

UNIVERSIDADE FEDERAL DE MINAS GERAIS
Instituto de Ciências Biológicas
Programa de Pós-graduação em Biologia Celular

LEONARDO ROSSI DE OLIVEIRA

**A importância do sistema nervoso simpático na unidade motora e a utilização
de seus agonistas no tratamento das Síndromes Miastênicas Congênitas**

Belo Horizonte

2024

LEONARDO ROSSI DE OLIVEIRA

**A importância do sistema nervoso simpático na unidade motora e a utilização
de seus agonistas no tratamento das Síndromes Miastênicas Congênitas**

Tese apresentada ao Programa de Pós-Graduação em
Biologia Celular do Departamento de Morfologia, do
Instituto de Ciências Biológicas, da Universidade Federal
de Minas Gerais, como requisito parcial para obtenção
do título de Doutor em Biologia Celular.

Área de concentração: Biologia Celular

Orientador(a): Dra. Cristina Guatimosim Fonseca

Belo Horizonte

2024

043

Oliveira, Leonardo Rossi de.

A importância do sistema nervoso simpático na unidade motora e a utilização de seus agonistas no tratamento das Síndromes Miastênicas Congênitas [manuscrito] / Leonardo Rossi De Oliveira. – 2024.
185 f. : il. ; 29,5 cm.

Orientadora: Dra. Cristina Guatimosim Fonseca.

Tese (doutorado) – Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas. Programa de Pós-Graduação em Biologia Celular.

1. Biologia celular. 2. Sistema nervoso simpático. 3. Síndromes Miastênicas Congênitas. 4. Tratamento. I. Fonseca, Cristina Guatimosim. II. Universidade Federal de Minas. Gerais. Instituto de Ciências Biológicas. III. Título.

CDU: 576

Belo Horizonte, 20 de maio de 2024.

Assinatura dos membros da banca examinadora:



Documento assinado eletronicamente por **Hermann Alecsandro Rodrigues, Usuário Externo**, em 20/05/2024, às 12:02, conforme horário oficial de Brasília, com fundamento no art. 5º do [Decreto nº 10.543, de 13 de novembro de 2020](#).



Documento assinado eletronicamente por **Walter Luis Garrido Cavalcante, Professor do Magistério Superior**, em 20/05/2024, às 14:17, conforme horário oficial de Brasília, com fundamento no art. 5º do [Decreto nº 10.543, de 13 de novembro de 2020](#).



Documento assinado eletronicamente por **Matheus Proença Simão Magalhães Gomes, Usuário Externo**, em 22/05/2024, às 12:37, conforme horário oficial de Brasília, com fundamento no art. 5º do [Decreto nº 10.543, de 13 de novembro de 2020](#).



Documento assinado eletronicamente por **Christopher Kushmerick, Professor do Magistério Superior**, em 23/05/2024, às 20:54, conforme horário oficial de Brasília, com fundamento no art. 5º do [Decreto nº 10.543, de 13 de novembro de 2020](#).



Documento assinado eletronicamente por **Cristina Guatimosim Fonseca, Professora do Magistério Superior**, em 24/05/2024, às 10:21, conforme horário oficial de Brasília, com fundamento no art. 5º do [Decreto nº 10.543, de 13 de novembro de 2020](#).



A autenticidade deste documento pode ser conferida no site https://sei.ufmg.br/sei/controlador_externo.php?acao=documento_conferir&id_orgao_acesso_externo=0, informando o código verificador 3251009 e o código CRC F41CDB2F.

AGRADECIMENTOS

Ao meus pais, pela educação primorosa, dentro e fora de casa e pelo exemplo cri
Sem eles nunca chegaria até aqui.

À professora Cristina, por me receber de braços abertos desde o primeiro dia que nos conhecemos, em 2018. Pela disponibilidade, entusiasmo, por confiar a mim a execução deste trabalho, pelos conhecimentos passados, pelos conselhos e pela amizade. Obrigado por ser um exemplo e a melhor orientadora que eu poderia ter e por sempre exigir a qualidade e o máximo de mim.

A todos os verdadeiros amigos, antigos e novos, que sempre me apoiaram nessa jornada, pelos conselhos e pelas risadas. Em especial às minhas sinceras amigas Priscila Valadão e Nathália Oliveira, que fizeram parte do dia a dia do laboratório.

À Jordane, cujo caminho se cruzou ao meu dentro dos laboratórios de pesquisa da UFMG e hoje estamos construindo uma linda história juntos.

À Bárbara, fiel companheira durante sua iniciação científica. Obrigado por toda a ajuda nos experimentos e cuidado com os camundongos. Se você minha trajetória teria sido muito mais difícil.

A todos os professores que passaram seus incontáveis conhecimentos e experiências durante as disciplinas, conversas e congressos. Em especial, à Profa. Aline pela sua enorme ajuda com esse e com outros trabalhos realizados em parceria, e também por prontamente me aceitar em seu laboratório para realizar meu pós-doutorado.

Aos colegas do CAPI – ICB – UFMG. Por toda a disponibilidade, ensinamentos e por fornecerem todos os equipamentos necessários para realização deste trabalho.

À ISN e IBRO por me concederem apoio financeiro na pesquisa e em dois congressos memoráveis.

À FAPEMIG, CAPES E CNPq, pelo auxílio financeiro.

“Equipado com seus cinco sentidos, o homem explora o universo ao seu redor e dá à
aventura o nome de ciência.”

Edwin Hubble

RESUMO

A acetilcolina e seus receptores são os responsáveis pela comunicação do Sistema Nervoso Central e a musculatura esquelética em mamíferos, através da Junção Neuromuscular. Distúrbios nessa comunicação podem causar doenças que afetam o sistema neuromuscular, como as Síndromes Miastênicas Congênitas (SMCs). As SMCs são um conjunto de doenças raras que afetam a JNM sendo que o tratamento usual é a utilização de fármacos anticolinesterásicos. Entretanto, os seus efeitos são reduzidos ao longo do tempo. Recentes descobertas têm evidenciado a importância do sistema nervoso simpático e seus receptores na fisiologia e em doenças que afetam a unidade motora. Em condições normais os receptores β_2 -adrenérgicos estão envolvidos em importantes processos na placa motora e na fisiologia muscular esquelética. Pesquisas anteriores e do nosso grupo têm evidenciado a importância da utilização de agonistas β_2 -adrenérgicos para o tratamento de SMCs. Apesar dos resultados serem positivos, ainda não se sabe ao certo qual é a função do sistema simpático e dos receptores β_2 -adrenérgicos tanto na fisiologia da unidade motora quanto no tratamento das SMCs. Nessa tese, nós avaliamos as alterações motoras a nível comportamental e da unidade motora em animais *knockout* para receptores β_2 -adrenérgicos, demonstrando que a falta desses receptores causa diversos prejuízos aos animais. Também avaliamos a utilização de um agonista β_2 -adrenérgico no tratamento de animais mnVAcHT-KD, um novo modelo de SMC de origem pré-sináptica, através da deleção do VAcHT (transportador vesicular de acetilcolina) especificamente em α -motoneurônios. Observamos que apesar de não ser capaz de reverter o quadro miastênico, o agonista β_2 -adrenérgico causou diversas alterações nos componentes da unidade motora dos animais tratados. Em conjunto, esses achados demonstram a importância do sistema simpático e os receptores β_2 -adrenérgicos na homeostase e no tratamento de doenças neuromusculares.

