroni post hoc; $F(2,14) = 21,98$, $p < 0.005$).

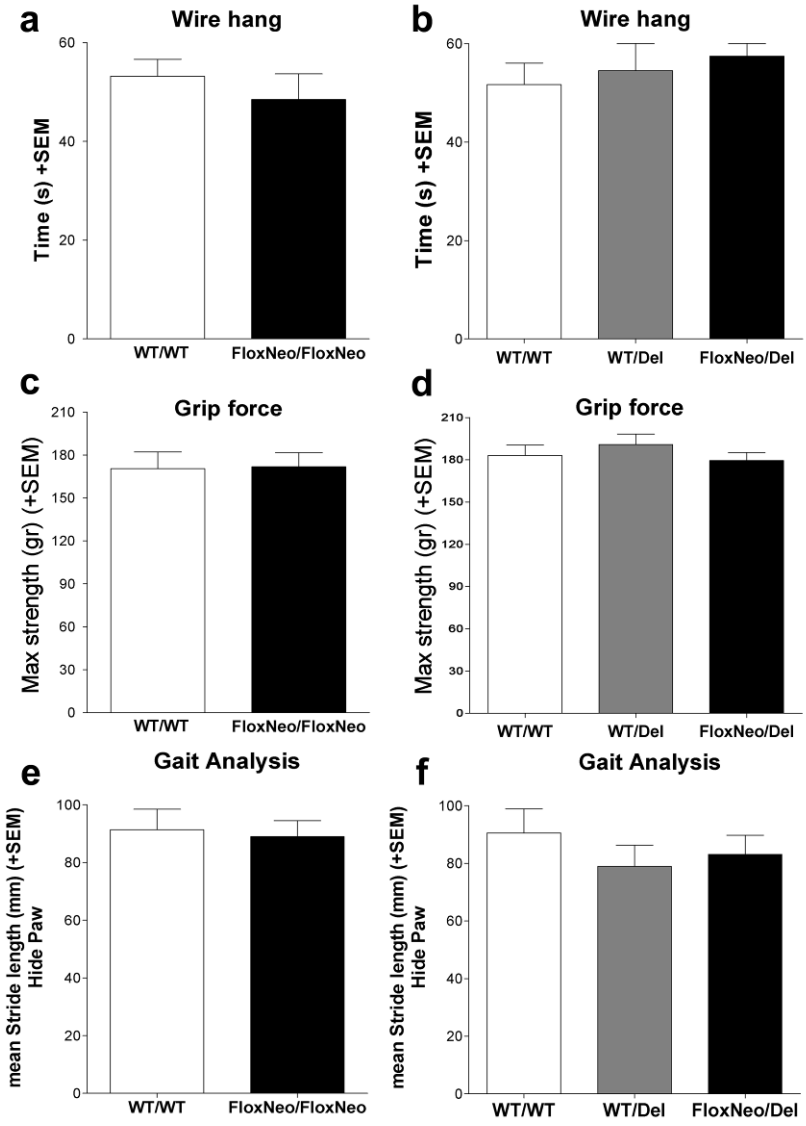


Figure 6: Neuromuscular function in VAChT mutant mice. a) Time spent hanging upside down from a wire netting for WT and VAChT$^{FloxNeo/FloxNeo}$ mice. No significant difference was observed [$T_{(33)}$ = 295, P = 0.728]. b) Wire Hang for WT, VAChT$^{WT/Del}$ and VAChT$^{FloxNeo/Del}$ mice. No significant difference was observed [Kruskal-Wallis, $H_{(2)}$ = 2.604, P = 0.272]. c) Grip force measured for WT and VAChT$^{FloxNeo/FloxNeo}$ mice. There is no significant difference between the two genotypes [$T_{(21)}$ = 125, P = 0.689]. d) Maximal force expressed in gram. No difference was observed between WT, VAChT$^{WT/Del}$ and VAChT$^{FloxNeo/Del}$ mice [One way ANOVA, $F_{(2)}$ = 0.600, P = 0. 507]. e) Gait analysis for WT, VAChT$^{FloxNeo/FloxNeo}$ mice. No significant difference between genotypes was revealed [Student test, $t_{(13)}$ = 0.263 P = 0. 797] f) Gait analysis for WT, VAChT$^{WT/Del}$ and VAChT$^{FloxNeo/Del}$. No significant difference between genotypes was observed [One way ANOVA, $F_{(2)}$ = 0.699, P = 0. 559].

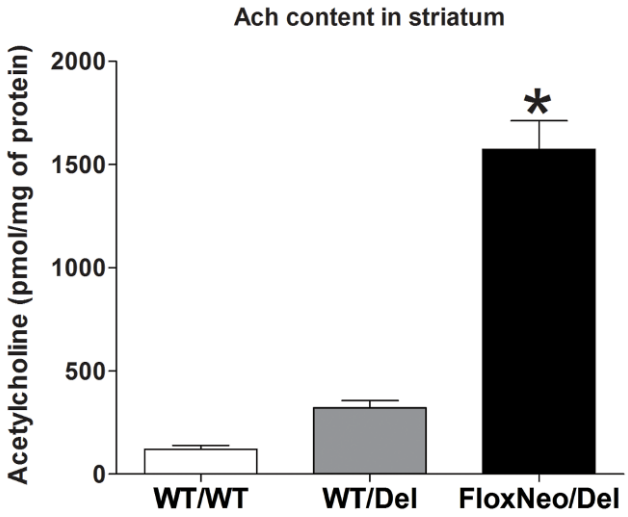


Figure 7: Acetylcholine content in the striatum VAChT mutant mice . Striatal tissue ACh levels for WT, VAChT$^{WT/Del}$ and VAChT$^{FloxNeo/Del}$ mice were assayed by chemiluminescent detection. Data represent 4-9 experiments (mean ± SEM). (One-way Anova with Bonferroni post hoc, $F_{(2,14)} = 21{,}98$, (*)p< 0. 05* for wild-type controls.

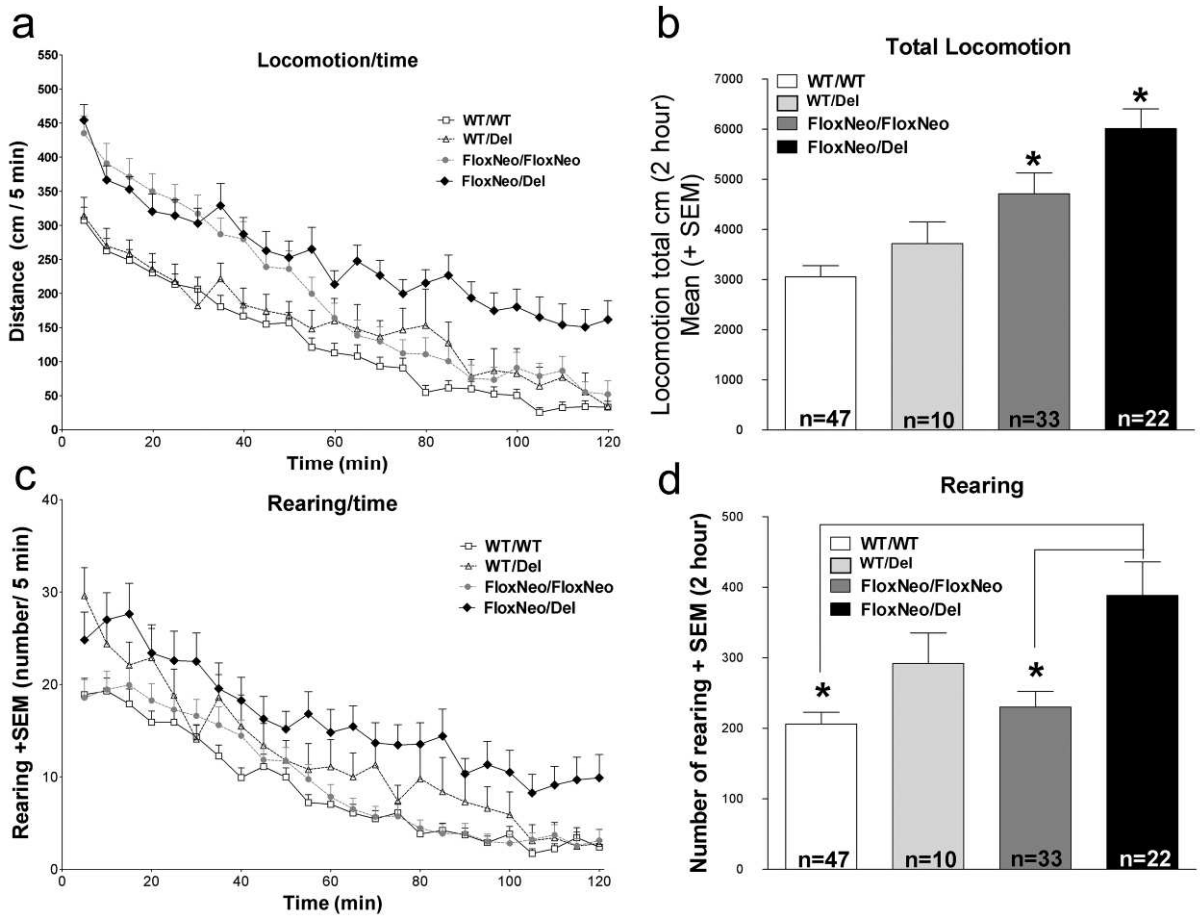


Figure 8: **VAChT mutant mice are hyperactive**. a) Spontaneous horizontal activity during two hours in the open field for WT, VAChT$^{WT/Del}$, VAChT$^{FloxNeo/FloxNeo}$ and VAChT$^{FloxNeo/Del}$ mice. b) Total spontaneous horizontal activity during the two hour was increased for VAChT$^{FloxNeo/FloxNeo}$ and VAChT$^{FloxNeo/Del}$ mice compared to WT/WT. But no difference between WT and VAChT$^{WT/Del}$ was observed. c) Spontaneous vertical activity during two hours in the open field for WT, VAChT$^{WT/Del}$, VAChT$^{FloxNeo/FloxNeo}$ and VAChT$^{FloxNeo/Del}$ mice. d) Total number of rearings during the two hour period. Rearings for VAChT$^{FloxNeo/Del}$ were significantly higher when compared to WT (Kruskal-Wallis test; ($H_{(3)}$ =13.764, post-hoc Dunn $p<0.05$). (*) indicate $p<0.01$.

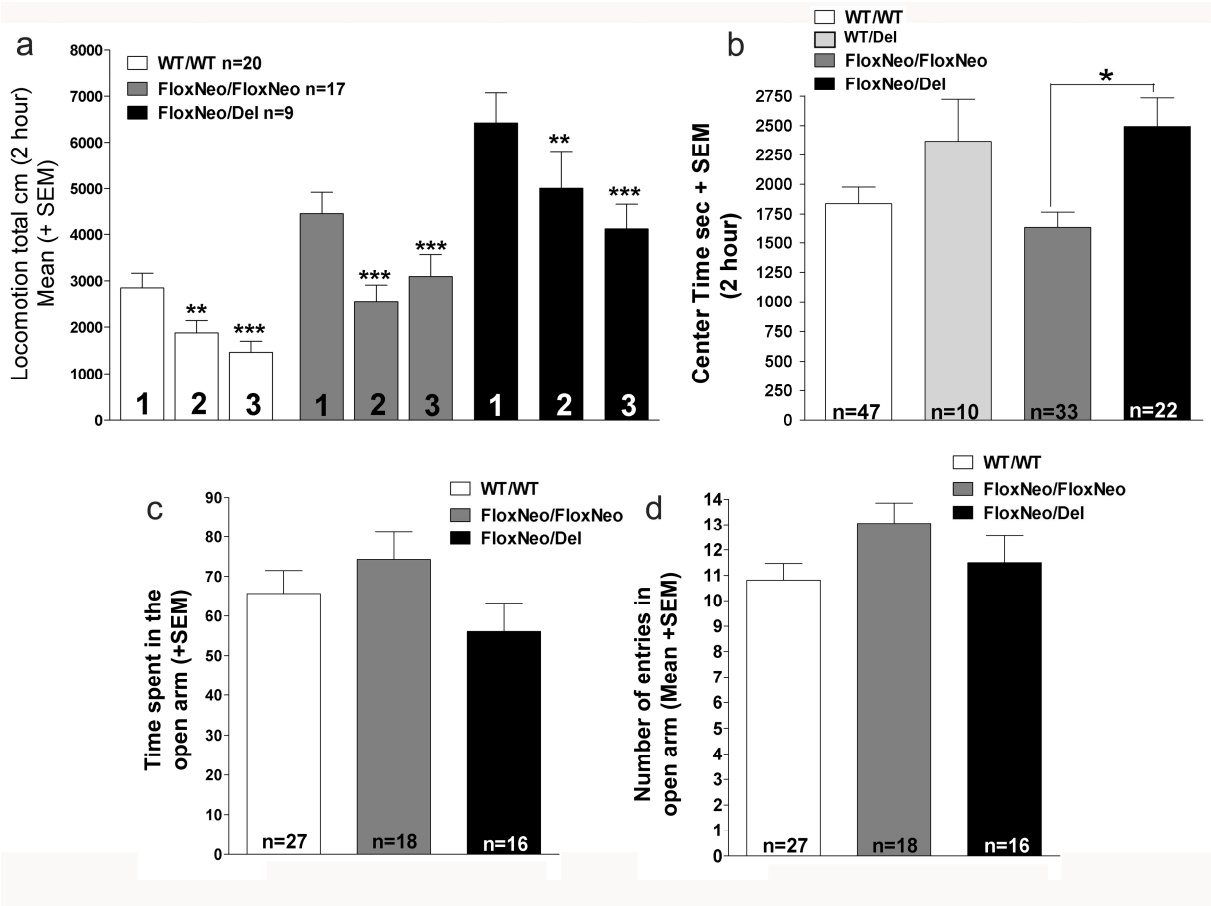


Figure 9: Habituation and anxiety are not changed in VAChT mutant mice. a) Habituation to open field during 3 consecutive days for WT, VAChT$^{FloxNeo/FloxNeo}$ and VAChT$^{FloxNeo/Del}$ mice. Mice showed no impairment in habituation in the novel environment. Two-way repeated measures ANOVA- main effect of genotype,$F_{(2, 86)} = 15.825$, $p < 0.001$, day $F_{(2, 86)} = 35.318$, $p < 0.001$ and interaction genotype x day $F_{(4, 86)} = 2.505$, $p < 0.05$]. b) Time spend in the centre during the 2 hour in the open field for WT, VAChT$^{WT/Del}$, VAChT$^{FloxNeo/FloxNeo}$ and VAChT$^{FloxNeo/Del}$ mice. VAChT$^{FloxNeo/del}$ mice spent significant more time in the center of the open field apparatus (Kruskal-Wallis test, $H_{(3)} = 11.537$, $p<0.05$; post-hoc Dunn's method, $p < 0.05$). c) Time spend in the open arm of elevated plus maze for WT, VAChT$^{FloxNeo/FloxNeo}$ and VAChT$^{FloxNeo/Del}$ mice was not significantly affected. One Way Analysis of Variance $F_{(2,58)} = 1,603$, NS). d) Number of entries in the open arm of elevated plus maze for WT, VAChT$^{FloxNeo/FloxNeo}$ and VAChT$^{FloxNeo/Del}$ mice was not significantly affected. One Way Analysis of Variance ($F_{(2,58)} = 1,845$, NS). (*), (**) and (***) indicate $p<0.05$, $p<0.01$ and $p<0.001$ respectively.

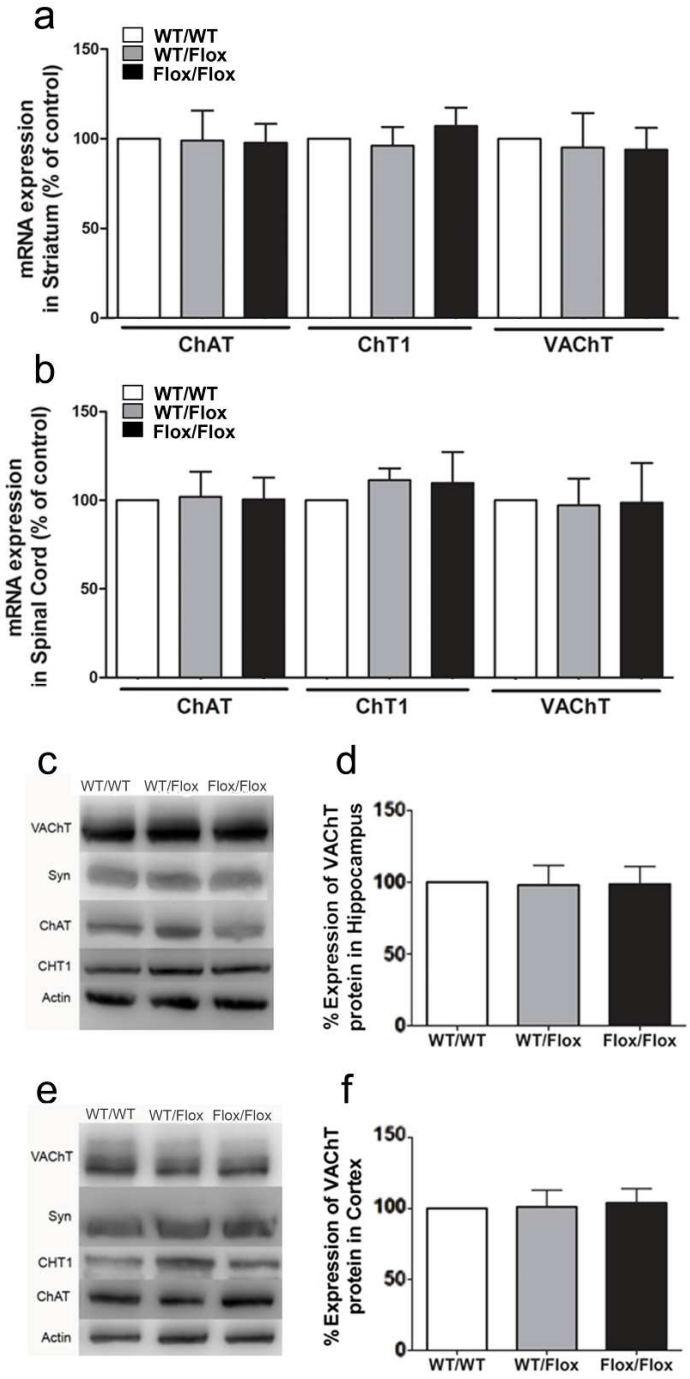


Figure 10: Genetic rescue of VAChT-mutant mice. a) VAChT, ChAT, and CHT1 mRNA levels were measured by qPCR in the striatum of WT mice (white bar), $VAChT^{WT/Flox}$ (grey bar) and $VAChT^{Flox/Flox}$ (black bar) mice. b) VAChT, ChAT, CHT1 mRNA levels in the spinal cord of WT mice (white bar), $VAChT^{WT/Flox}$ (grey bar) and $VAChT^{Flox/Flox}$ (black bar) mice. c) Representative immunoblot of control, $VAChT^{WT/Flox}$ and $VAChT^{Flox/Flox}$ mice in striatum. d) Quantification of protein levels. Actin immunoreactivity was used to correct for protein loading between experiments. Data are presented as a percentage of wild-type levels. e) Representative immunoblot of control, $VAChT^{WT/Flox}$ and $VAChT^{Flox/Flox}$ mice in spinal cord. f) Quantification of protein levels. Actin immunoreactivity was used to correct for protein loading between experiments. Data are presented as a percentage of wild-type levels.

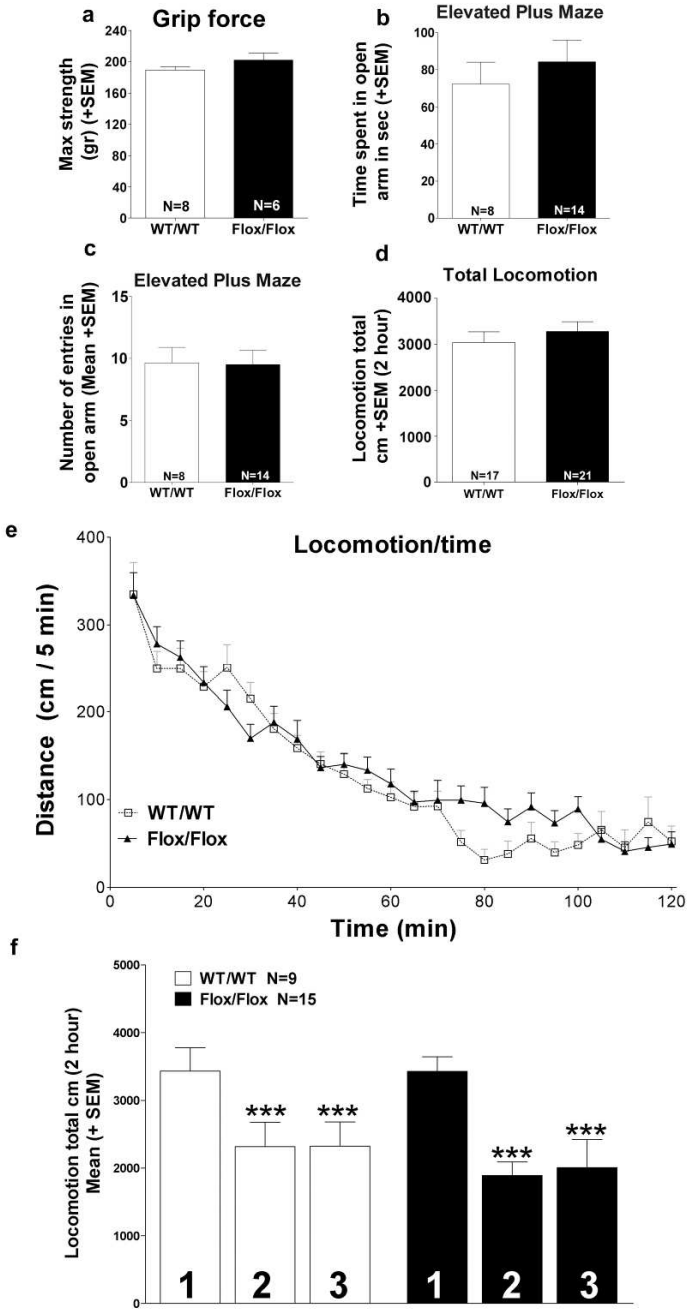
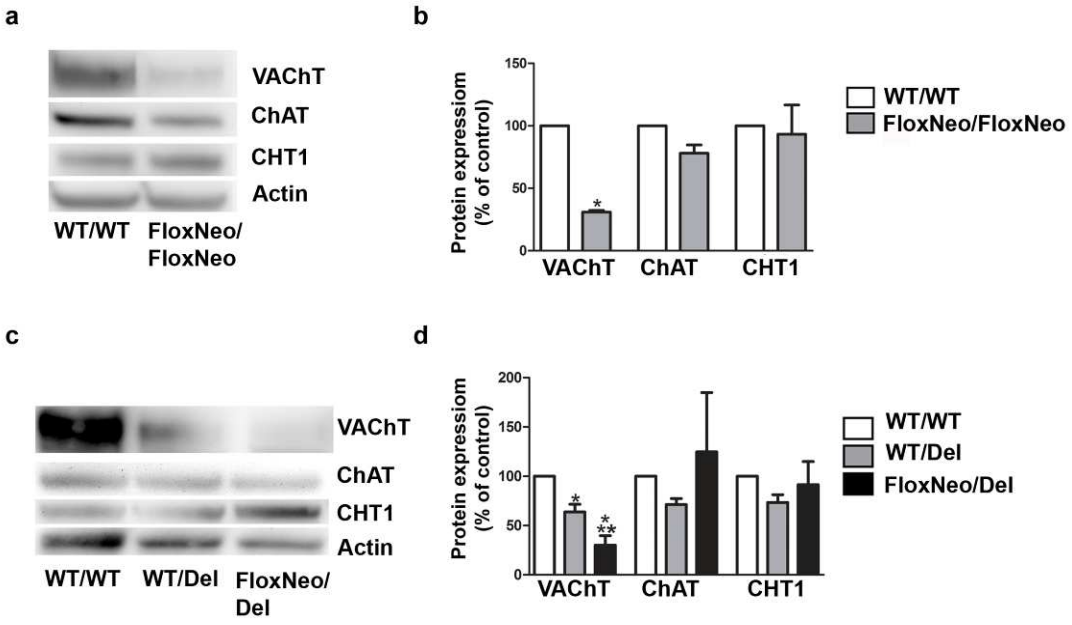


Figure 11: Restoration of normal phenotype by removing of the Neo-cassette. a) Spontaneous horizontal activity during two hours in the open field for VAChT$^{Flox/Flox}$ mice. The total locomotion is similar in both genotype ($t_{(36)}$ = -0.769 P = 0.447). b) Grip force for VAChT$^{Flox/Flox}$ mice. ($t_{(12)}$= -1.414 P = 0.183) c) Time spent in the open arm of elevated plus maze for VAChT$^{Flox/Flox}$ mice. No difference in anxiety level was observed ($t_{(20)}$ = -0,670, P = 0,510). d) Number of entries in the open arm of elevated plus maze for WT and VAChT$^{Flox/Flox}$ mice. e) Spontaneous horizontal activity during two hours in the open field for VAChT$^{Flox/Flox}$ mice. f) Habituation to open field during 3 consecutive days. The ANOVA reveal no effect of genotype ($F_{(1,44)}$ = 0.475, P = 0.498), a significant effect of the factor day ($F_{(2,44)}$ = 16.733, P < 0.001) and no interaction genotype x day ($F_{(2,44)}$ = 0.364, P = 0.697). Post-hoc showed difference between the Day1 and Day2, 3. (***) indicate p<0.001.



Supp 1: Protein expression is changed in VAChT mutant mice. a) Western blot analysis of VAChT in the striatum of $VAChT^{FloxNeo/FloxNeo}$ mice compared to WT control and b) quantification of protein levels. c) Western blot analysis of VAChT in the striatum of $VAChT^{FloxNeo/Del}$ mice, $VAChT^{WT/Del}$ and $VAChT^{WT/WT}$. d) quantification of protein levels. Actin immunoreactivity was used to correct for protein loading between experiments. Data are presented as a percentage of wild-type levels. Graphs represent average of 4-6 different mice. (*) indicates statistically different from WT/WT control (Student test, $p<0.05$), (**) indicates statistically different from $VAChT^{WT/Del}$ (Student test, $p<0.01$).

Artigo 5 Antidepressant-like effect of striatal-selective elimination of the vesicular acetylcholine transporter

Nesse trabalho testamos o efeito de uma deleção específica de VAChT no estriado sobre as funções cognitivas. Fui responsável pela realização dos experimentos de immunofluorescência para figura 1. Realizei também os testes neuromusculares de grip-force e wire-hang dos animais adulto $VAChT^{D2\text{-}Cre\text{-}flox/flox}$ para a figura 3 a,b. E fui responsável pelo estudo da atividade locomotora e dos testes cognitivos como nado forçado, cruz elevada e beliscadura da cauda desses animais (Fig 4-6, Fig 7c e Fig 8) .

Antidepressant-like effect of striatal-selective elimination of the vesicular acetylcholine transporter.

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Abstract:

Acetylcholine-dopamine balance is thought to play major roles in the striatum. Although we know in detail the roles dopamine plays for striatal function, there still poor understanding of striatal cholinergic functions in behaviour. To address the role of acetylcholine for striatal function we generated a striatal-selective vesicular acetylcholine transporter (VAChT) mouse knockout line, using the Cre/lox system. Striatal-selective elimination of VAChT does not affect motor learning or physical fitness in mice. Because overactivity of the cholinergic system may have an important role in depression-like behaviour, we tested this new mouse line in the forced-swimming test. VAChT-eliminated mice showed decreased immobility and increasing swimming distance compared to control mice, suggesting an antidepressant-like phenotype. This increased activity was selective for forced-swimming, locomotor activity, habituation, cocaine-induced hyperlocomotion, object recognition-memory and stress-related locomotion were not altered in these mutant mice. Moreover, the effect of VAChT elimination was additive to the effect of the clinically-relevant antidepressant fluoxetine, suggesting a distinct mechanism of action. We propose that inhibition of striatal cholinergic activity may be of benefit to improve depression-like symptoms.

Key words: acetylcholine, depression, Parkinson's disease, synaptic vesicle, conditional knockout.

Introduction

The striatum is the major input gateway in the basal ganglia. Striatal activity plays important roles in controlling motor functions, goal-directed and reward-related behaviours. The striatum is the brain region mostly affected in several motor diseases such as Parkinson's disease (PD), Huntington's disease and dystonia (Pisani et al., 2007). Medium spine neurons, activated by corticostriatal glutamatergic inputs, are the major output neurons for the striatum; these neurons are regulated extensively by the classical neurotransmitters dopamine and acetylcholine (ACh) (Aosaki et al., 2010;Calabresi et al., 2006;Lovinger, 2010;Shen et al., 2007). MSN neurons consist of two classes that form the direct and indirect pathways to the Globus pallidus, regulated respectively by D1 and D2 dopaminergic receptors. Regulation of MSNs by dopamine has received considerable attention, owing to the well known effects of reduced dopamine levels for motor symptoms in PD (Rodriguez-Oroz et al., 2009). In addition to the fall in striatal dopamine levels in PD there is also increased activity of cholinergic neurons (Calabresi et al., 2000;Ding et al., 2006;Pisani et al., 2001;Pisani et al., 2007;Spehlmann and Stahl, 1976), which could contribute to motor symptoms in PD. In addition to motor symptoms, chemical unbalance in the striatum may also cause non-motor symptoms. For example, a large proportion of individuals affected with PD show clinical signs of depression (Rodriguez-Oroz et al., 2009).

Dopamine-acetylcholine balance in the striatum is believed to be critical for proper motor function and behaviour (Aosaki et al., 2010;Kaneko et al., 2000;Pisani et al., 2007). These neurotransmitters have a reciprocal relationship regulating each other's release, and they have opposing actions in the direct and indirect striatal pathways (Aosaki et al., 2010;DeBoer et al., 1996;DeBoer and Abercrombie, 1996;MacKenzie et al., 1989;Pisani et al., 2007). Attenuation of muscarinic signalling is of clinical benefit to control motor symptoms in PD. However, in comparison with the known roles of dopaminergic neurotransmission in the striatum, much less is known about the physiological functions of released ACh in this brain region.

Striatal cholinergic interneurons fire in a fast rate (Bennett and Wilson, 1999;Wilson, 2005) and therefore can keep high levels of extracellular released ACh, which is believed to have a strong influence in the striatum (Kaneko et al., 2000). To maintain high levels of transmitter release striatal cholinergic neurons require to effective generate and release ACh. Choline acetyltransferase (E.C. 2.3.1.6) synthesizes ACh by using the precursor acetyl-CoA and choline (Dobransky and Rylett, 2005), the later derived from the uptake by a cholinergic specific high-affinity choline transporter [SLC5A7, (Okuda et al., 2000)]. Transport of

acetylcholine synthesized in the cytoplasm into synaptic vesicles requires the activity of the vesicular acetylcholine transporter [SLC18A3, VAChT (Parsons, 2000;Prado et al., 2008)]. The VAChT provides the last cholinergic-specific step for ACh-mediated neurotransmission; there is no other transport activity to refill cholinergic synaptic vesicles during neurotransmission and embryos null for VAChT cannot release ACh effectively (de Castro et al., 2009a). Muscarinic receptors (Wess et al., 2007), as well as several nicotinic–type of receptors (Drenan et al., 2008;Drenan et al., 2010;Zhou et al., 2001;Zhou et al., 2003) can be activated in the striatum to influence MSN function, dopamine and ACh release, adding to the complexity of understanding the roles of endogenous ACh in the striatum.