Palavras-chave: Biologia celular; Sistema nervoso simpático; Síndromes miastênicas congênitas; Tratamento.

ABSTRACT

Acetylcholine and its receptors are responsible for the communication between the Central Nervous System and skeletal muscles in mammals, through the Neuromuscular Junction. Disturbances in this communication can cause diseases that affect the neuromuscular system, such as Congenital Myasthenic Syndromes (CMSs). CMSs are a group of rare diseases that affect the NMJ and the usual treatment is the use of anticholinesterase drugs. However, its effects are lost over time. Recent discoveries have highlighted the importance of the sympathetic nervous system and its receptors in the physiology and diseases that affect the motor unit. Under normal conditions, β 2-adrenergic receptors are involved in important processes in the motor endplate and in skeletal muscle physiology. Previous works from others and from our group have highlighted the importance of using β 2-adrenergic agonists for the treatment of CMSs. Although the results are positive, the role of the sympathetic system and β 2-adrenergic receptors in both the physiology of the motor unit and the treatment of CMSs is still unknown. In this work, we evaluated motor changes at the behavioral and motor unit level in knockout animals for β 2-adrenergic receptors, demonstrating that the lack of these receptors causes several alterations in the animals. We also evaluated the use of a β 2-adrenergic agonist in the treatment of mnVACHT-KD animals, a new model of SMC of presynaptic origin, through the deletion of VACHT (vesicular acetylcholine transporter) specifically in α -motoneurons. We observed that despite not being able to reverse the myasthenic condition, the β 2-adrenergic agonist caused several changes in the components of the motor unit of the treated animals. Taken together, these findings demonstrate the importance of the sympathetic system and β 2-adrenergic receptors in homeostasis and in the treatment of neuromuscular diseases.

Keywords: Cellular biology; Sympathetic nervous system; Congenital Myasthenic Syndromes; Treatment.

LISTA DE FIGURAS

Figura 1- Esquema ilustrativo da unidade motora.	18
Figura 2 - Esquema ilustrativo dos tipos de motoneurônios e os tipos de fibras musculares esqueléticas que cada um inerva.....	21
Figura 3 - Fotomicrografias de fluorescência, de microscopia eletrônica e esquema ilustrativo da Junção Neuromuscular.	25
Figura 4 - Esquema ilustrativo da estrutura e funcionamento do VACHT.....	26
Figura 5 - Esquema ilustrativo das divisões da musculatura esquelética..	29
Figura 6 - Gel de genotipagem dos animais mnVACHT-KD.....	39
Figura 7 - Esquema ilustrativo demonstrando a geração dos animais mnVACHT-KD.....	40
Figura 8 - A deleção completa dos β_2-AR em camundongos β_2-comprometeu a força de agarre comparação com animais WT.....	49
Figura 9 - Mesmo com a deleção completa de β_2-AR o tamanho dos α-MNs de animais β_2-KO é similar ao de animais WT.....	50
Figura 10 - Painel representativo evidenciando que os músculos EDL, SOL e ETM dos animais β_2-KO apresentam drástica redução de tamanho de seus elementos sinápticos em relação aos animais WT.....	53
Figura 11 - A quantificação da área dos elementos pré- e pós-sináptico dos animais WT e β_2-KO demonstra a diminuição da área das JNMs em todos os músculos	54
Figura 12 - Painel representativo demonstrando o aumento da fragmentação do elemento pós-sinápticos (α-bungarotoxina em vermelho) dos músculos EDL, SOL e ETM nos animais β_2-KO.....	57
Figura 13 - A análise da fragmentação e colocalização da JNM dos animais WT e β_2-KO confirma o observado nas micrografias representativas, uma desestabilização da JNM nos animais β_2-KO.....	58
Figura 14 - A deleção dos β_2-AR causa atrofia das fibras de músculo de contração lenta, mas não de músculos com perfil de contração rápida.....	60
Figura 15 - Animais fêmeas mnVACHT-KD heterozigotas não apresentam fenótipo miastênico em comparação com animais WT.....	62
Figura 16 - Animais fêmeas mnVACHT-KD homozigotas apresentam fenótipo miastênico em comparação com animais WT a três meses de idade.....	63

Figura 17 - Animais fêmeas mnVACHT-KD homozigotas não apresentam alterações cognitivas quando comparadas a animais WT	65
Figura 18 - Curva de comportamentos motores antes (0), e ao final (30) do tratamento com formoterol. O formoterol não foi capaz de reverter os déficits motores dos animais mnVACHT-KD	67
Figura 19 - O formoterol causou redução na área dos motoneurônios do segmento cervical de camundongos WT e mnVACHT-KD	69
Figura 20 - Comparação da área dos motoneurônios sem deleção e com deleção do VACHT em animais mnVACHT-KD.	71
Figura 21 - Painel contendo micrografias representativas evidenciando o aumento da área dos elementos pré e pós-sináptico dos animais mnVACHT-KD em comparação com animais WT.....	73
Figura 22 - A quantificação da área dos elementos pré- e pós-sináptico dos animais WT e mnVACHT-KD mostrou que o formoterol foi capaz de hipertrofiar a JNM de animais WT e mnVACHT-KD.....	74
Figura 23 - A análise da área dos elementos pré- e pós-sinápticos mostrou que a deleção do VACHT afeta a morfologia da JNM de forma distinta de acordo com a musculatura e o formoterol foi capaz de hipertrofiar as JNMs deletadas....	76
Figura 24 - Análise da colocação dos elementos sinápticos dos animais WT e β_2-KO.....	78
Figura 25 - Animais mnVACHT-KD não apresentam fragmentação do terminal pós-sináptico dos músculos EDL, SOL e ETM.....	79
Figura 26 - Análise da área de secção transversa dos músculos EDL, SOL e ETM dos animais WT e mnVACHT-KD mostra que o formoterol foi capaz de promover hipertrofia na musculatura esquelética	82
Figura 27 - Quantificação do perfil do tipo de fibras dos músculos EDL, SOL e ETM dos animais WT e mnVACHT-KD.....	85

LISTA DE TABELAS

Tabela 1- Tabela evidenciando os diferentes tipos de cadeia pesada de miosina (MyHC) e algumas características bioquímicas e morfológicas de cada uma. 31