There has been increased interest in understanding how cholinergic neurons regulate striatal function, due to their potential to influence striatal responses in pathological states (Pisani et al., 2007). Previous work used an ablation strategy (immunotoxin-mediated cell targeting) to probe the functions of cholinergic interneurons in the nucleus accumbens (Kaneko et al., 2000). A comprehensive body of work using this strategy implicated striatal cholinergic neurons in reward-related learning, control of locomotor activity and rewarding responses to cocaine (Hikida et al., 2001;Hikida et al., 2003;Kakiuchi et al., 2001;Kaneko et al., 2000;Kitabatake et al., 2003). These results suggested that striatal cholinergic neurotransmission could have critical roles in striatal regulation of locomotor behaviour. However, these experiments were performed before the discovery that the vesicular glutamate transporter 3 (VGLUT3) is also expressed in striatal cholinergic neurons (Fremeau, Jr. et al., 2002;Gras et al., 2002;Herzog et al., 2004;Schafer et al., 2002); therefore ablation of striatal cholinergic interneurons lead to loss of cholinergic, but also VGLUT3-mediated glutamatergic responses.

In addition to the possibility of regulating motor functions there is evidence that cholinergic signalling in the striatum may modulate depression-like symptoms. It has long been suggested that increased activity of cholinergic neurons may be related to depression (Janowsky et al., 1972b;Janowsky et al., 1972a). Evidence from human and animal research suggest that overall decreased cholinergic activity improves depression-like symptoms (Mineur and Picciotto, 2010). Moreover, antagonism of both nicotinic (Andreasen et al., 2009;Andreasen and Redrobe, 2009;Mineur et al., 2007a;Mineur et al., 2007b;Picciotto et al., 2002;Rabenstein et al., 2006;Rollema et al., 2009) and muscarinic receptors has been shown to be antidepressant (Drevets and Furey, 2010;Furey and Drevets, 2006). Interestingly, injection of muscarinic antagonists in the Nucleus accumbens improves the performance of

mice in the forced-swimming test, used to assess behavioural despair (Chau et al., 2001), pointing to a possible role of striatal cholinergic neurotransmission in antidepressant activity .

Previously we have generated mice with distinct expression levels of VAChT and consequently ACh release to probe for functional roles of synaptic vesicle filling in cholinergic activity (de Castro et al., 2009b;de Castro et al., 2009a;Prado et al., 2006). Here we used a novel mouse line in which we selectively eliminated VAChT in the striatum to understand the contribution of these neurons to phenotypes associated with striatal dysfunction. We found surprisingly that mice with selective deletion of VAChT in the striatum do not show changes in motor performance and motor learning. However, decreased cholinergic function in the striatum seems to specifically improve the response of mice in the forced-swimming task, suggesting that striatal VAChT might be a novel target for treatment of depression. Because in PD cholinergic neurons are overactive, our data may have implications for treatment of depression associated to this motor disorder.

MATERIAL AND METHODS

Animals:

The isolation of a VAChT genomic clone was described previously (Prado et al., 2006). The genomic clone was used to construct a gene-targeting vector in which we added LoxP sequences flanking the VAChT open reading frame and a TK-Neo cassette. Generation of $VAChT^{flox/flox}$ mice is described elsewhere (De Jaeger et al., submitted) and the construct is shown on supplementary figure 1. Briefly, after removal of the TK-Neo cassette, one LoxP sequence was present 260 bp upstream from the VAChT translational initiation codon and a second LoxP sequence was located approximately 1.5 kb downstream from the VAChT stop codon and within the second ChAT intron. Note that this is a vector distinct from that we previously reported for generation of VAChT KD mice (47).

D2-Cre mice (Drd2, Line ER44) were obtained from the GENSAT project via the mutant mouse regional resource centers. $VAChT^{D2\text{-}Cre\text{-}flox/flox}$ mice were generated by crossing $VAChT^{flox/flox}$ with the D2-Cre mouse line. We then intercrossed $VAChT^{D2\text{-}Cre\text{-}flox/wt}$ to obtain $VAChT^{D2\text{-}Cre\text{-}flox/flox}$ mice. Because these mice were apparently normal and fertile, we bred $VAChT^{D2\text{-}Cre\text{-}flox/flox}$ mice and $VAChT^{flox/flox}$ to obtain all the mice used in the present study. These mice were backcrossed to C57BL/6J mice for 5 generations. Unless otherwise stated all control mice used were $VAChT^{flox/flox}$ mice without the cre transgene but with the same background. Rosa26-YFP mice (B6.129X1-Gt(ROSA)26Sor$^{tm1(EYFP)Cos/J}$, stock number 006148) was obtained from Jackson Laboratories. Dopamine transporter knockout mice

(DAT-KO) have been previously described (Giros et al., 1996). Animals were housed in groups of three to four mice per cage in a temperature controlled room with 12-h light–12-h dark cycles, and food and water were provided ad libitum. Mouse stocks were maintained in SPF conditions, however experimental subjects were kept in a conventional mouse facility. All studies were conducted in accordance with the NIH and the Canadian Council of Animal Care (CCAC) guidelines for the care and use of animals with approved animal protocol from the Institutional Animal Care and Use Committees at the University of Western Ontario (protocol number 2008-089) and Duke University. In order to reduce the number of animals used for behaviour experiments the same group of mice were tested in distinct tasks, but at least one-week interval between demanding tasks was observed. However, we always had a smaller naive group of mice being tested in all the behaviour experiments and the results were pooled together. Mice were assigned to distinct experimental groups in random way.

Immunofluorescence, qPCR and Western-Blot.

For the immunofluorescence experiments we followed the protocol described previously (de Castro et al., 2009b). Briefly, mice were anesthetized and transcardically perfused with 4% paraformaldehyde (PFA) in PBS for 15 min. Perfused brains were immediately postfixed in 4% PFA in PBS overnight at 4°C. Following cryoprotection in 4% PFA with 10% sucrose, tissues were rapidly frozen in isopentane over dry ice and kept at -80°C. Serial sections (40 µm) were cut on a Cryostat and immersed in PBS. Sections from control and test mice were processed simultaneously for all experiments. Brain slices were permeabilized in 1.2% Triton/PBS and rinsed in PBS. Tissues were blocked for 1 h in 10% normal goat serum/PBS and immunonstained with primary antibodies, CHT1 (rabbit polyclonal 1:250, kindly provided by R. Jane Rylett, University of Western Ontario, London, Canada) and GFP (Goat polyclonal FITC from Abcam, 1:1000) in incubation buffer (2% normal goat serum; 0.2% Triton; PBS) for 48 h. After washing three times with PBS for 20 min each, tissues were incubated with Alexa Fluor 546 goat anti-rabbit (1:500, Invitrogen) in incubation buffer for 1 h. Slices were rinsed and, mounted in a coverslip. Sections were mounted on slides and visualized with a Zeiss LSM 510 META - NLO mulitophoton laser-scanning microscope with a Zeiss 5X and 20X lens and appropriate filters. Control experiments were done in D2-Cre mice in which no GFP staining was observed (not shown).

For mRNA analysis tissues were frozen in a mixture of dry ice/ethanol and kept at -80ºC until use. For real-time quantitative PCR (qPCR), total RNA was extracted using the Aurum Total RNA for fatty and fibrous tissue kit from Biorad. Quantification and quality

analysis of RNA in the extracted samples was done by microfluidic analysis (Agilent Technologies' Bioanalyzer). First-strand cDNA was synthesized using the iSCRIPT cDNA SYNTHESIS KIT from Biorad. cDNA was subsequently subjected to qPCR on a CFX-96 Real Time System (Biorad) using the iQ SYBR GREEN SUPERMIX (Biorad). For each experiment, a nontemplate reaction was used as a negative control. In addition, the absence of DNA contaminants was assessed in reverse transcription-negative samples and by melting-curve analysis. Relative quantification of gene expression was done with the $\Delta\Delta CT$ method using β-actin gene expression to normalize the data.

For preparation of extracts for protein analysis, tissues were homogenized in a solution containing 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and protease inhibitor cocktail (Sigma). Extracts remained on ice for 15 min and were then centrifuged (10,000 x g x 20 min) at 4°C. Protein concentration in the supernatant was determined using the method of Bradford (Bradford, 1976). Samples were boiled for 5 minutes, and equal amounts of proteins (50µg) were resolved by 4-12% SDS-PAGE and transferred to nitrocellulose membranes (Millipore). Immunobloting was performed as described elsewhere (Prado et al., 2006). When necessary, blots were stripped using Restore Stripping buffer (Thermo Scientific) for 20 minutes and then reprobed again. The antibodies and respectively dilution used were: anti-VAChT (Synaptic Systems Gottingen, Germany1:2000), anti-synaptophysin (Sigma, 1:2000), anti-actin (Chemicon, CA,1:5000), anti-ChAT (1:1000), and anti-CHT1 (Ribeiro et al, 2005, 1:1000). Blots were developed using the Enhanced Chemiluminescence Kit (GE Healthcare) according to the supplier's instructions. All blots were quantified using a CCD-camera device running the software FluoChem (Alpha Innotech).

Measurements of locomotor activivity

All behaviour experiments were performed between 9AM-4PM in the light cycle. Mice that had not experienced any cage change for at least 24 hours were acclimated to the room for 20 minutes prior to the beginning of the test. To measure locomotor activity the animals were introduced in an open field box (20x20 cm) for two hours. Data were collected automatically using an infrared system (Accuscan, Columbus, Ohio) running the software Versamax. Horizontal activity was recorded. In addition time spent in the center and in the corners of the box was evaluated to assess anxiety. For habituation experiments, the mice were exposed for 120 min to the same open field during 3 consecutive days.

In order to test the interaction of galantamine and cocaine on the locomotor activity, mice were pre-injected with cocaine and immediately placed in the open field for 2 hours. They received a galantamine injection 15-20 min after their introduction in the boxes. DAT-KO mice were injected with galantamine or saline 30 min after being placed in the open filed.

Grip-force and Wire-hang

A Grip Strength Meter from Columbus Instruments (Columbus, OH) was used to measure forelimb grip strength essentially as described (Prado et al., 2006). The test was repeated 10 consecutive times and the highest value from the 10 trials was recorded as the grip strength for that animal. Mice are not trained prior to testing and each mouse was tested once (10 trials equal one test session).

For the wire-hang each mouse was placed on a metal wire-grid, which was slowly inverted and suspended 30 cm above a piece of foam as previously described (Prado et al., 2006). The time it took for each mouse to fall from the cage top was recorded with a 60 s cut-off.

Rotarod

The rotarod task followed a previously described protocol (Prado et al., 2006). Briefly, mice were placed on the rotarod apparatus (SD instruments), facing away from the experimenter's view and rotation was increased gradually from 5 to 35 rpm. Latency to fall was recorded automatically. Ten trials were given on the first day and four trials on the second day, with a 10 min intertrial interval. In the time between trials, mice were placed in their home cage.

Elevated plus maze

Animals were placed in the center of the elevated plus maze (Med Associate, San Diego) and activity was recorded for five minutes with a webcam connected to a computer. Total amount of time spent in the closed and in the open arms of the maze was calculated with the Anymaze software; an animal is considered to be completely within an arm of the maze when its center of gravity is inside the arm. The result is expressed as time spent in the open arm in seconds.

Forced-swimming test

Mice were placed individually for 10 min in an glass cylinder containing water (diameter 14 cm; height 18 cm; water depth 14 cm; 25 to 30°C). The experiment was recorder with a video camera and `Immobility' was scored with the Anymaze software during the interval 2-6min using 800 ms as the cut-off time. In addition the software allowed for recording of the distance swam. Fluoxetine (20mg/Kg) was injected 30 min before the test. After the test, mice were removed and dried with paper towel before returning them to their home cage.

Tail pinch

The tail pinch stressor involved placing mice in a novel cage with a small fold-back binder clip fastened at the base of their tail for a period of 5 min as previously described (Poulter et al., 2010). A gauze pad was placed between the tail and the clip to avoid tissue damage. Following this the mice were placed immediately or 24 hours later in the open field for 20 min. The tail pinch clearly stressed the mice; subjects tried to remove the clip shacking their tail, biting and manipulating the clip.

Object recognition memory

The general procedure was previously described (De Jaeger et al.). Here, the apparatus used was an open box made of white PVC (50 x 50 x 40 cm) under a video system connected to a computer and a compact fluorescent light (equivalent 60 Watts, input 800 lumens). In the first day, mice were individually submitted to a single familiarization session of 10 min, during which they were introduced to the empty arena. On the second day (24 h later), animals were place in the open field with two identical objects (A1 and A2) during 10 min. 24h after mice were tested for 10 min in the presence of a familiar object and a novel object (A and B). For each session, mice were always placed in the apparatus facing the wall. The position and the object were randomly assign. The data was collected with AnyMaze Video Tracking System software, which automatically detects the nóse of the mice. Exploration was defined by position of the nóse to the object at a distance of no more than 1cm.

Data Analysis

Result was expressed as mean ± SEM. The Sigmastat 3.1 software was used for statistical analysis. Comparison between two experimental groups was done by Student’s t Test or Mann-Whitney Rank Sum Test in case the data did not follow a normal distribution. When several experimental groups were analyzed we used two-way analysis of variance

(ANOVA). For locomotion experiments we used ANOVA with repeated measures and when appropriate, a Tukey post-hoc comparison test was used.

Results:

To address the role of ACh in specific brain regions, we generated a VAChT floxed mouse line ($VAChT^{flox/flox}$, De Jaeger et al., submitted). The addition of lox P sites did not change VAChT expression at the mRNA and protein levels when compared to wild-type control mice (De Jaeger et al., submitted and data not shown). $VAChT^{flox/flox}$ mice have normal levels of VAChT and other pre-synaptic cholinergic markers, locomotor activity, grip-strength and fatigue when compared to wild-type mice (not shown).

In order to selectively eliminate VAChT in the forebrain, we used the D2-Cre mouse line generated by GENSAT (Gong et al., 2007), which express the enzyme Cre recombinase under the control of regulatory elements of the D2 dopamine receptor, by using a modified BAC construct. We crossed the D2-Cre mouse line with Rosa26-YFP mice to confirm the pattern of expression previously described for the Cre recombinase in the brain in this mouse line (Fig. 1a). YFP staining in D2-Cre x Rosa26-YFP mice was detected in over 90% of striatal cholinergic neurons (Fig. 1b) identified with an antibody against CHT1. We did not detect co-localization of YFP in cholinergic neurons in the penduculopontine nucleus or in motor neurons in the brainstem. However, localization of YFP in cholinergic neurons was detected in the basal forebrain, albeit this was not as extensive as in the striatum (Fig. 1b). We therefore proceeded to cross D2-Cre mice with $VAChT^{flox/flox}$ mice and generated $VAChT^{D2\text{-}Cre/flox/flox}$ mice to eliminate VAChT in the striatum. Genotyping for these lines is shown in Supplementary figure 1.

In a next set of experiments we evaluated the expression of VAChT in the striatum in $VAChT^{D2\text{-}Cre\text{-}flox/flox}$ to assess the degree of Cre-mediated recombination. As expected based on the observations with Rosa26-YFP cross, both mRNA and protein levels for VAChT were almost abolished in the striatum of $VAChT^{D2\text{-}Cre\text{-}flox/flox}$. In contrast, CHT1 and ChAT levels were not altered (Fig. 2). There was no difference in VAChT protein expression levels in the hippocampus of $VAChT^{D2\text{-}Cre\text{-}flox/flox}$ mice when compared to controls (Supplementary Figure 2). Moreover, we did not find any alteration in mRNA levels for VAChT in the spinal cord of $VAChT^{D2\text{-}Cre\text{-}flox/flox}$ mice (Supplementary Figure 2). However, we detected an increase in ChaT mRNA levels and protein levels in the spinal cord. There was also about 50% decrease in VAChT protein levels in the spinal cord. We found no gross morphological difference in the striatum or other brain regions in $VAChT^{D2\text{-}Cre\text{-}flox/flox}$ mice (not shown).

Previous experiments from our laboratory have shown that up to 50% decrease in the expression of VAChT in the spinal cord is well supported in mice and does not alter motor function (de Castro et al., 2009b;Prado et al., 2006). In agreement with these previous results, $VAChT^{D2\text{-}Cre\text{-}flox/flox}$ showed no difference in grip-force strength [Fig. 3a, ($t_{(47)}$ = 1.702; P = 0.095)] or fatigue (detected by the Wire-hang task, Fig. 3b, Mann-Whitney, $T_{(13)}$ = 49; P = 0.710). Interestingly, we also found that $VAChT^{D2\text{-}Cre\text{-}flox/flox}$ mice had no deficit in motor performance or motor learning tested using the rotarod when compared to control mice [Fig. 3c, Two-Way Repeated Measures ANOVA reveal no difference between the two genotypes $F_{(1,261)}$=0.0000409, P=0.995; both mice improved their performance $F_{(9,261)}$=41.614, P<0.001; and there was no interaction between genotype and session, $F_{(9,261)}$=1.333, P=0.220]. These results suggest that despite a decrease in the levels of VAChT in the spinal-cord this did not reflect in changes in motor function. The rotarod experiments also suggest that $VAChT^{D2\text{-}Cre\text{-}flox/flox}$ mice are physically fit and that motor learning does not seem to depend on striatal cholinergic activity. We also tested $VAChT^{D2\text{-}Cre\text{-}flox/flox}$ mice in object recognition memory. The mice performed identically to controls in this cognitive task (not shown).

In order to test if striatal cholinergic neurons would have any role in depressive-like behaviour, we used the forced-swimming task, which in mice is a task effective to assess the effect of antidepressants in behaviour-despair (Porsolt et al., 1977;Porsolt et al., 1978b;Porsolt et al., 2001). Surprisingly, we found that $VAChT^{D2\text{-}Cre\text{-}flox/flox}$ mice performed significantly better in the forced-swimming task than control mice when we analysed both immobility time [T-Test, $t_{(31)}$= 3.765, P<0.001] and distance of swimming (Fig. 4a and b, Mann-Whitney, T= 126.000, df= 14, P <0.001). We attempted to confirm these results using a second task for depression-like behaviour, the tail-suspension test. Unfortunately, in our hands all control and $VAChT^{D2\text{-}Cre\text{-}flox/flox}$ mice climbed on their tails, in a behaviour similar to C57BL/6 mice (Cryan et al., 2005;Mayorga and Lucki, 2001), and making it impossible to perform these experiments. Nonetheless, the forced-swimming data suggest that mice lacking striatal VAChT and consequently ACh release may show improved responses to “behavioural-despair”. However, other interpretations are warranted and it is possible that $VAChT^{D2\text{-}Cre\text{-}flox/flox}$ mice may have higher general activity and because of that they maintain higher levels of swimming during the task. Indeed, previous experiments in which cholinergic neurons in the nucleus accumbens were ablated indicated that loss of these neurons causes hyperlocomotion and increased sensitivity to the locomotor effects of cocaine (Hikida et al., 2001;Kaneko et al., 2000;Kitabatake et al., 2003).

Therefore, to test if the improved performance of VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice in the forced-swimming test is due to higher general activity, we performed locomotor experiments in automated activity boxes. Surprisingly, we found no difference in locomotor activity when we compared VAChT$^{flox/flox}$ with VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice (Fig. 5a). Both, total horizontal activity [Fig. 5b, $t_{(24)}$ = -0.315 ; P = 0.755)] or vertical activity [Fig. 5c, ($t_{(24)}$ = 1.027; P = 0.315)] were not statistically different. We also tested habituation by investigating locomotor activity in 3 consecutive days in the open field (Fig. 5d). We observed that both genotypes habituate to the open field. Two-Way Repeated Measures ANOVA confirmed that the general activity is the same for both genotypes [genotype factor, $F_{(1,58)}$ = 0.932, p=0.342]. The activity decreases over the day [day factor, $F_{(2,58)}$ = 10.244, p<0.001] and both genotypes habituate to the environment [Interaction between genotype and day, $F_{(2,58)}$ = 1.506, p=0.230]. We conclude that deletion of VAChT in the striatum did not affect general spontaneous activity or compromise the capacity to habituate to a new environment.

The above results suggest that decreased striatal cholinergic neurotransmission does not cause hyperlocomotion. Would VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice be more sensitive to increased dopaminergic tone that can be achieved by the psychostimulant cocaine? We tested this possibility by assessing locomotor activity using two distinct doses of cocaine. Both 5mg/kg or 20 mg/kg increased locomotor activity of VAChT$^{flox/flox}$ mice and VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice (Fig. 6a, b Turkey test between 20min and 25 min was significant for both concentrations). There was no difference between the two genotypes in their increased activity in response to cocaine injected i.p. in either concentration {Fig. 6a, 5 mg/kg, ANOVA show no effect of genotype [$F_{(1,322)}$ = 0.201, p = 0.661], significant effect of time [$F_{(23,322)}$ = 12.820, p <0.001] and no interaction time x genotype [$F_{(23,322)}$ = 1.373, p = 0.121] ; Fig. 6b, 20 mg/kg, ANOVA show no effect of genotype [$F_{(1,299)}$ = 0.279, p = 0.606], significant effect of time [$F_{(23,299)}$ = 11.458, p <0.001] and no interaction time x genotype [$F_{(23,299)}$ = 1.135, p = 0.306}, albeit there seems to be a slightly decreased response to cocaine for VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice.

Previous experiments have suggested that increased cholinergic tone, by inhibition of acetylcholinesterase, can reverse the locomotor effects of cocaine (Hikida et al., 2003). Given that in our experiments we see no change in the response to cocaine due to reduced striatal cholinergic function, we decided to revisit this point and to test further if lack of striatal cholinergic neurotransmission would affect cocaine-mediated locomotion. First, we treated wild-type mice with cocaine (20 mg/kg) and transferred them to locomotor activity boxes. After 15-20 min of sustained and higher locomotor exploratory behaviour we injected a group of mice with saline and another with galantamine (1mg/kg, i.p), a cholinesterase inhibitor. As

observed before for cholinesterase inhibitors, galantamine decreased the locomotor activity induced by cocaine when compared to saline treated mice (Figure 7a). Mice presented a decrease in their activity during the session [Two-Way Repeated Measures ANOVA, $F_{(23, 322)} = 26.950$, $p<0.001$]. A significant interaction between treatment and time was detect [$F_{(23, 322)} = 26.950$, $p<0.001$]. This result indicates that after the injection, mice treated with galantamine diminished their horizontal activity faster than mice injected with saline. A Tukey's post-hoc test revealed difference in treatments between 20 and 50 min. Identical results were obtained when dopamine transporter KO mice, which are hyperactive in a new environment (Giros et al., 1996), were treated with galantamine (Figure 7b). These results confirm that treatment with the cholinesterase inhibitor galantamine does decrease the locomotor effects of hyperdopaminergia. In order to test if this effect of galantamine is mediated by increasing cholinergic neurotransmission from striatal cholinergic neurons, we repeated the experiments of Figure 7a in $VAChT^{D2\text{-}Cre\text{-}flox/flox}$ mice. Two groups of mice representing the two distinct genotypes ($VAChT^{flox/flox}$ and $VAChT^{D2\text{-}Cre\text{-}flox/flox}$ mice) were injected with cocaine and after 20 min of measuring their locomotor activity the two groups were injected both with galantamine (fig. 7c). Two-Way Repeated Measures ANOVA shows a significant decrease of activity during the time [$F_{(23, 276)} = 27.214$, $p<0.001$], no significant difference between genotype [$F_{(1, 276)} = 0.197$, $p=0.665$] and no significant interaction between treatment and time was observed [$F_{(23, 276)} = 0.866$, $p=0.645$]. Galantamine was therefore similarly effective to decrease the locomotor activity in both genotypes (compare with data on Fig. 7a), suggesting that the effect of galantamine was not due to increased cholinergic neurotransmission in the striatum.

After establishing that lack of VAChT in the striatum, and consequently ACh release, does not increase general locomotor activity or response to the psychostimulant cocaine, we wondered if the improved response in the forced-swimming task was related to an increased activity of $VAChT^{D2\text{-}Cre\text{-}flox/flox}$ mice during stress. M1-muscarinic KO mice have increased locomotor activity and improved behaviour in the forced-swimming task due to both increased general activity and activity under stress (Miyakawa et al., 2001). Therefore, we tested if $VAChT^{D2\text{-}Cre\text{-}flox/flox}$ mice have higher activity when stressed. For these experiments we stressed the mice with a tail-pinch for 5 min and evaluated locomotor activity. There was no significant alteration in locomotor activity in stressed $VAChT^{D2\text{-}Cre\text{-}flox/flox}$ mice when compared to control mice immediately after the pinch or 24 hours later [Fig. 8a and b, respectively $t_{(17)} = -0.990$, $P = 0.336$ and $t_{(17)} = 0.798$, $P = 0.436$]. In addition, we found that $VAChT^{D2\text{-}Cre\text{-}flox/flox}$ mice did not present higher anxiety measured either using the elevated-

plus maze task [Fig. 8c, $t_{(30)} = -0.180$, $P = 0.859$] or by evaluating the amount of time mice spent in the centre of the locomotor box during ambulance tests [Fig. 8d, $t_{(24)} = -0.327$, $P = 0.747$].

Taken together the present experiments indicate that VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice show improved performance in the forced-swimming task, but behaved normally in terms of motor function, locomotor activity and locomotor response to cocaine. Is the improved performance of VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice in the forced-swimming task due to a common pathway activated by antidepressant drugs? The role of monoamines in depression and in particular of serotonin is well established. To test if lack of VAChT in the striatum activates neuronal circuits that are also activated by antidepressants, we repeated the forced-swimming experiments in mice treated with fluoxetine. Fluoxetine improved the performance of control mice by decreasing their immobility [$t_{(22)} = 2.259$ $P = 0.034$] and increasing the distance that mice swam [compare Fig. 4 and Fig 8e, f ; $t_{(22)} = 103$, $P = 0.007$]. Importantly, the performance of VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice was also improved by fluoxetine [time immobility: $t_{(28)} = 4.066$, ($P = <0.001$) ; distance: $T_{(28)} = 112.000$, $P = 0.002$], suggesting that targeting VAChT improved the performance of mutant mice by a mechanism distinct from that activated by fluoxetine.

Discussion

Our experiments provide a new intake on the role of cholinergic neurons for striatal function. We found that elimination of VAChT in the striatum does not cause overt disruptions or alterations in some behavioural tasks previously thought to depend on ACh, such us open-field activity. However, we find improved performance of these novel mutant mice in the forced-swimming test, suggesting that decrease of striatal cholinergic neurotransmission decreases behavioural-despair in mice. These results support inhibition of VAChT activity in the striatum as a potential target in controlling depression-like symptoms.

VAChT$^{D2\text{-}cre\text{-}flox/flox}$ mice have normal motor performance and motor learning

Our assessment of motor function and motor learning in VAChT$^{D2\text{-}cre\text{-}flox/flox}$ mice indicate that at least in the rotarod test decreased release of ACh in the striatum does not seem to have a role. We found previously that general reduction of ACh release as observed in VAChT KD^{HET} mice affects motor learning in the rotarod (Prado et al., 2006). The present experiments suggest that this deficit is not related to striatal cholinergic function. This is in agreement with previous experiments in striatal cholinergic neuron-ablated mice that also showed no deficit in rotarod performance (Kitabatake et al., 2003).

VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice have selective improvement in forced-swimming performance

The cholinergic system has been classically linked to attention, learning and memory (Hasselmo and Giocomo, 2006). Decreased cholinergic function has been suggested to have a role in cognitive symptoms in Alzheimer's disease (Bartus et al., 1982;Perry et al., 1977) and in dementia associated with PD (Perry et al., 1985). In addition to these classical roles of ACh in the brain, it has been suggested that cholinergic activity may be related to symptoms of mood disorders. Initial data suggest that an overactive cholinergic system may play a role in depressive symptoms (Janowsky et al., 1972b). Experiments using a selectively bred line of rats, the Flinders sensitive line, with increased cholinergic sensitivity, support a role for increased ACh in depressive-like symptoms (Overstreet et al., 1986;Overstreet, 1993;Overstreet et al., 2005). Interesting, despite some initial controversy regarding whether activation of inhibitions of nicotinic receptors has antidepressant-like effects, more recent evidence suggests that antagonists of nicotinic receptors have antidepressant activity (Andreasen et al., 2009;Rabenstein et al., 2006). Interesting, multiple nicotinic receptor subunits have been linked to these antidepressant effects (Rabenstein et al., 2006).

In addition to nicotinic receptors, antagonism of muscarinic receptors has also been show to effectively produce antidepressant-like effects (Furey et al., 2010;Furey and Drevets, 2006). Importantly, local infusion of muscarinic agonists in the accumbens suggests a role for the striatal cholinergic system in depressant-like behaviour, an effect blocked by specific M1-antagonists (Chau et al., 2001). Taken together these observations point to multiple roles of cholinergic neurons in depression-type behaviour and lead us to ask the question of whether mice with reduced cholinergic neurotransmission in the striatum present antidepressant-like behaviour. Our experiments suggest that indeed inactivation of cholinergic activity can improve the performance of mice in the forced-swimming task, a widely used test to measure the activity of clinically effective antidepressants (Porsolt et al., 1977;Porsolt et al., 1978b;Porsolt et al., 1978a). Importantly, the increased activity of VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ in the forced-swimming task is not a consequence of an overall hyperactivity. Both, spontaneous locomotor activity and locomotor activity after a tail-pinch stress were not altered in VAChT$^{D2\text{-}Cre\text{-}flox/flox}$. In addition, there is not increase in anxiety in these mice assessed by elevated-plus maze and time spent in the center of the open-field.

Previous assessment of the role of cholinergic neurons in striatal function was done by ablation of cholinergic neurons using immunotoxin-mediated cell targeting. Injection of

toxin targeting cholinergic neuron in the accumbens lead to 80% decrease in ChAT-positive neurons, albeit independent measurements of ACh release, to assess the degree of ACh inhibition, were not performed in these experiments (Kaneko et al., 2000). These previous experiments suggest that decreased ACh release in the striatum lead to inhibition of reward-related learning, hyperactivity and increased sensitivity to the locomotor effects of cocaine (Hikida et al., 2001;Hikida et al., 2003;Kitabatake et al., 2003), but these mice were not tested for antidepressant-type of behaviour.

We were surprised to find that VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice did not show the increased locomotor phenotype previously described by Nakanishi and collaborators in mice with inactivated striatal cholinergic neurons. The reason for this discrepancy is not entirely clear at the moment. One possibility is the more chronic nature of genetic manipulation compared to ablation in adult mice. However, perhaps a more parsimonious explanation might be related to the fact that ablation of cholinergic neurons eliminates both VAChT and VGLUT3. It is known now that in addition to VAChT, VGLUT3 is also present in synaptic vesicles of striatal cholinergic interneurons, where it can mediate glutamate uptake into synaptic vesicles (Gras et al., 2008) and likely glutamate release.

The role of VGLUT3 in striatal function is only starting to be understood. Interestingly, VGLUT3-null mice show hyperactivity and increased response to the locomotor effects of cocaine (Gras et al., 2008). Therefore, mice lacking VGLUT3 show a phenotype that is remarkably similar to that of mice in which the cholinergic neurons in the accumbens were targeted by immunotoxin (Hikida et al., 2001;Kaneko et al., 2000). Experiments in VGLUT3-null mice have led to the interpretation that the absence of VGLUT3 causes a decrease in striatal cholinergic tone, due to a “synergism” between VGLUT3 and VAChT that facilitates ACh storage in synaptic vesicles. However, measurements of ACh release in striatal slices from VGLUT3-null mice showed only modest decrease [(30%), (Gras et al., 2008)]. Although we do not dispute the fact that VGLUT3 can indeed synergize ACh storage, we argue, based on our results, that the final alteration in ACh release in these mice cannot be solely responsible for their locomotor phenotype.