LISTA DE ABREVIATURAS

ACh – Acetilcolina

AChE – Acetilcolinesterase

AChR – Receptores para ACh

ChaT – Colina acetiltransferase

CHT1 – Transportador de colina de alta afinidade-

EDL – Extensor longo dos dedos

ETM – Esternomastoideo

FF - *fast-twitch fatigable*

FFR - *fast-twitch fatigue-resistance*

JNM – Junção neuromuscular

KD – *Knockdown*

mAChR – Receptores muscarínicos para ACh

MEPPs – *Miniature end plate potentials*

MNs – Motoneurônios

MuSK – Quinase específica do músculo

MyHC – Cadeia pesada de miosina

nAChR – Receptores nicotínicos para ACh

OPN – Osteopontina

PFA – Paraformaldeído

SFR - *slow-twitch fatigue-resistant*

SMCs – Síndromes Miastênicas Congênitas

SNC – Sistema Nervoso Central

SOL – Sóleo

TH – Tirosina hidroxilase

VACHT – Transportador vesicular de ACh

WGA – *Wheat Germ Agglutinin*

WT – *Wild type*

α -MNs – Motoneurônios do tipo α

β_2 -AR – Receptores β_2 -adrenérgicos

β_2 -KO – *knockout* para β_2 -AR

SUMÁRIO

1	INTRODUÇÃO	17
1.1	A unidade motora	17
1.2	O motoneurônio	19
1.3	A Junção Neuromuscular	22
1.3.1	O VACHT	26
1.4	O músculo esquelético	28
1.5	As Síndromes Miastênicas Congênicas	32
1.5.1	Agonistas β_2 -adrenérgicos no tratamento das SMCs	34
2	OBJETIVO GERAL	37
2.1	Objetivos específicos	37
3	METODOLOGIA	38
3.1	Animais	38
3.2	Avaliação comportamental motora e cognitiva	41
3.3	Tratamento Farmacológico	42
3.4	Avaliação da integridade das placas motoras	42
3.5	Avaliação da área de secção transversa dos músculos	43
3.6	Avaliação do tipo de fibras dos músculos	44
3.7	Avaliação da integridade dos motoneurônios	45
3.8	Análise estatística	46
4	RESULTADOS	47
4.1	A deleção completa dos β_2 -AR compromete parcialmente a capacidade motora de camundongos	47
4.2	Os motoneurônios de camundongos <i>knockout</i> para o β_2 -AR são semelhantes aos de animais WT	49
4.3	A ausência dos β_2 -AR promove profundas alterações na morfologia da JNM de diferentes músculos dos animais β_2 -KO	51
4.4	A deleção completa do β_2 -AR gera atrofia em músculo de contração lenta 59	
4.5	Camundongos mnVACHT-KD homozigotos fêmeas possuem fenótipo miastênico	61
4.6	O tratamento de camundongos fêmeas mnVACHT-KD de três meses com formoterol não foi capaz de melhorar o fenótipo miastênico	66
4.7	Camundongos fêmeas mnVACHT-KD tratadas com formoterol exibem alterações nos α -MN	68

4.8	O tratamento de camundongos fêmeas mnVACHT-KD de três meses com formoterol promoveu alterações na JNM.....	72
4.9	O tratamento de camundongos fêmeas mnVACHT-KD de três meses com formoterol foi capaz de hipertrofiar a musculatura esquelética	80
4.10	O tratamento de camundongos fêmeas mnVACHT-KD de três meses com formoterol alterou a proporção de fibras do músculo esquelético	82
5	DISCUSSÃO	86
6	CONCLUSÃO	96
7	REFERÊNCIAS	97
8	ANEXOS	110
8.1	Artigo 1: Influence of β_2 -adrenergic selective agonist formoterol on the motor unit of a mouse model of a congenital myasthenic syndrome with complete VACHT deletion.....	110
8.2	Artigo 2: Motoneuron-specific loss of VACHT mimics neuromuscular defects seen in congenital myasthenic syndrome.....	121
8.3	Artigo 3: Neuromuscular defects after infection with a beta coronavirus in mice. 140	
8.4	Artigo 4: A Biosafety Level 2 Mouse Model for Studying Betacoronavirus-Induced Acute Lung Damage and Systemic Manifestations.	152
8.5	Artigo 5: A suitable model to investigate acute neurological consequences of coronavirus infection.	170

1 INTRODUÇÃO

1.1 A unidade motora

A unidade motora é composta pelo neurônio motor, seu axônio e seus dendritos e a fibra muscular que é inervada pelo axônio (DUCHATEAU, 2022). O conceito de unidade motora foi introduzido pela primeira vez em 1925 pelos pesquisadores britânicos Edward Lidell e Charles Sherrington. Em seus trabalhos, estudavam os mecanismos envolvidos na inibição do reflexo em gatos descerebrados e definiram a unidade motora como motoneurônios e suas fibras musculares adjuntas (LIDELL e SHERRINGTON, 1925).

Após os estudos conduzidos por Lidell e Sherrington, outros pesquisadores prosseguiram com novas descobertas a respeito da unidade motora, assim como o desenvolvimento de novas tecnologias para estudá-la. Adrian e Bronk, por exemplo, realizaram trabalhos inovadores nos quais desenvolveram pela primeira vez a tecnologia necessária para observar e gravar a atividade de uma unidade motora em humanos (ADRIAN e BRONK, 1929). Pela primeira vez foi demonstrado que a força gerada por um músculo por meio de um movimento voluntário era proveniente do recrutamento conjunto de unidades motoras e pela modulação da propagação de potenciais de ação.

A unidade motora é formada por grandes neurônios, os motoneurônios (MNs), localizados no corno anterior da medula espinhal, cujos prolongamentos axonais (axônios motores) podem se projetar a longas distâncias. Esses prolongamentos, ao chegarem na fibra muscular esquelética, perdem sua bainha de mielina e se ramificam, formando a Junção Neuromuscular (JNM), outro componente da unidade motora. Por fim, têm-se as fibras musculares esqueléticas, a porção final da unidade motora (Figura 1). O neurotransmissor acetilcolina (ACh) é responsável pela comunicação entre o sistema nervoso e o músculo via JNM em mamíferos. Nas próximas sessões vamos aprofundar em cada componente da unidade motora.