Our previous experiments with distinct mouse lines with decreased VAChT expression have shown that ACh release is directly proportional to the amount of VAChT expressed (de Castro et al., 2009a;Lima et al., 2010;Prado et al., 2006). Importantly, in heterozygous VAChT KD mice with 40-50% reduction of VAChT, striatal ACh release in freely moving mice is reduced by close to 50% (Prado et al., 2006). Both the VAChT KD^{HET} mouse line and heterozygous VAChT KO mice (50% reduction in VAChT levels, VAChT$^{wt/del}$) do not show

increased locomotor activity in the open-field (de Castro et al., 2009b;de Castro et al., 2009a). Moreover, the new mouse line which selectively eliminates VAChT from striatal cholinergic neurons shows a much more pronounced deficit in striatal ACh release (80%) than the VGLUT3-null mice, yet they are not hyperactive. Therefore, in three distinct lines of mice in which VAChT expression was decreased to 50% globally (VAChT KD^{HET} and $VAChT^{wt/del}$) or to more than 90% in the striatum ($VAChT^{D2\text{-}cre\text{-}flox/flox}$) there was no change in locomotor activity. We suggest that the locomotor phenotypes observed previously in striatal cholinergic neuron-ablated mice (Hikida et al., 2001;Kitabatake et al., 2003) and in VGLUT3-null mice (Gras et al., 2008) might be related to the elimination of VGLUT3-mediated neurotransmission or the combination of reduced glutamatergic and cholinergic activity. Future experiments using $VAChT^{D2\text{-}Cre\text{-}flox/flox}$ mice can provide an assessment of independent effects of VGLUT3-mediated neurotransmission in the striatum.

Although elimination of cholinergic neurotransmission in the striatum does not cause hyperlocomotion, other cholinergic neuronal groups may play important roles in locomotor activity. Pharmacological modulation of cholinergic receptors can modulate locomotor activity. It is long been known that muscarinic antagonists increase locomotor activity and M1-muscarinic receptor KO mice are hyperactive (Gerber et al., 2001;Miyakawa et al., 2001). M4-muscaring receptor KO mice are also hyperactive, likely due to lack of inhibitory effects on D1-expressing neurons in the striatum (Gomeza et al., 1999;Guo et al., 2010;Jeon et al., 2010). Because G-protein coupled receptor may present agonist-independent intrinsic activity, these experiments assess the roles of specific receptors. The present experiments present complementary evidence for the role of the neurotransmitter ACh in the striatum, indicating that inhibition of VAChT or its decreased expression does not cause overt motor consequences. This may be particular important when considering that in Huntington's disease VAChT levels are decreased in the striatum (Smith et al., 2006). Our experiments would suggest that this alteration will not participate in motor symptoms in Huntington's disease.

In agreement with the normal locomotor activity observed in $VAChT^{D2\text{-}cre\text{-}flox/flox}$, but distinct from previous experiments in which was concomitantly VGLUT3 inactivated (Kitabatake et al., 2003), we found that $VAChT^{D2\text{-}cre\text{-}flox/flox}$ are not more sensitive to the locomotor effects of cocaine. Interestingly, we do find that hyperdopaminergic mice, that were treated with galantamine present an immediate decrease in locomotion when in a new environment, as previously observed for other cholinesterase inhibitors (Hikida et al., 2003).

Importantly, this effect of galantamine could also be observed in VAChT$^{D2\text{-}cre\text{-}flox/flox}$ mice, suggesting that it was not related to increased cholinergic tone in the striatum.

VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice respond to fluoxetin in the forced-swimming task

Is the improvement in performance detected in the forced swimming-task a result of an indirect effect of suppression of ACh release in the striatum in other neurotransmitter systems? Performance in the forced-swimming task can be significantly improved by clinically used antidepressants, such as serotonin uptake inhibitors. To test if VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice performed better in the forced-swimming test because indirectly the serotonin system was affected, we repeated the forced-swimming experiments in animals treated with fluoxetine. These experiments indicated that fluoxetine was effective to improve performance in the forced-swimming task in control and VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice, suggesting that the effects of inactivation of striatal VAChT and inhibition of serotonin transporter are additive.

Conclusions

We introduced here a new experimental model, the VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice, to understand the consequences of cholinergic neurotransmission and dopaminergic-cholinergic balance in the striatum. Our data suggest the need for re-evaluation of previous attributed functions of cholinergic neurons and indicate the possibility that VGLUT3-mediated glutamatergic neurotransmission may have a larger influence in striatal function than previously envisioned. We provide evidence that mice with selective elimination of VAChT in the striatum are remarkably normal. The improved performance of these mice in the forced-swimming test suggests that VAChT inhibition in the striatum may have antidepressant effects. Because in PD there is increased activation of cholinergic neurons in the striatum, in addition to the fall of dopamine levels, our experiments open the possibility that targeting VAChT may improve hypercholinergic-related symptoms in PD.

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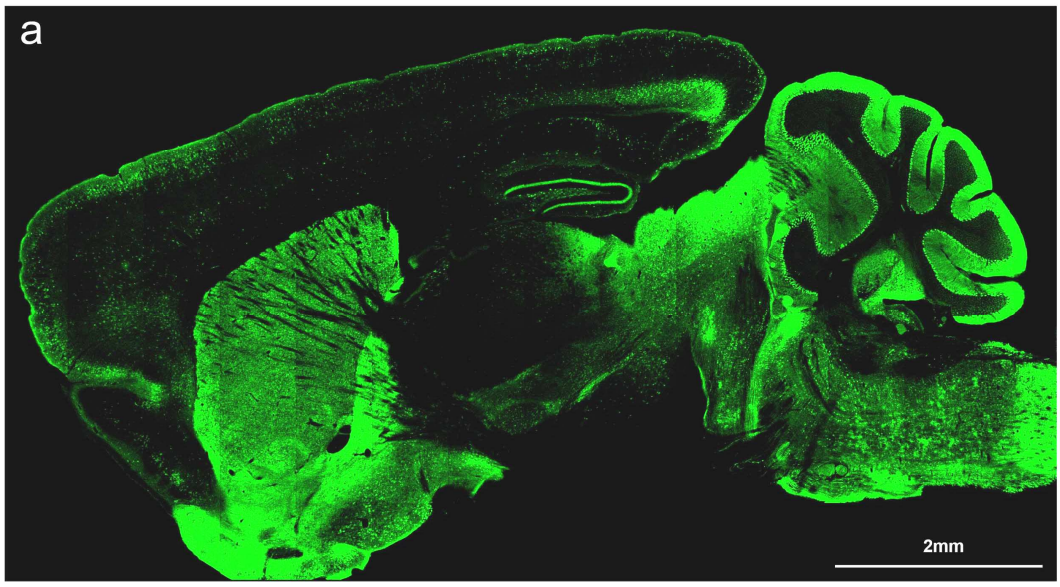
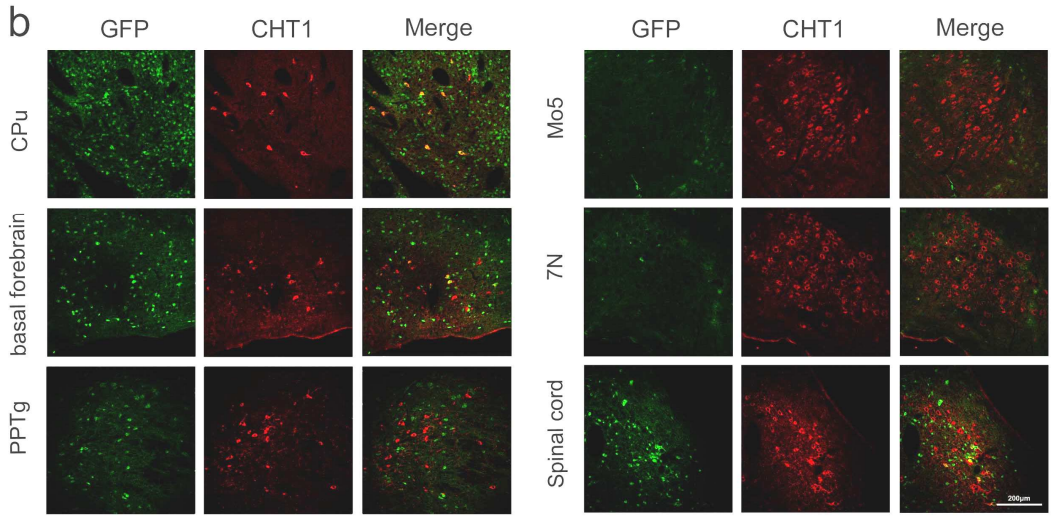
Figures and legends :





c

Brain region	Percentage colocalization d2Cre and CHT1
Caudate Putamen (striatum)	≈ 96 %
Basal Forebrain (HDB/SI/MCPO)	≈ 58%
Pedunculopontine Tegmental nucleus	≈ 6%
Motor Trigeminal nucleus	≈ 1%
Facial nucleus	≈ 4%

Figure 1. D2-Cre drives the expression of Cre in striatal cholinergic neurons. (a) Expression pattern of Cre detected by staining of YFP in the brain of D2-Cre x Rosa26-YFP mice. (b) Sections from different regions of the central nervous system were immunóstained for CHT1 (Red) and YFP (Green) in D2-Cre x Rosa26-YFP mice. (c) Percentage of Cre expression (YFP) in cholinergic neurons (CHT1 staining). These images are representative of 3 mice that were double stained for CHT1 and YFP.

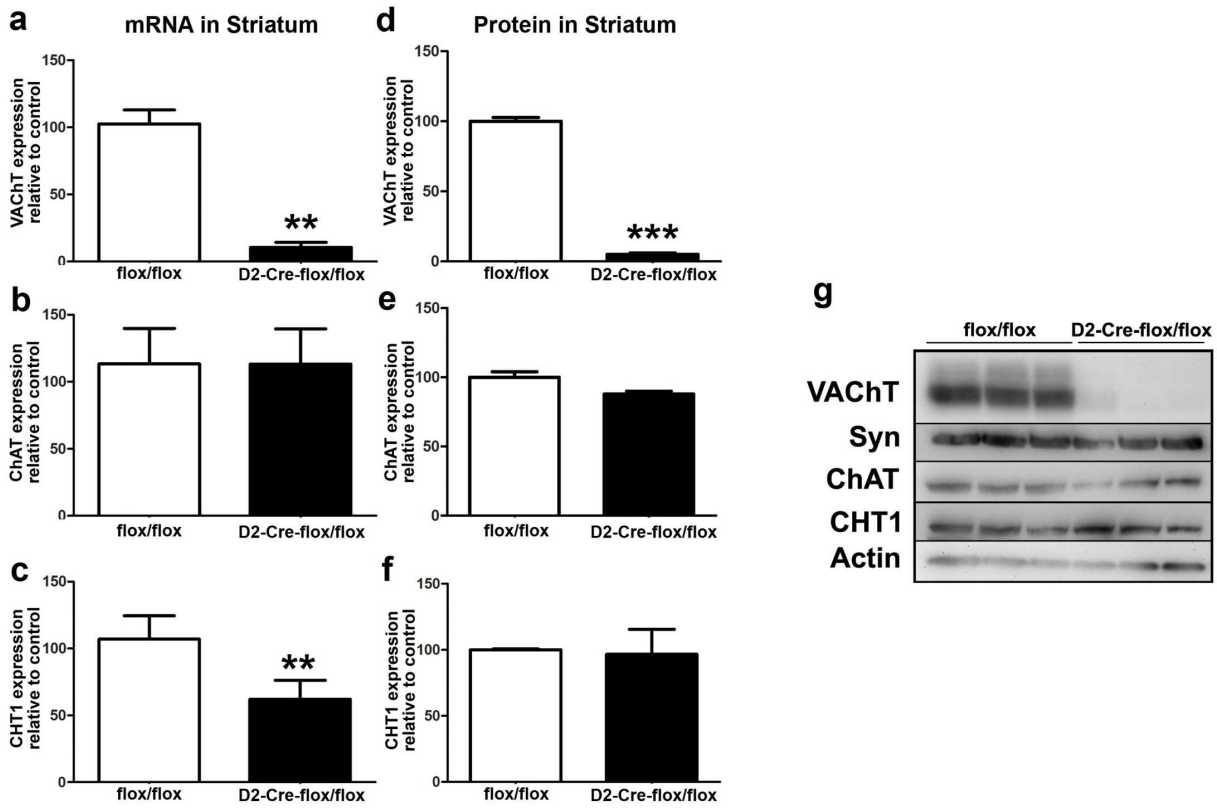


Figure 2. Expression of VAChT in the striatum of VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice.

(a) VAChT mRNA expression (b) ChAT mRNA expression (c) CHT1 mRNA expression. (d) VAChT protein expression (e) ChaT protein expression (f) CHT1 protein expression (g) Representative immunioblot of control and VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice. ** and *** indicate $p<0.01$ and $p<0.001$ respectively. mRNA expression levels was quantified by qPCR using actin to normalize the data and figures represent N=5 mice. Protein levels were quantified using synaptophysin as a loading control. N=5

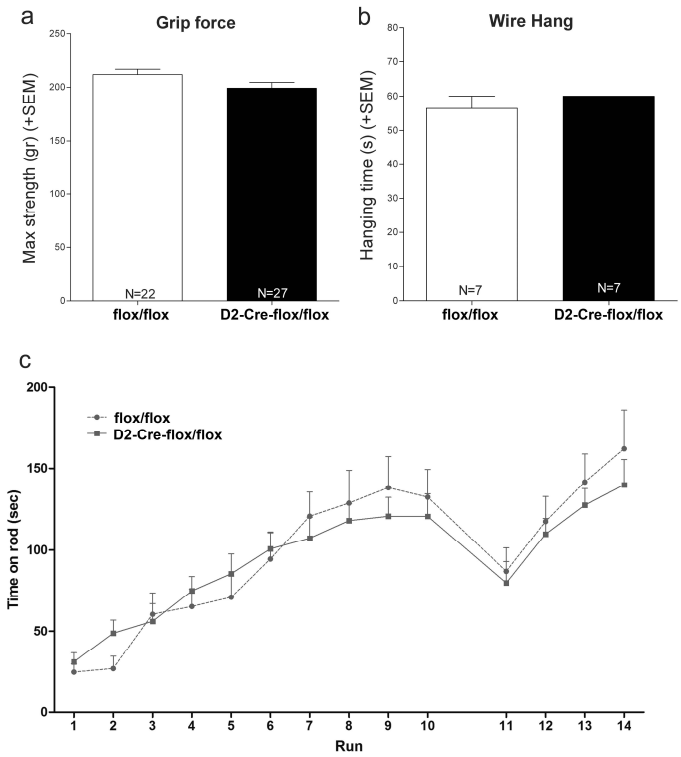


Figure 3. Motor function is not altered in VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice. (a) grip-force analysis of VAChT$^{flox/flox}$ and VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice. (b) Time spent hanging upside-down from a grid, to measure fatigue, for VAChT$^{flox/flox}$ mice and VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice. Cut-off time 60s. (c) Motor learning and acrobatic motor skills of VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice determined using the rotarod**.**

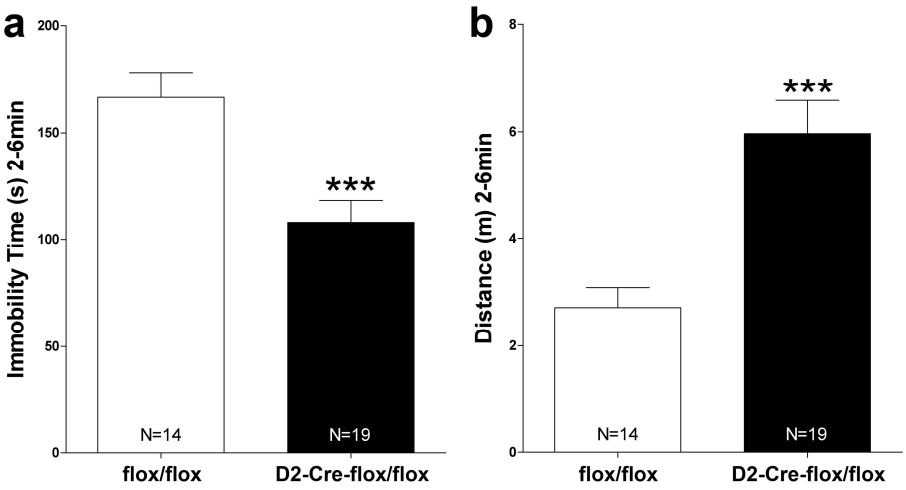


Figure 4 VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice perform better than control mice in the forced-swimming task. (a) Immobility time in the forced swimming-test automatically detected using Anymaze. (b) Swimming distance calculated using Anymaze. *** represents $p<0.001$. Mice were considered immobile if their centre of gravity did not move for 800 ms.