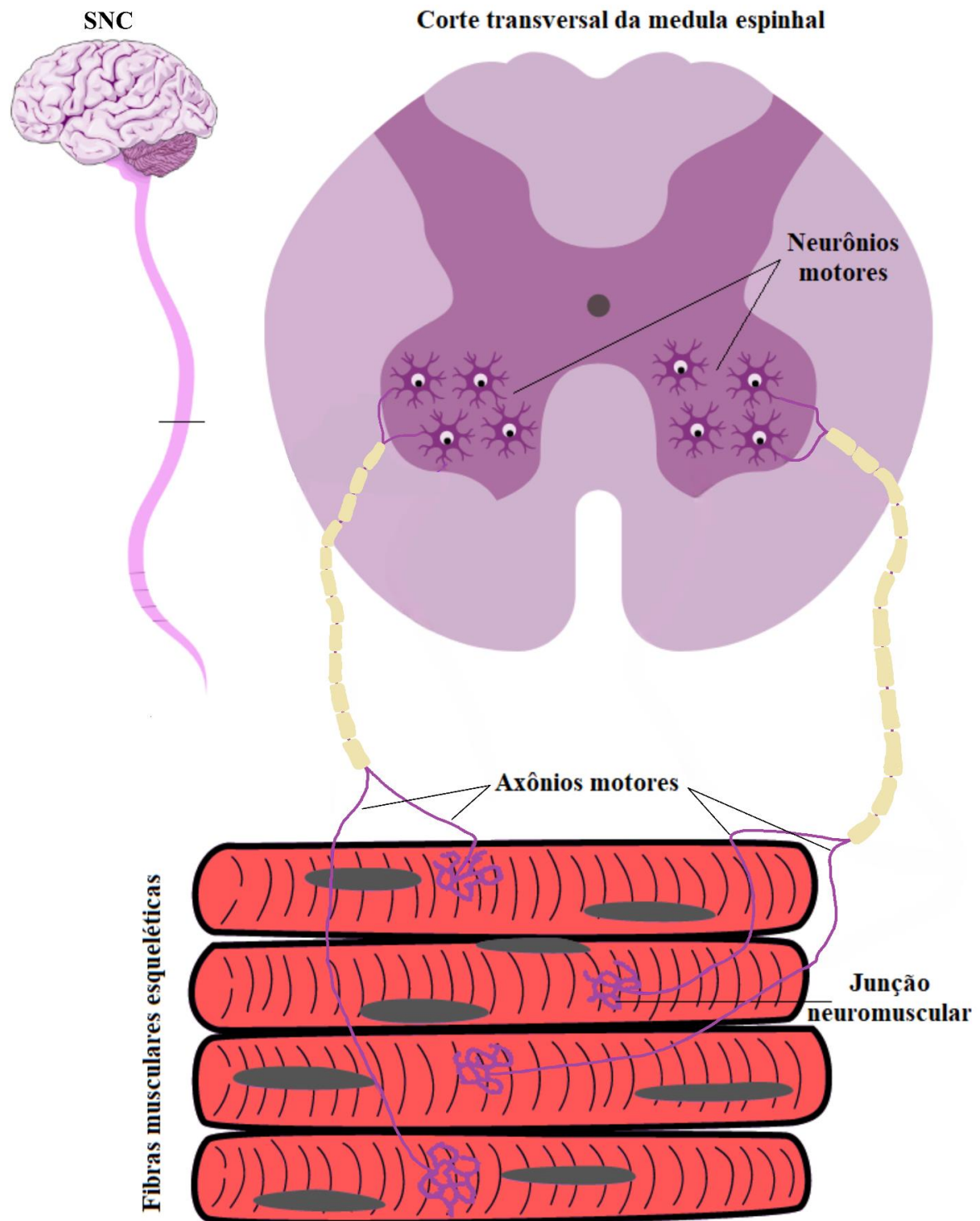


Figura 1- Esquema ilustrativo da unidade motora. Podemos identificar neste desenho ilustrativo um corte transversal da medula espinhal, no qual identificamos a localização dos motoneurônios no corno ventral da medula espinhal. Eles projetam seus axônios para a musculatura esquelética, onde se comunicam e controlam a contração muscular através da Junção Neuromuscular. A liberação do neurotransmissor vai ocasionar uma série de eventos que culminarão na contração muscular esquelética. É válido ressaltar que, apesar de não representado neste esquema, os motoneurônios inervam e controlam diversas fibras musculares ao mesmo tempo. Fonte: próprio autor.

1.2 O motoneurônio

Podemos separar os motoneurônios em dois tipos principais: i) os motoneurônios superiores, que têm origem no córtex cerebral e; ii) os motoneurônios inferiores, que estão localizados no tronco cerebral e na medula espinhal (STIFANI, 2014). Os motoneurônios inferiores são intrínsecos para os movimentos voluntários, sendo responsáveis por controlar as contrações musculares esqueléticas. Sendo assim, de agora em diante quando me referir a motoneurônios será sobre os motoneurônios inferiores da medula espinhal.

Dentre todos os neurônios do Sistema Nervoso Central (SNC) apenas os motoneurônios têm a capacidade de projetar seus axônios para fora do SNC, sendo as células mais longas conhecidas. Estão localizados no corno anterior (ou ventral) da medula espinhal e se organizam em *clusters*, de acordo com os músculos que inervam, sendo que seus axônios deixam a medula pela raiz anterior (FLOETER, 2010, STIFANI, 2014). A quantidade de motoneurônios inervando um músculo pode variar bastante, desde algumas dezenas a centenas de neurônios.

Em mamíferos foram identificados três tipos de motoneurônios: o alfa, o gama e o beta. Os motoneurônios alfa inervam fibras musculares extrafusais e são os responsáveis pelo controle do movimento voluntário muscular (STIFANI, 2014; ZAYIA, 2023). Os motoneurônios alfa são células grandes (FRIESE, 2009; FLOETER, 2010), diferentemente dos outros motoneurônios. Podem ainda serem divididos em três subtipos, de acordo com o tipo de fibra muscular que inervam: i) SFR (*slow-twitch fatigue-resistant*); FFR (*fast-twitch fatigue-resistance*) e; FF (*fast-twitch fatigable*) (STIFANI, 2014; ZAYIA, 2023). Nas próximas seções veremos em mais detalhes as características bioquímicas e contráteis dos diferentes tipos de fibras musculares. De antemão, os motoneurônios SFR inervam fibras musculares de contração lenta, resistentes à fadiga (tipo I). Os FFR inervam fibras musculares de contração rápida, porém são razoavelmente resistentes à fadiga (tipo IIa). Por último, os FF inervam as fibras musculares de contração muito rápida, que são pouco resistentes à fadiga (tipo IIb [IIx em humanos]) (Figura 2a).

Os motoneurônios gama são responsáveis por controlar a sensibilidade do fuso muscular, e inervam as fibras intrafusais (Figura 2b). Sua atividade aumenta a tensão das fibras intrafusais, mimetizando o alongamento da musculatura (STIFANI, 2014; ZAYIA,

2023). Eles não possuem função motora como os motoneurônios alfa, ficando restritos a receber *inputs* sensoriais e contribuir para a modulação da contração muscular (STIFANI, 2014; ZAYIA, 2023).

Por fim, os motoneurônios beta são células menores e bem menos abundantes, o que torna sua caracterização mais difícil e menos estudada. Os motoneurônios beta são uma exceção, uma vez que inervam tanto fibras intrafusais quanto extrafusais (Figura 2c), contribuindo para a contração muscular e para a parte sensorial (STIFANI, 2014; ZAYIA, 2023). Aparentemente, os motoneurônios beta podem modular a atividade de algumas fibras sensoriais, tanto na sua taxa de disparo quanto na sensibilidade ao alongamento da fibra (STIFANI, 2014; ZAYIA, 2023).