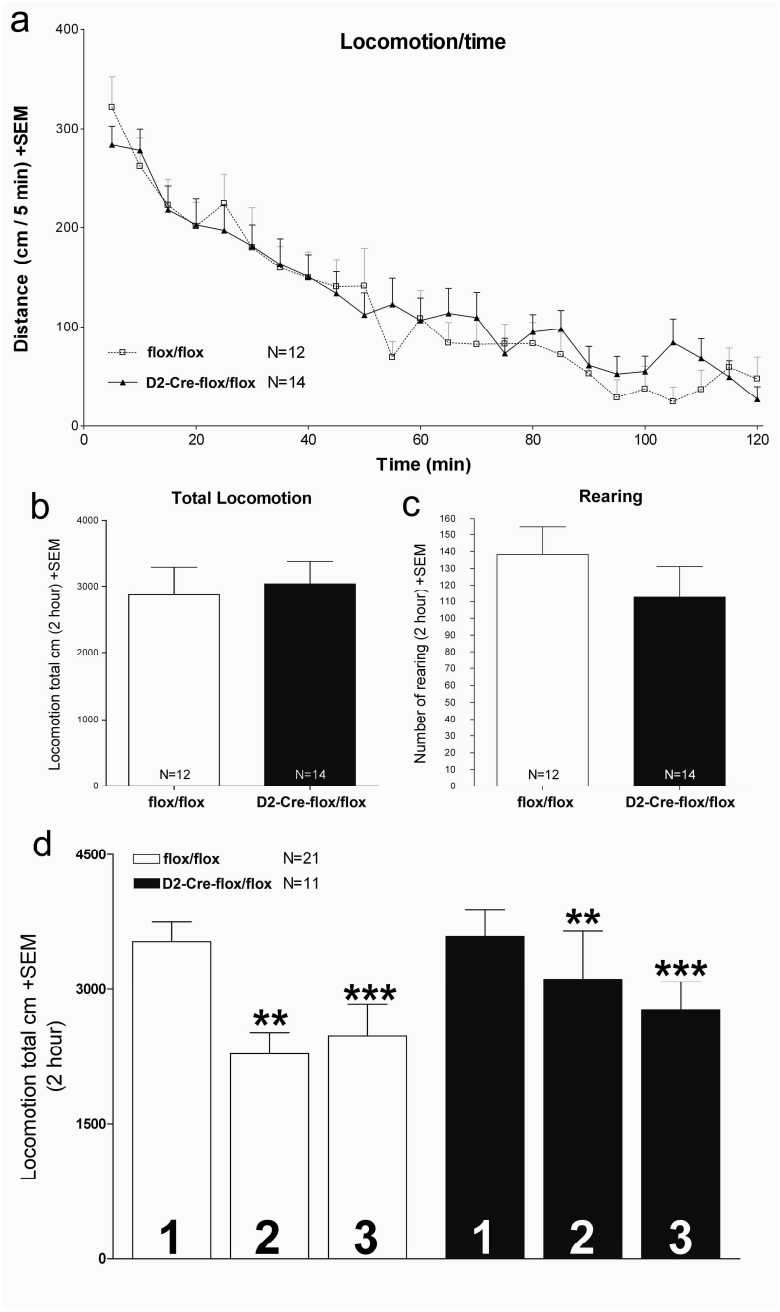


Figure 5 Locomotor activity of VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice. (a) Horizontal locomotor activity in an open-field for VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ and control mice (b) Cumulative 2 h locomotion VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ and control mice (c) Cumulative rearing activity (2h) for VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ and control mice (d) Habituation in the open-field measured in three consecutive days. ** $p<0.01$, *** $p<0.001$ compared to the first day.

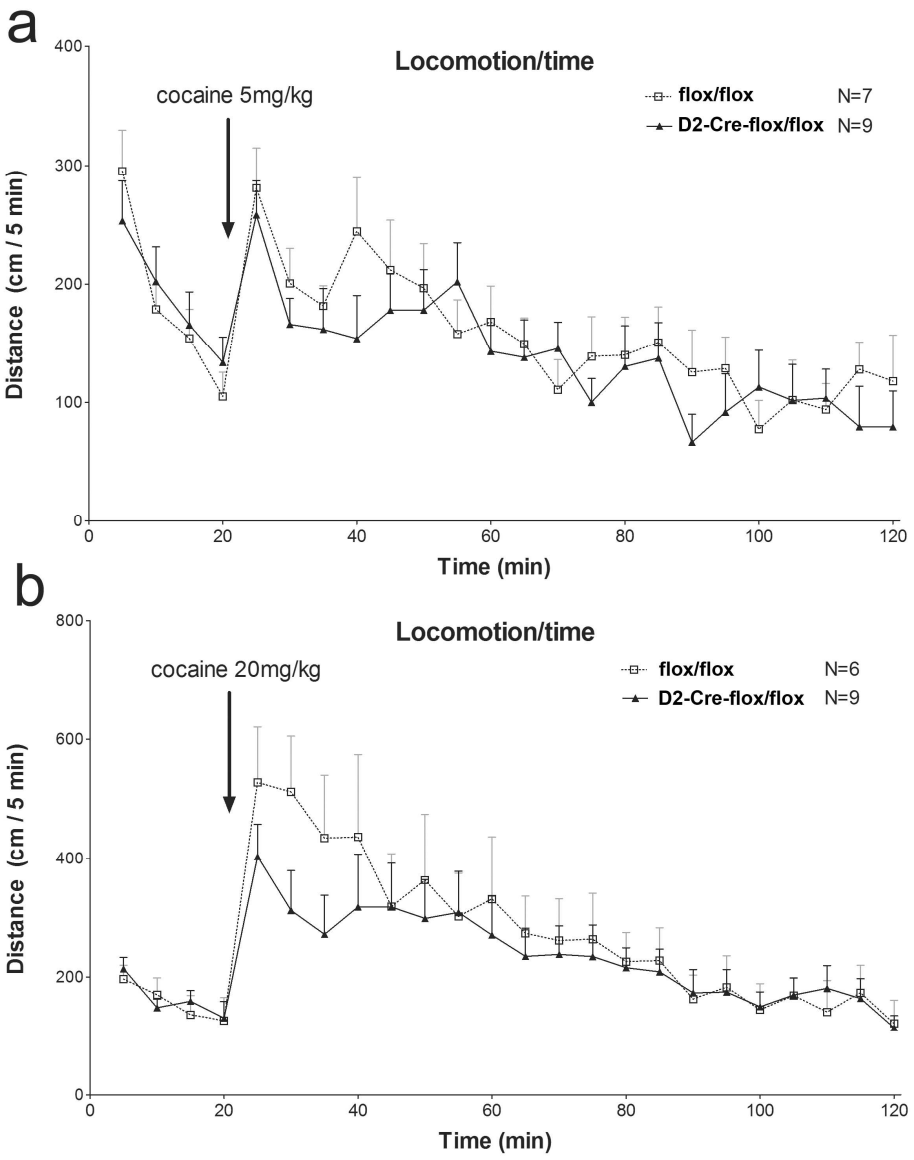


Figure 6: Cocaine-mediated locomotor activity in VAChT$^{D2\text{-}Cre\text{-}flox/flox}$. (a) Mice were injected with 5mg/kg of cocaine after 20 min in the open-field and horizontal locomotor activity was measured (b) Mice were injected with 20 mg/kg of cocaine after 20 min in the open-field and horizontal locomotor activity was measured open field. Injection of saline did not change locomotor activity for either genotype (not shown).

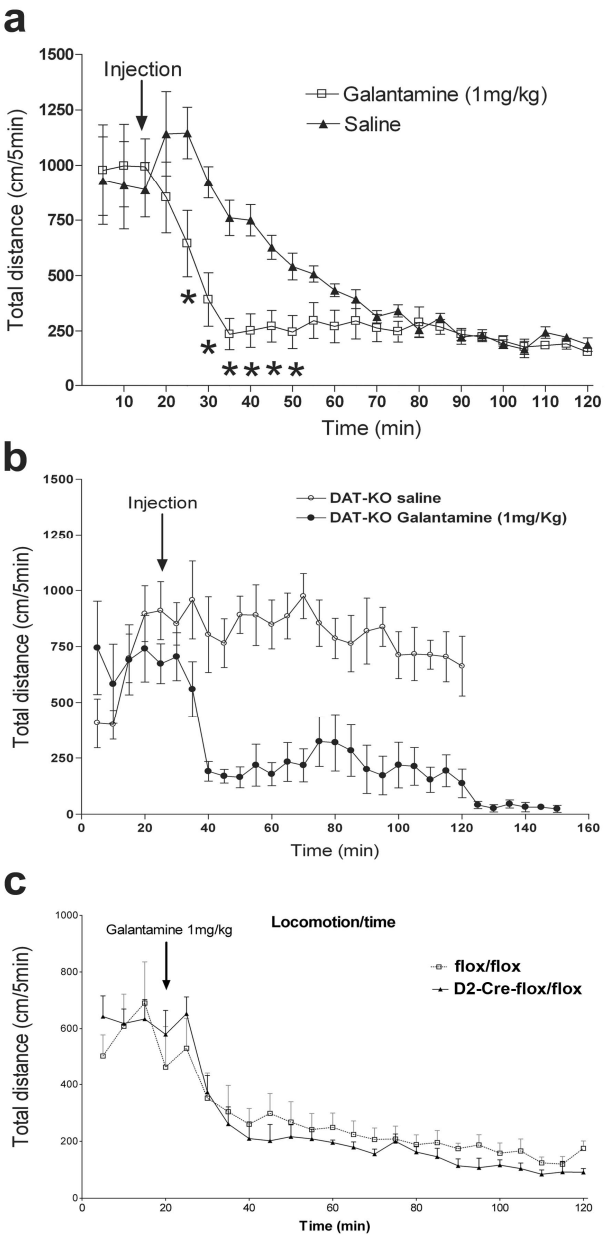


Figure 7: Galantamine decreases hyperlocomotion evoked by cocaine independently of functional cholinergic neurons in the striatum. (a) Wild-type mice were injected with cocaine (20 mg/kg) 5 min before being introduced to the open-field. After 15 min to estimate ambulance mice were injected with either saline or galantamine (1 mg/kg, i.p.). N= 8 galantamine and 8 saline . (b) DAT KO mice were introduced to the open-field and after 25 min they were injected with either saline or galantamine (1 mg/kg, i.p.). N= (c) $VAChT^{D2\text{-}Cre\text{-}flox/flox}$ and control mice were injected with cocaine (20 mg/kg) and introduced to the open-field. After 20 min they were injected with galantamine (1mg/kg, i.p.) * $p<0.05$ compared to saline injected mice.

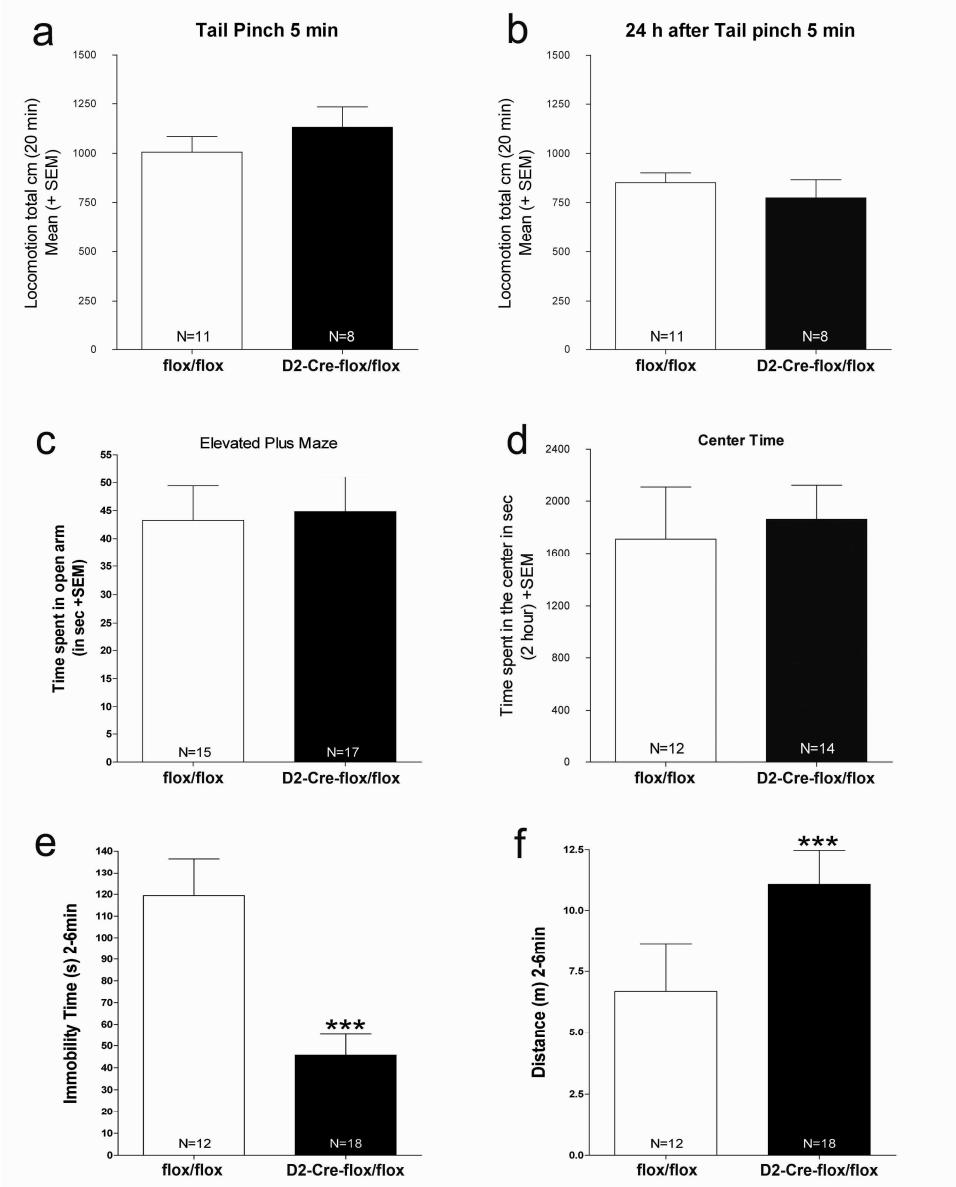
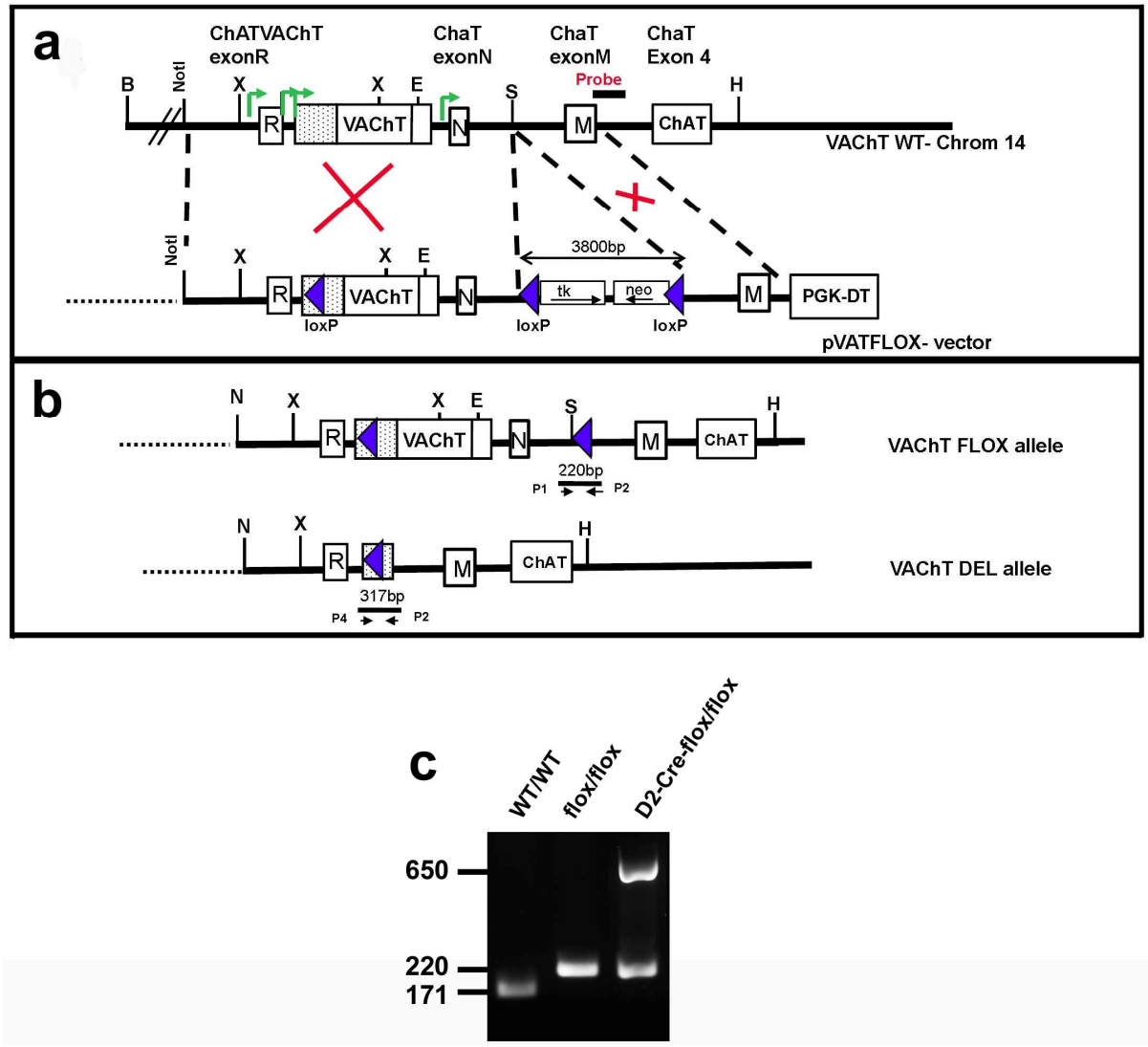
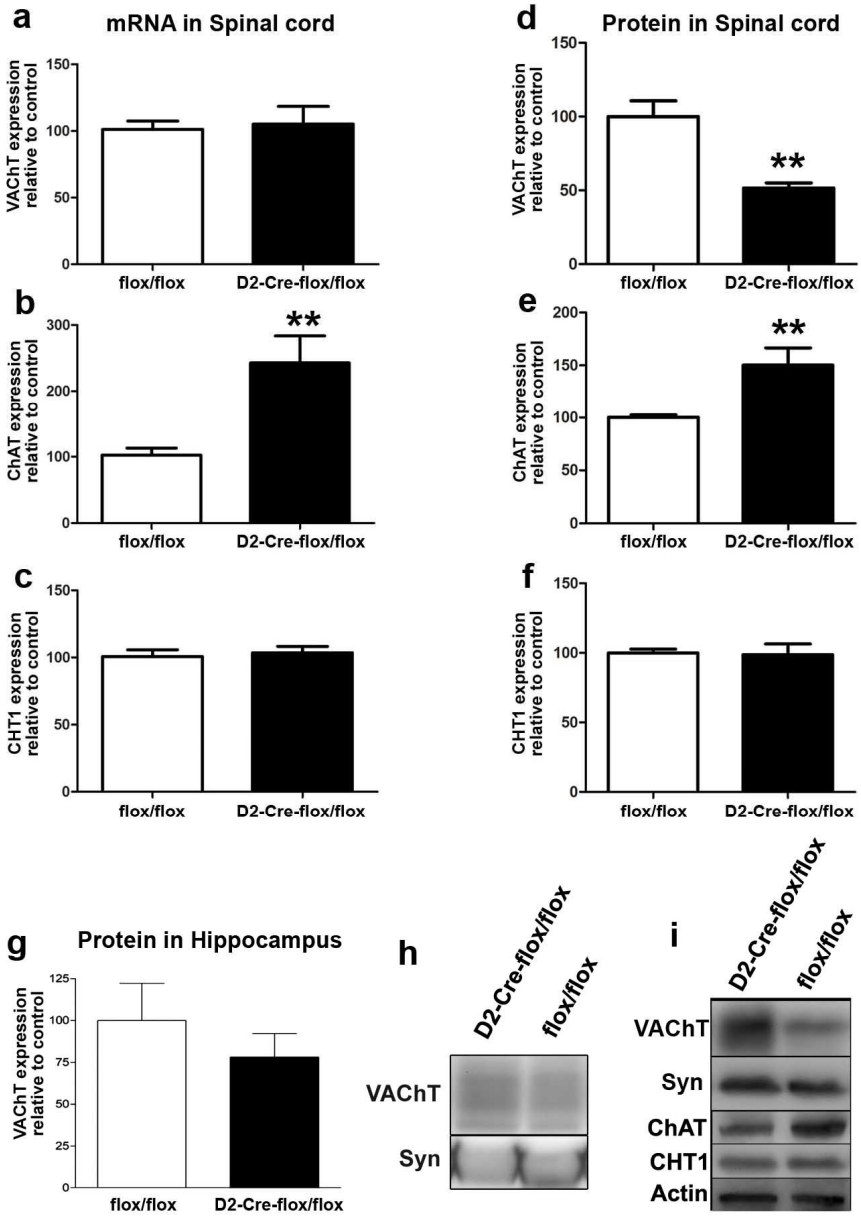