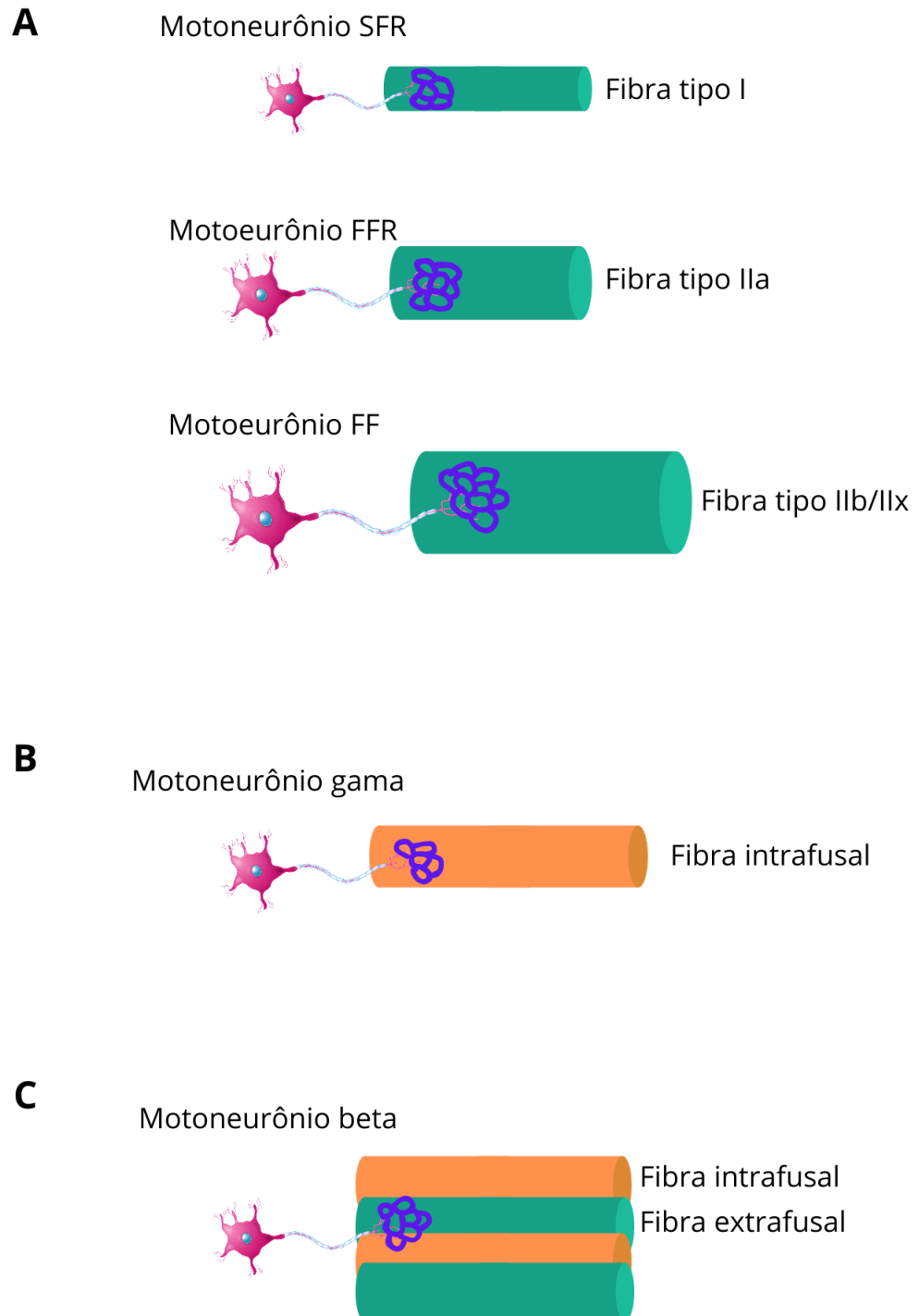


Figura 2 - Esquema ilustrativo dos tipos de motoneurônios e os tipos de fibras musculares esqueléticas que cada um inerva. A- Os α -motoneurônio SFR (*slow-twitch fatigue-resistant*) são responsáveis por inervar e controlar fibras musculares de contração lenta do tipo I. Possuem corpo celular e terminal axonal reduzidos em tamanho, assim

como a fibra muscular do tipo I possui menor diâmetro; os α -motoneurônios FFR (*fast-twitch fatigue-resistance*) são responsáveis por inervar e controlar fibras musculares de contração relativamente rápida, porém resistentes à fadiga (IIa). Possuem corpo celular e junção neuromuscular maiores do que os SFR e fibra muscular um pouco maior; já os α -motoneurônios FF (*fast-twitch fatigable*) são responsáveis por inervarem as fibras musculares de contração rápida do tipo IIb/IIx. Ao contrário dos anteriores, possuem corpo celular grande, assim como junção neuromuscular e fibra muscular de maior diâmetro. **B-** Os motoneurônios gama controlam a sensibilidade dos fusos musculares inervando as fibras intrafusais. **C-** Os motoneurônios beta inervam tanto fibras extrafusais quanto intrafusais e ainda não possuem função totalmente elucidada. Possui corpo celular de menor diâmetro. Fonte: próprio autor.

1.3 A Junção Neuromuscular

Acredita-se que a JNM apareceu na literatura científica pela primeira vez em meados de 1840, pelos zoologistas Doyère e Quatrefages. O primeiro estudava o curioso animal microscópico tardígrado (*Milnesium tardigradum*) (DOYÈRE, 1840), e o segundo, estudava o caramujo *Eolidina paradoxum* (QUATREFAGES, 1843). Ambos demonstraram que havia uma continuidade entre a placa motora e a fibra muscular. Desde então diversas descobertas a respeito da JNM foram realizadas, especialmente com a evolução de técnicas de microscopia.

Hoje é bem estabelecido que a JNM representa a comunicação entre o sistema nervoso e a musculatura esquelética. É uma região especializada, uma sinapse química que permite que o sistema nervoso controle processos musculares essenciais para a vida, como a respiração e movimentos voluntários (RUDOLF, 2019; LEPORE, 2019), e são envolvidas pelas células de Schwann (LI, 2018).

A JNM representa a melhor forma de se estudar as sinapses, devido à sua localização fora do SNC, portanto sem as complexidades das inúmeras conexões sinápticas e celulares e ao seu tamanho, privilegiado em relação a outras sinapses. A formação da JNM envolve a diferenciação dos elementos pré-sináptico, pós-sináptico e das células de Schwann terminais (LI, 2018). Um processo chamado *pre-patterning* acontece, sendo que o elemento pós-sináptico se desenvolve antes da sua inervação pelo axônio do neurônio

motor (LEGAY, 2017; LI, 2018). Inicialmente, os *clusters* de receptores para acetilcolina (AChR) se organizam como placas ovais, normalmente invadidas por mais de um axônio. Ao longo de um processo denominado de eliminação sináptica, as placas vão adquirindo uma forma de *pretzel* (forma clássica das JNMs) (Figura 3A) e cada fibra muscular perde a inervação de múltiplos axônios (MARQUES, 2000). Na figura 3 B podemos observar uma JNM em corte de micrografia eletrônica de transmissão.

A ACh possui importância intrínseca em diversos eventos fisiológicos, não se limitando à processos do sistema nervoso, sejam eles centrais ou periféricos (PRADO, 2013). A síntese de ACh é um processo de suma importância, e ocorre no citoplasma das terminações sinápticas. O ciclo da transmissão colinérgica ocorre da seguinte maneira: a molécula de colina é internalizada pelo transportador CHT1 (*Choline High-affinity Transporter 1*) e, uma vez no citoplasma, a enzima ChaT (*Choline acetyl Transferase*) une uma molécula de colina a uma molécula de acetil-CoA, proveniente da mitocôndria, formando assim a ACh; uma vez sintetizada, a ACh possui característica catiônica e necessita de um transportador para entrar na vesícula sináptica, sendo este transportador o VACHT (*Vesicular Acetylcholine Transporter*), que internaliza a ACh em vesículas sinápticas que, por sua vez, com a chegada do impulso nervoso, serão exocitadas e seu conteúdo vai ser liberado na fenda sináptica; a ACh se liga aos seus receptores, sejam eles nicotínicos (nAChR) ou muscarínicos (mAChR), dependendo da célula alvo, estimulando-os, e, ao final, a enzima AChE (*acetylcholinesterase*) é responsável quebrar a molécula de ACh em colina e acetato, sendo possível iniciar o ciclo novamente (PRADO, 2013). A Figura 3C mostra um desenho esquemático do processo descrito acima.

O VACHT é uma importante molécula limitadora e intrínseca para a neurotransmissão colinérgica. Sendo assim, o próximo tópico será dedicado a explorar esse transportador devido tanto à sua importância para o processo quanto para o presente trabalho.