Figure 8: VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice are not more active because of stress and can respond to fluoxetine in the forced-swimming task. (a) Cumulative horizontal activity (20 min) immediately after a 5 min of tail-pinch for VAChT$^{flox/flox}$ and VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice (b) Cumulative horizontal activity (20 min) for VAChT$^{flox/flox}$ and VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice 24 h after tail-pinch (c) Elevated-plus-maze performance for VAChT$^{flox/flox}$ and VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice (d) Time spent in the centre of the open-field for VAChT$^{flox/flox}$ and VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice (e) Immobility time in the forced swimming-test for VAChT$^{flox/flox}$ and VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice injected 30 min prior to the test with fluoxetine (20 mg/kg, i.p.) automatically detected using Anymaze. (f) Swimming distance calculated using Anymaze in the same condition as in (e). *** represents $p<0.001$. Mice were considered immobile if their centre of gravity did not move for 800 ms.



Supplementary figure 1: (a) Constructs used for generation of VAChT$^{flox/flox}$ and VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice (b) Representation of the VAChT$^{flox/flox}$ allele before (FLOX allele) and after Cre-mediated recombination (Del allele) and VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice (c) Genotyping for WT, VAChT$^{flox/flox}$ and VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice



Supplementary figure 2: (a, b, c) Quantification of mRNA expression for VAChT, ChAT and CHT1 in the Spinal cord. (d, e, f) Quantification of protein levels in the Spinal cord for VAChT, ChAT and CHT1. Synaptophysin immunoreactivity was used to correct for protein loading between experiments. (g) Quantification of protein levels in the hippocampus for VAChT, protein loading was corrected with Synaptophysin. (h) Representative hippocampus Western- blot for VAChT and synaptophysin. ** and *** indicates respectively $p<0.01$ and $p<0.001$) (i) Representative spinal cord Western-blot of VAChT, synaptophysin, ChAT, CHT1 and actin.

Discussão

Nósso grupo gerou, através de técnicas de recombinação homóloga, camundongos com diferentes níveis de redução na expressão do VAChT. Além disso, fomos capazes de gerar animais com deleção do VAChT em regiões específicas do cérebro, abrindo uma nova área de investigação para compreender o papel da ACh no sistema nervoso central. Considerando-se que a expressão do VAChT é capaz de regular a quantidade de ACh presente em vesículas sinápticas, estes animais representam modelos únicos para o estudo do impacto da hipofunção colinérgica sobre funções fisiológicas, além de permitirem a compreensão do papel do VAChT no sistema nervoso central e periférico.

I- Modelos de animais de hipofunção colinérgica

A geração de modelos animais de disfunção colinérgica apresenta vários desafios. Os animais VAChT KD permitem o estudo do papel da armazenagem da ACh nas funções cognitivas e neuromuscular. Porém, o déficit muscular dos animais VAChT KD^{HOM} se revelou um problema em alguns testes que envolvem exercício físico (como a natação). Animais nocaute $VAChT^{WT/DEL}$ nós permitem estudar as funções cognitivas, porém, esses animais apresentam uma diminuição da liberação de ACh de apenas 50%. O estudo de animais adultos homozigotos é impossível, uma vez que os animais $VAChT^{DEL/DEL}$ nascem mortos. Portanto, uma análise da deleção de VAChT em regiões específicas do cérebro se revelou necessária. Para a geração de animais nocaute específicos, foi utilizado o sistema cre/loxP. O sistema Cre/loxP permite a geração de camundongos nocaute condicionais em que há um controle espacial da expressão de um gene alvo (Morozov et al., 2003). A possibilidade de utilizar animais que expressam a enzima Cre sobre o controle de diferente promotores nós permite estudar a função da liberação de ACh controlada por VAChT em um região determinada como o estriado, no caso dos animais $VAChT^{D2\text{-}Cre\text{-}flox/flox}$.

II- Reconhecimento de objeto

Nóssos resultados demonstram a importância do sistema colinérgico para as funções cognitivas. Após analisarmos duas linhagens de animas mutados para VAChT (VAChT KD^{HET} e $VAChT^{WT/DEL}$), e animais selvagens tratados com atropina, vimos que a liberação de ACh é essencial para a memória de longo prazo, mas não para a memória remota.

A injeção após o treino de um antagonista de receptores muscarínicos em camundongos selvagens e de galantamina nós camundongos mutantes para VAChT, demonstrou a importância da ACh na consolidação celular da memória. De fato, injetando-se os animais imediatamente após o treino, excluímos qualquer efeito decorrente da injeção sobre a aquisição e podemos estudar unicamente a consolidação da memória, mecanismo essencial para conservar uma informação a longo prazo. Esse tipo de injeção nós permite também, excluir um possível efeito da ACh sobre atenção como foi sugerido por Ennaceur (Ennaceur and Meliani, 1992).

Usando camundongos mutados em regiões específicas do cérebro, nós observamos que a presença de VAChT no estriado não é importante para a memória de reconhecimento de objeto. Ao contrário, uma deleção mais acentuada de VAChT nós neurônios colinérgicos do prosencéfalo basal observado nós camundongos $VAChT^{Six3\text{-}CreFlox/Flox}$ (anexo 1, figura 1) leva a um déficit de memória de longo prazo (Anexo 1, Figura 2). O déficit dos animais $VAChT^{Six3\text{-}CreFlox/Flox}$ pode ser devido a diminuição de liberação da ACh no hipocampo ou no córtex perirhinal, que foi demonstrado ser especialmente importante no teste de reconhecimento de objeto (Warburton et al., 2003; Abe et al., 2004; Winters and Bussey, 2005c; Barker and Warburton, 2009).

A memória remota depende de regiões corticais, enquanto a memória de longo prazo depende de regiões mais internas, como o hipocampo. No teste de reconhecimento de objeto, o hipocampo é envolvido apenas durante um tempo curto após a aquisição. De fato, dois dias após o treino, a reativação da memória envolve regiões corticais como o córtex enthorinal e prefrontal (Romero-Granados et al., 2010). Considerando o modelo descrito em (Romero-Granados et al., 2010), nós podemos sugerir que a ACh é necessária para a consolidação celular da memória de longo prazo no hipocampo e não é envolvida na consolidação sistêmica da memória remota nas regiões corticais.

III- Atividade locomotora

Nóssos resultados mostram que uma redução de 75% da expressão do VAChT no prosencéfalo como visto nós camundongos $VAChT^{FloxNeo/FloxNeo}$ e 85% nós camundongos $VAChT^{FloxNeo/Del}$ produz um grande aumento da atividade locomotora. É sabido que os antagonistas de receptores muscarínicos causam hiperatividade (Molinengo et al., 1989; Shannon and Peters, 1990; Ukai et al., 1994; Chintoh et al., 2003). Da mesma forma, o

aumento da atividade locomotora foi observado nós camundongos KO para os receptores M1 e M4 (Gomeza et al., 1999b; Miyakawa et al., 2001) e nós camundongos mutantes para receptor nicotínico Beta 2 (Granon et al., 2003).

Apesar da diminuição do tônus colinérgico no estriado nós animais $VAChT^{D2\text{-}Cre\text{-}flox/flox}$ nós não observamos o aumento da locomoção. Foi observado que uma injeção de imunotoxina seletiva para os neurônios colinérgicos que remove perto de 80 % do neurônios positivos para ChAT no estriado provoca um aumento da locomoção (Kaneko et al., 2000; Kitabatake et al., 2003). A diferença entre nóssos resultados com os precedentes pode ser explicada pela presença de VGLTU3 nós neurônios colinérgicos (Fremeau et al., 2002; Gras et al., 2002; Schafer et al., 2002). Os camundongos KO para VGLUT3 apresentam uma hiperlocomoção que os autores acreditam estar relacionada a uma redução de 40% da liberação de ACh que foi observada. Porém, nós observamos anteriormente com os camundongos VAChT KD^{HET} e $VAChT^{WT/Del}$ que uma redução de 50% na liberação de ACh não altera a locomoção (de Castro et al., 2009b; de Castro et al., 2009a). Isso nós leva a sugerir que a hiperlocomoção observada nós animais KO para VGLUT3 não é devida a uma diminuição da liberação da ACh mas, provavelmente, à falta de liberação de glutamato. A hiperlocomoção observada nós animais $VAChT^{FloxNeo/FloxNeo}$ e $VAChT^{FloxNeo/Del}$, que não foi vista nós animais $VAChT^{D2\text{-}Cre\text{-}flox/flox}$ deve ser devida a uma região do cérebro diferente do estriado.

Uma diminuição de VAChT nós neurônios da região do prosencéfalo basal presente nós animais $VAChT^{Six3\text{-}CreFlox/Flox}$ induz um aumento da atividade locomotora (Anexo 1 Figura 3a e 3c). Da mesma forma, uma injeção de imunotoxina saporina p75 provoca nós camundongos com uma lesão dos neurônios colinérgicos do prosencéfalo basal hiperlocomoção (Moreau et al., 2008). Isto nós sugere que o tônus colinérgico dos neurônios do prosencéfalo basal poderia ser importante para manter a locomoção normal.

Nós mostramos que o aumento de locomoção induzido pela cocaína pode ser reduzido com o aumento da disponibilidade de ACh (Prado et al., 2006). A injeção de galantamina reduziu a atividade locomotora tanto em animais selvagem pré-injetados com cocaína quanto em animais DAT-KO que apresentavam hiperlocomoção basal. Desta maneira, nós mostramos que a ACh tem um efeito hipolocomotor, ao contrário da hiperlocomoção provocada pela dopamina. Os animais $VAChT^{D2\text{-}Cre\text{-}flox/flox}$ pré-injetados com cocaína apresentam uma diminuição da atividade locomotora após injeção de galantamina (1mg/kg), portanto, o efeito da Ach sobre a hiperlocomoção induzida pela cocaína é independente do tônus colinérgico do estriado.

Os animais VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ apresentaram um aumento similar aos controles na atividade locomotora após tratamento com 5 mg/kg de cocaína. Por outro lado, camundongos que tiveram os neurônios colinérgicos do estriado removidos apresentaram um grande aumento da atividade locomotora induzida pela cocaína (Hikida et al., 2001). Mais uma vez, podemos explicar essa diferença entre nóssos resultados e o que foi observado por Hikida e colaboradores através da presença do transportador VGLUT3 nós neurônios colinérgicos. Animais KO para o VGLUT3 também apresentam aumento de sensitividade aos efeitos locomotores da cocaina. Esse efeito seria, portanto, controlado no estriado pelo glutamato liberado pelos neurônios colinérgicos e não pela liberação da ACh.