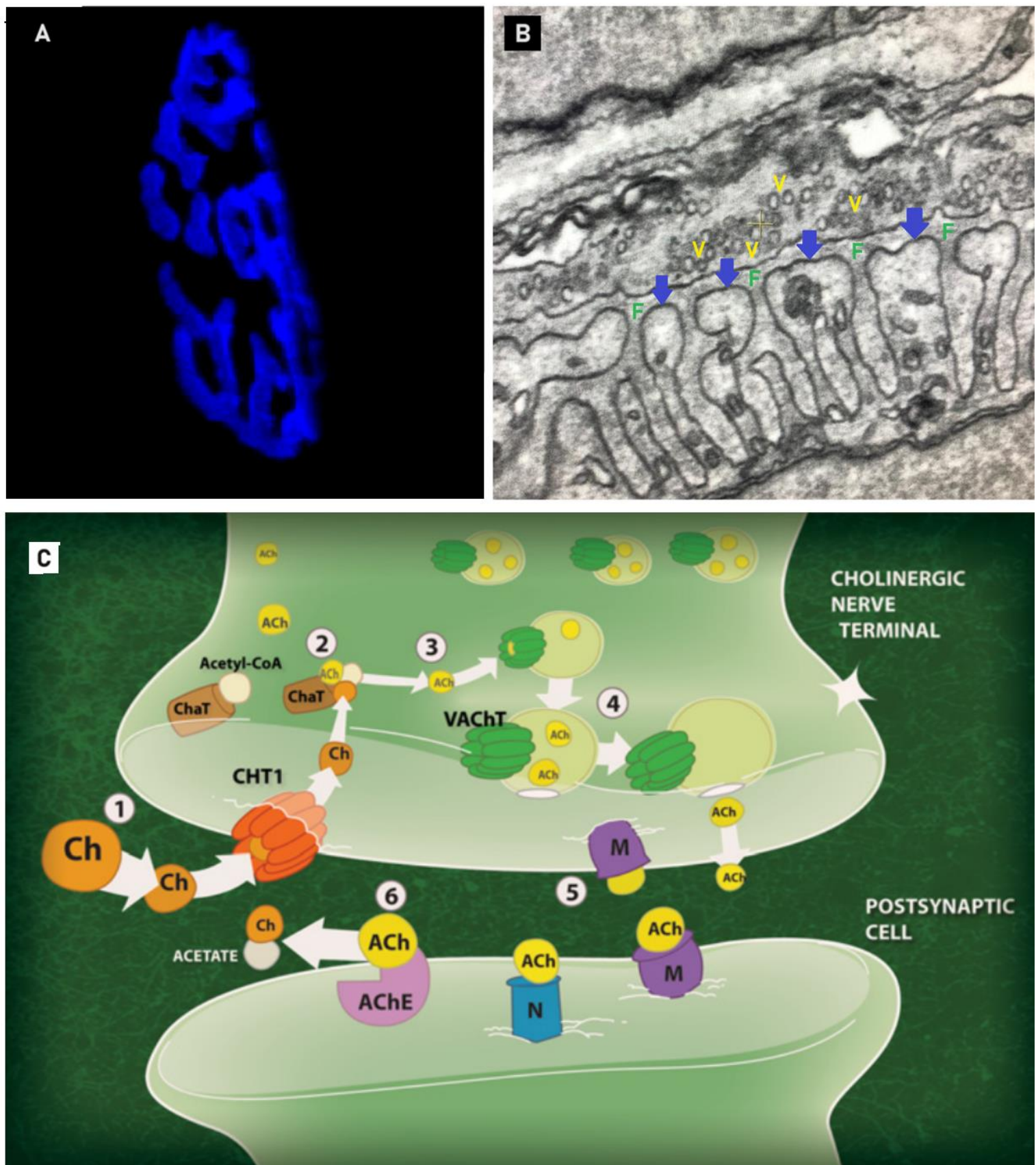


Figura 3 - Fotomicrografias de fluorescência, de microscopia eletrônica e esquema ilustrativo da Junção Neuromuscular. **A-** Fotomicrografia confocal evidenciando o elemento pós-sináptico de uma JNM de camundongo C57Bl6/J através da marcação de receptores nicotínicos pela α -bungarotoxina (647nm). Ao término da eliminação sináptica as JNMs adquirem a forma de *pretzel*. (Imagem do próprio autor). **B-** Fotomicrografia eletrônica de transmissão evidenciando a ultraestrutura de uma JNM de camundongo C57Bl6/J. Letra V (amarela) evidencia o *pool* de vesículas sinápticas, estruturas do terminal pré-sináptico. Com a chegada do potencial de ação há entrada de íons Ca^{2+} no terminal neuronal, que vai ser detectado pelas proteínas sensoras sinaptotagmina 1 e 2, o que vai culminar na exocitose de vesículas sinápticas e liberação do seu conteúdo de acetilcolina na fenda sináptica (F, verde), que por sua vez se liga aos receptores para acetilcolina presentes nas dobras pós-sinápticas (ponta das setas azuis; os receptores estão ancorados à membrana pós-sináptica e aparecem como uma faixa eletrondensa na micrografia eletrônica de transmissão) (imagem gentilmente cedida pela profa. Cristina Guatimosim). **C-** Esquema ilustrativo da neurotransmissão colinérgica. O precursor da acetilcolina, a colina, entra no terminal pré-sináptico através do seu transportador CHT1 (1), e dentro do citoplasma, através da ação da ChAT vai se unir a uma molécula de acetil-CoA proveniente do metabolismo mitocondrial e formar a acetilcolina (2). A acetilcolina possui carga positiva, sendo que não possui a capacidade de atravessar passivamente membranas celulares, sendo necessário que seja internalizada em vesículas sinápticas pelo seu transportador, o VACht (3). Uma vez dentro das vesículas sinápticas, com a chegada do potencial de ação, o conteúdo destas vesículas será liberado na fenda sináptica (4), sendo que a acetilcolina poderá se ligar aos seus receptores, sejam eles muscarínicos ou nicotínicos (no caso da JNM) (5). A ligação da ACh aos seus receptores causa a despolarização do terminal pós-sináptico e a consequente contração muscular. Por fim, o último passo da neurotransmissão colinérgica é o cessamento do estímulo pela acetilcolina, que é realizado pela enzima AChE (acetilcolinesterase), que vai clivar a molécula de acetilcolina em colina e acetato (6), dando início a um novo ciclo de síntese do neurotransmissor (Adaptado de Prado *et al.*, 2013).

1.3.1 O VACHT

Como mencionado anteriormente, o VACHT tem a função de internalizar a ACh recém-sintetizada dentro de vesículas sinápticas. Apesar de ainda não ser totalmente elucidada, a estrutura do VACHT é composta por 12 domínios transmembrana, e responsável por regular a liberação da ACh (PRADO, 2013) (Figura 4A). Assim como outros transportadores vesiculares de neurotransmissores, o VACHT depende de um gradiente eletroquímico para realizar sua função, sendo que ele troca dois prótons por cada molécula de ACh (NGUYEN, 1998; VAN LIEFFERINGE, 2013, PRADO, 2013) (Figura 4B).

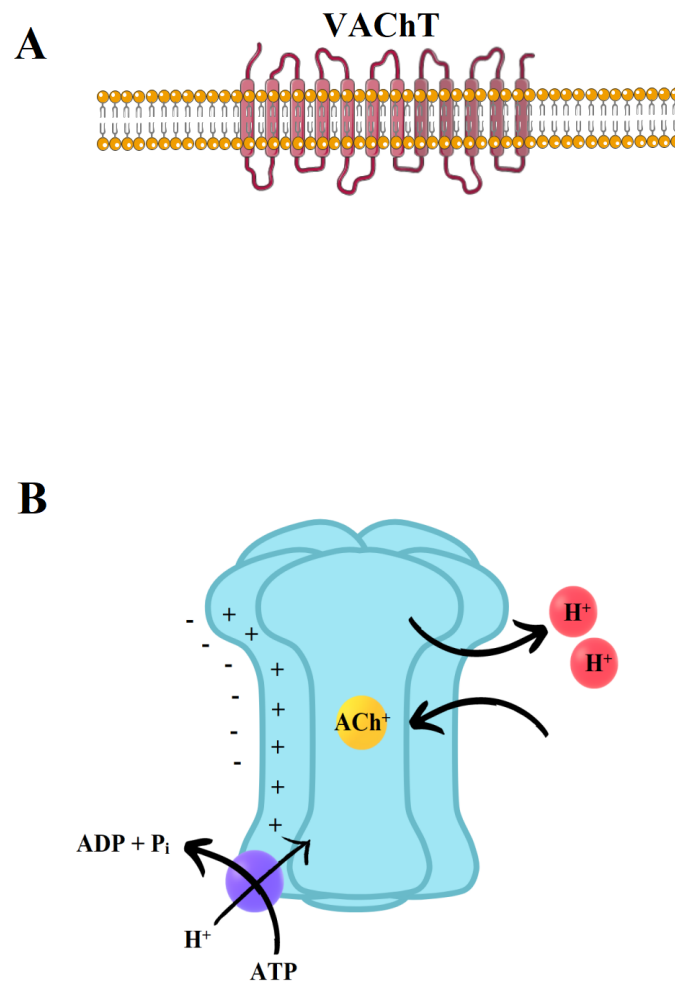


Figura 4 - Esquema ilustrativo da estrutura e funcionamento do VACHT. A- O VACHT é uma proteína transmembrana com 12 domínios e é responsável por internalizar a ACh recém-sintetizada em vesículas sinápticas. **B-** O VACHT troca dois prótons (H^+) para cada molécula de ACh internalizada. Fonte: próprio autor.

Já se sabe que alterações na expressão do VACHT, mesmo que pequenas, interferem com a quantidade de neurotransmissor liberado na fenda sináptica, impossibilitando a saturação dos receptores para ACh (EDWARDS, 2007; PRADO, 2013), o que é apoiado pelo fato de o VACHT ser um transportador de baixa velocidade, sendo crucial para a neurotransmissão colinérgica (VAROQUI, 1996). Além do mais, foi demonstrado através de animais *knockout* (K.O) homozigotos para este transportador, que o VACHT é essencial para a vida (de CASTRO, 2009a), uma vez que sobrevivem somente algumas horas após o nascimento e acabam vindo a óbito por falência respiratória.

Apesar da sua importância comprovada, surpreendentemente, os mesmos animais K.O para o VACHT apresentam MEPPs (*miniature-endplate potentials*; liberação espontânea de ACh, sem a necessidade de um potencial de ação) em JNM de embriões (de CASTRO, 2009a), sugerindo que uma fração da ACh, possivelmente, é liberada no terminal sem a necessidade do VACHT. Apesar disto, é nítido que não é o suficiente para sustentar uma atividade mínima compatível com a vida. Entretanto, em modelo *knock down* (KD) para o VACHT, no qual há uma redução parcial na expressão do VACHT e, conseqüentemente, na liberação de ACh, animais heterozigotos não apresentam qualquer prejuízo motor, tendo uma sobrevida normal (PRADO, 2006), porém, é interessante notar que estes mesmos animais apresentam distúrbios comportamentais cognitivos (PRADO, 2006; de CASTRO, 2009b; DE JAEGER, 2012). Isso demonstra que o SNC é mais dependente do VACHT e de uma fina regulação da neurotransmissão do que a JNM. Uma possível explicação para esse dado seria a menor quantidade de vesículas sinápticas nas sinapses colinérgicas do SNC comparado com a JNM, bem como a margem de segurança da JNM que é muito superior do que nas sinapses centrais (PRADO, 2013).

Como foi dito nos primeiros parágrafos deste tópico, a JNM e seus componentes são de suma importância para a vida, uma vez que ela controla diversas funções muito importantes como respiração e movimentos voluntários. Estas funções, por sua vez, são executadas pela musculatura esquelética, que está sob controle direto do motoneurônio e da JNM.