A teoria de Dale, que propõe que um mesmo neurotransmissor é apenas liberado para um tipo de neurônio (Dale, 1935), se revelou falsa no final dos anós 90. Jonas e colaboradores mostraram que um mesmo neurônio pode liberar os neurotransmissores glicina e GABA (Jonas et al., 1998). Mais tarde, foi também demonstrado que os neurônios colinérgicos podem liberar glutamato, além de acetilcolina (Li et al., 2004; Nishimaru et al., 2005; Allen et al., 2006). Nóssos resultados, somados aos de outros autores (Hikida et al., 2003; Kitabatake et al., 2003; Gras et al., 2008), reforçam a idéia de que um mesmo neurônio possa liberar diferentes neurotransmissores, alem de atribuir uma função aos diferentes neurotransmissores liberados por neurônios colinérgicos do estriado.

IV- Estresse e Depressão

Nós verificamos se a diminuição da liberação da ACh poderia afetar a ansiedade nós animais mutantes ao determinar o tempo em que eles ficavam no centro do campo aberto e através do teste da cruz elevada. Nóssos resultados mostraram que a eliminação do VAChT no estriado ou a diminuição deste transportador no cérebro inteiro não modifica o nível de ansiedade, a resposta dos camundongos no teste da cruz elevada e no tempo em que eles passam no centro do campo aberto são similar aos selvagens. Assim como nóssos animais mutantes, camundongos KO heterozigotos para CHT1 também não apresentaram diferença em relação aos selvagens no teste da cruz elevada (Bazalakova et al., 2007).

A incidência de depressão nós pacientes com Parkinson é muito alta. Sendo a doença de Parkinson caracterizada por um distúrbio do balanço Acetilcolina-Dopamina no estriado, nós estudamos os animais VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ para testar se os mesmos apresentam um comportamento tipo depressivo diferente dos selvagens. Nós observamos que os animais knockout para VAChT que apresentam uma redução de 90% do VAChT no estriado

apresentam uma redução do comportamento depressivo. Os animais VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$, que apresentam 60%-70% de redução de VAChT no estriado, apresentam uma diminuição não significativa do tempo de imobilidade no teste de natação forçada (Anexo 1, Figura 4). Isto nós sugere que o estriado e uma região do cérebro que tem um papel central no mecanismo de depressão. Mais específicamente especulamos que o núcleo accumbens poderia ser a estrutura do estriado responsável por este comportamento, uma vez que injeções de agonista e antagonista colinérgicos nessa região modifica o tempo de imobilidade no teste de Porsolt (Chau et al., 2001). A participação da ACh no controle da depressão foi também confirmada através da geração de camundongos KO para os receptores nicotínicos Beta 2 (Caldarone et al., 2004).

A diminuição do tempo de imobilidade no teste da natação forçada, nós animais VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ não pode ser relacionado a um aumento de atividade locomotora, uma vez que eles não apresentam aumento de locomoção no campo aberto. Porém, a diminuição do tempo de imobilidade pode ser explicada por uma resposta comportamental diferente dos animais em condição de estresse provocada pelo teste de Porsolt. Os animais VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ não apresentam diferença na atividade locomotora quando estressados com beliscadura da cauda antes de serem colocados no campo aberto. Com isso, demonstramos que uma redução da liberação de ACh no estriado provoca uma diminuição do tempo de imobilidade no teste de Prosolt. Isto ocorre devido a uma diminuição do comportamento depressivo e não um aumento da atividade locomotora.

A Fluoxetina, um antidepressivo clássico, é um inibidor seletivo da recaptação de serotonina e provoca uma redução da imobilidade no teste de Porsolt em camundongos VAChT$^{flox/flox}$ e VAChT$^{D2\text{-}Cre\text{-}flox/flox}$. Contudo, mesmo após tratamento com fluoxetina, camundongos VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ apresentam um comportamento menós depressivo que os controles. Podemos concluir portanto que o efeito anti-depressivo provocado pela diminuição de ACh no estriado é independente do sistema serotoninérgico.

Foi observado anteriormente que o tratamento com benztropine (antagonista dos receptores muscarínicos) de um paciente com Parkinson tratado previamente com fluoxetina teve um efeito antidepressivo maior (Huszonek, 1995). Assim, os camundongos deletados para VAChT no estriado poderiam ser uma ferramenta importante para o estudo de novas drogas antidepressivas para os pacientes do Parkinson. Além disso, ≈50% de pacientes com depressão não respondem ao tratamento clássico com inibidor da recaptação da serotonina (Mineur and Picciotto, 2010). Portanto, o estudo de droga colinérgicas antidepressivas poderia fornecer uma alternativa nós tratamentos desses pacientes.

V- Conclusão

A ACh é necessária para o processo de consolidação celular da memória de longo prazo, mas sua ausência não bloqueia a formação da memória remota. Os neurônios colinérgicos do prosencéfalo basal aparentemente participam deste processo. Nóssos resultados sugerem que o tônus colinérgico no estriado não é necessário para manter uma atividade locomotora normal, e que a hiperlocomoção induzida pela cocaína é independente desse tônus. Porém, nóssos resultados sugerem indiretamente que a presença de VGLUT3 nós neurônios colinérgicos do estriado é indispensável para manter uma locomoção normal. Nós podemos sugerir que a hiperlocomoção dos animais com diminuição geral da liberação da ACh é devida à diminuição de liberação da ACh dos neurônios colinérgicos do prosencéfalo basal. Os camundongos mutantes para o $VAChT^{D2\text{-}Cre\text{-}flox/flox}$ nós permitiram sugerir que o balanço entre a acetilcolina e a dopamina no estriado é essencial para a expressão do comportamento depressivo. Esses camundongos se revelaram um modelo único para o estudo da depressão, busca por novas drogas antidepressivas e o estudo específico do papel da ACh no estriado nós processos cognitivos.

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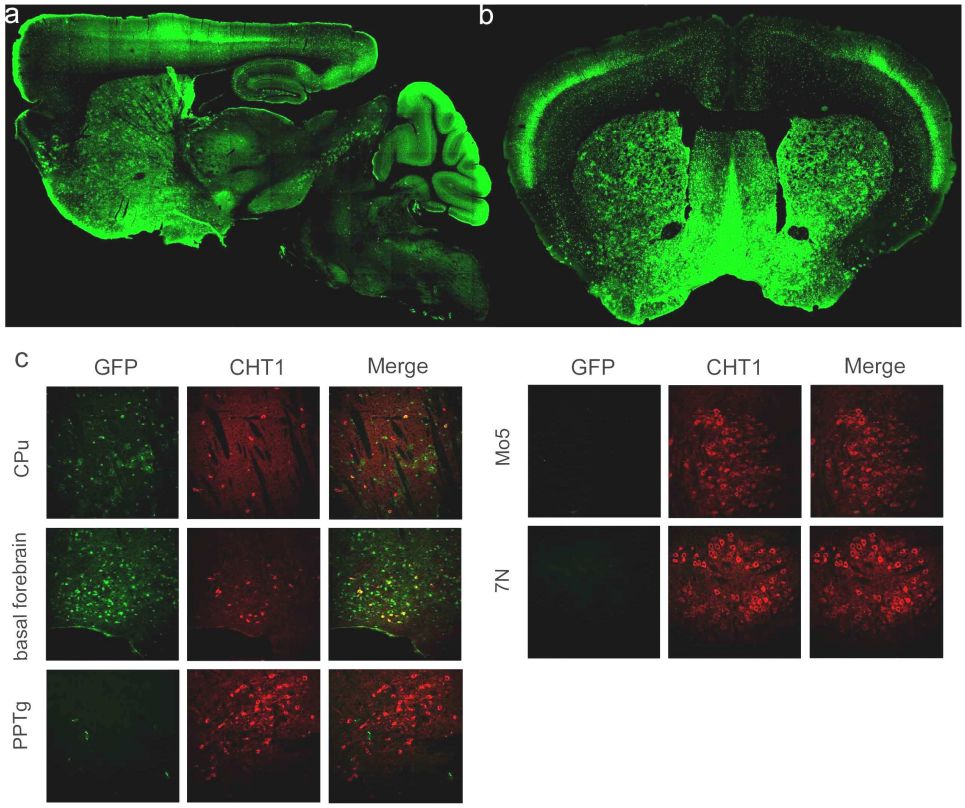
Anexo 1



d

Brain region	Percentage colocalization d2Cre and CHT1
Caudate Putamen (striatum)	≈ 58 %
Basal Forebrain (HDB/SI/MCPO)	≈ 84%
Pedunculopontine Tegmental nucleus	≈ 3%
Motor Trigeminal nucleus	≈ 4%
Facial nucleus	≈ 0%

Figura 1. a,b) Padrão da expressão de Cre marcada com YFP no cérebro de camundongos Rosa26-YFP x Six3-Cre. c) Diferentes regioes do cérebro foram marcadas para YFP (verde) e CHT1 (vermelho) em camundongos Six3-Cre x Rosa26-YFP. d) Porcentagem de expressão de Cre (YFP) nós neurônios colinérgicos (marcados para CHT1) .

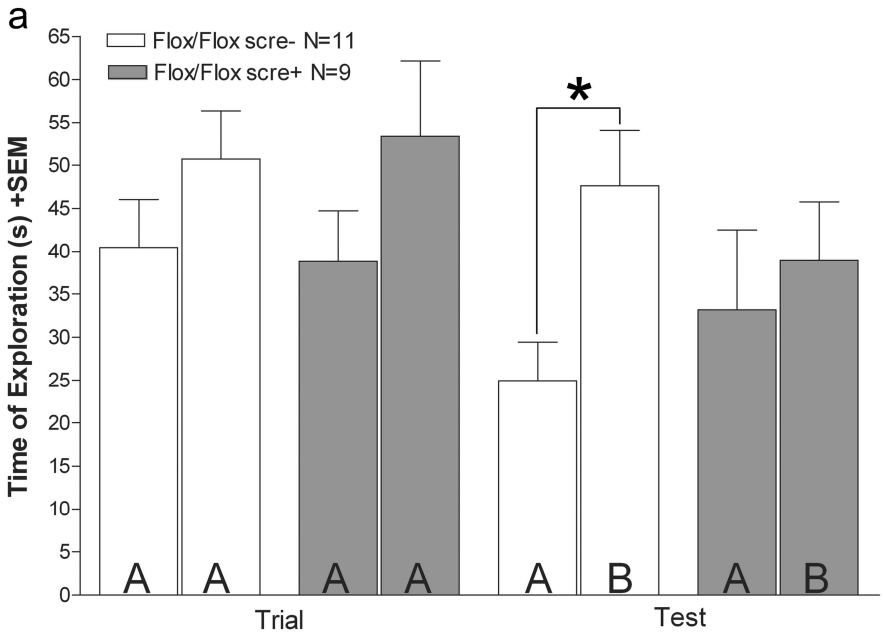


Figura 2. a) Tempo de exploração no reconhecimento de objeto durante o treino e o teste para os camundongos VAChT$^{Flox/Flox}$ e VAChT$^{six3\text{-}Cre\text{-}Flox/Flox}$. A e B representam respectivamente o objeto familiar e novo. * refere a uma diferença significativa entre o objeto A e B.

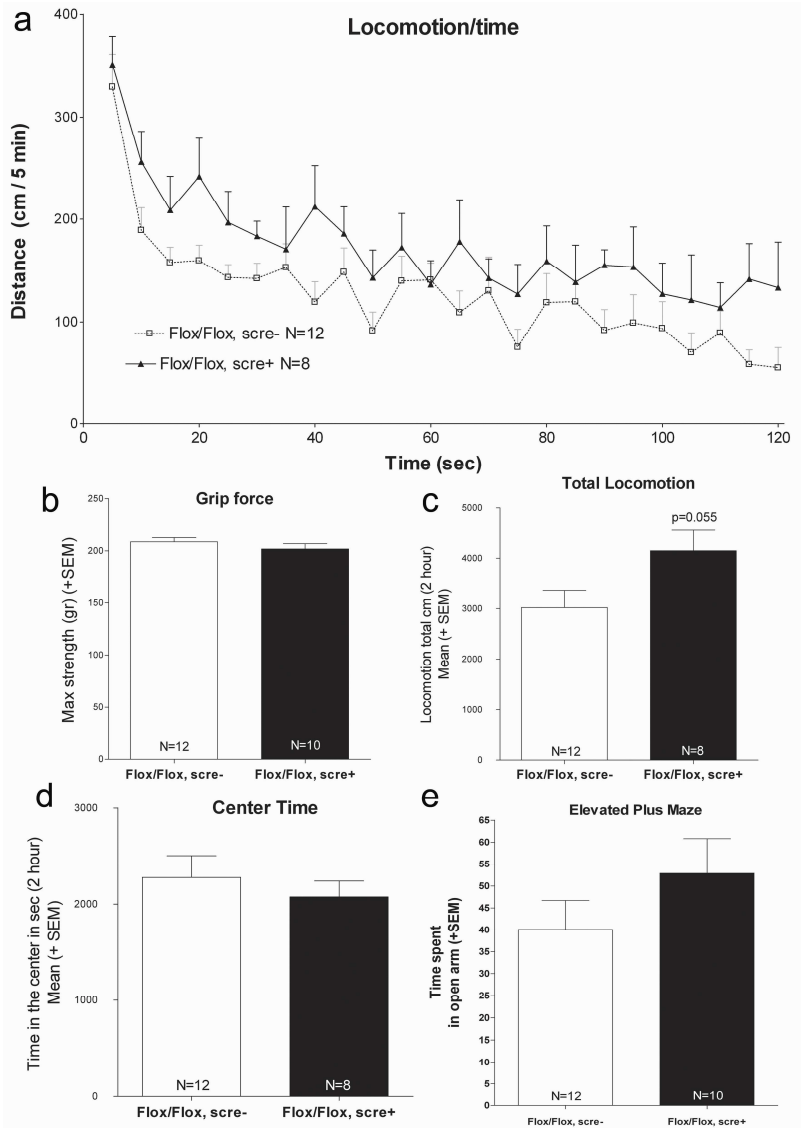


Figura 3. a) Atividade Locomotora de camundongos controle (quadrados brancos) e camundongos VAChT$^{six3\text{-}Cre\text{-}Flox/Flox}$ (quadrados pretos). Os camundongos foram expostos ao campo aberto pela primeira vez durante 2 horas. b) Teste de força realizado nós animais VAChT$^{Flox/Flox}$ e VAChT$^{six3\text{-}Cre\text{-}Flox/Flox}$. c) Soma da atividade locomotora durante 2 horas nós camundongos VAChT$^{Flox/Flox}$ e VAChT$^{six3\text{-}Cre\text{-}Flox/Flox}$. d) Tempo passado no centro do campo aberto durante 2 horas para camundongos VAChT$^{Flox/Flox}$ e VAChT$^{six3\text{-}Cre\text{-}Flox/Flox}$. e) O Tempo que os camundongos VAChT$^{Flox/Flox}$ e VAChT$^{six3\text{-}Cre\text{-}Flox/Flox}$ ficam no braço aberto no teste da cruz elevada.

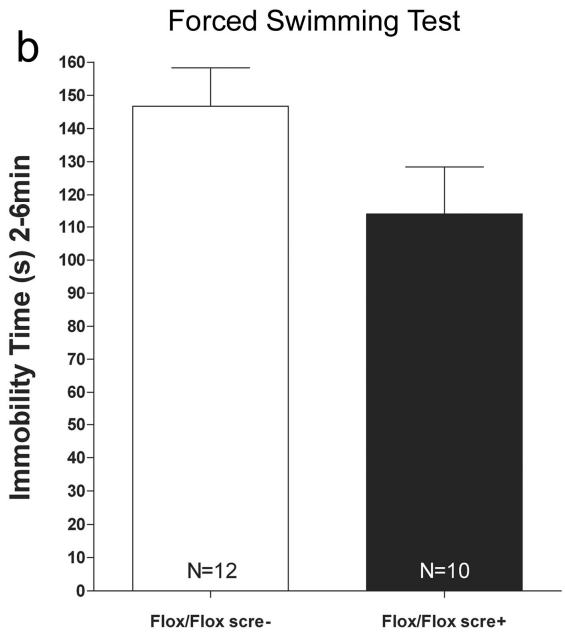


Figura 4. a) Tempo de imobilidade no teste de nado forçado dos camundongos VAChT$^{Flox/Flox}$ e VAChT$^{six3\text{-}Cre\text{-}Flox/Flox}$.