1.4 O músculo esquelético

A última parte da unidade motora é a musculatura esquelética. O músculo esquelético de mamíferos é um órgão muito heterogêneo e possui uma capacidade única de se modificar e adaptar a diversas situações. A musculatura contribui para diversas funções como a manutenção da postura, locomoção, fonação, mastigação e respiração, além de funções metabólicas como a manutenção do metabolismo basal servindo de depósito de aminoácidos e glicogênio muscular (KRAEMER, 2016; MCARDLE, 2016).

O corpo humano possui cerca de 660 músculos esqueléticos. São caracterizados por serem formados por um feixe de células longas, cilíndricas, apresentando miofibrilas e múltiplos núcleos. A forma mais didática de se entender a estrutura básica do músculo é decompô-lo em suas subseqüentes subunidades. Geralmente o músculo está ligado a um tendão (existem músculos que não apresentam tendão) e é envolvido pelo epimísio (também conhecido como fáscia), que mantém os fascículos no lugar; os fascículos, que são revestidos pelo perimísio, são responsáveis por organizar e manter as fibras musculares unidas; fibras musculares que, por sua vez, são revestidas pelo endomísio; logo abaixo do endomísio está localizado o sarcolema, que é a membrana celular da fibra muscular, que recobre as miofibrilas em seu interior (KRAEMER, 2016; MCARDLE, 2016) (Figura 5).

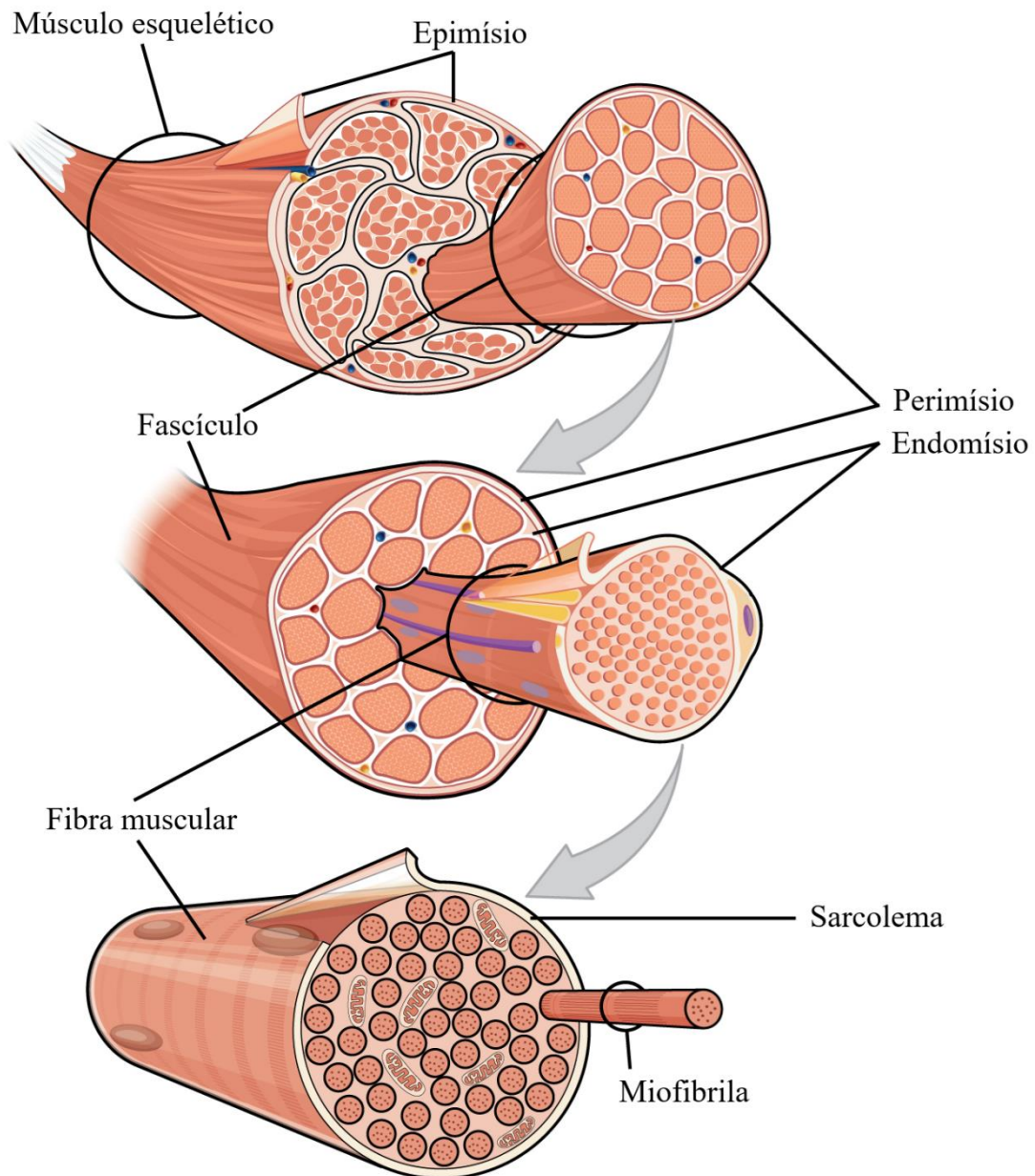


Figura 5 - Esquema ilustrativo das divisões da musculatura esquelética. A musculatura esquelética é organizada em uma série de estruturas cilíndricas até chegar nas miofibrilas. Na maioria das vezes, o músculo esquelético está ligado ao osso por meio de tendões. Sua totalidade é recoberta externamente pelo epimísio ou fáscia. Dentro da fáscia estão localizados os fascículos, que são revestidos e mantidos firmemente organizados pelo perimísio. No interior dos fascículos estão presentes as fibras musculares, que por sua vez estão recobertas pelo endomísio. O sarcolema é a membrana celular da fibra muscular e é responsável por cobrir as miofibrilas que são compostas pelas proteínas de contração actina e miosina (Adaptado de Betts et al., 2022).

As miofibrilas são compostas pelos sarcômeros, a unidade funcional, contrátil dos músculos estriados, que são organizados em série e em paralelo (esta última dá o aspecto estriado para a musculatura esquelética). Cada sarcômero é formado em arranjos de miosina (filamentos grossos) e actina (filamentos finos), que se sobrepõem (SCHIAFFINO, 2011).

Uma importante característica da musculatura esquelética é a presença de diferentes tipos de fibras musculares. Como citado anteriormente que existem diferentes fibras musculares com propriedades bioquímicas e contráteis distintas. Isso ocorre, pois, a cadeia pesada da miosina (MyHC) pode ter isoformas, que vão influenciar diretamente no comportamento daquele músculo. As MyHC podem ser divididas em: MyHC-I; MyHC-IIA; MyHC-IIB/IIX; ainda existem outros dois tipos de isoformas que são as transitórias: MyHC-emb (embrionária) e MyHC-neo (neonatal) (SCHIAFFINO, 2011). Cada isoforma de MyHC possui características intrínsecas, que serão também refletidas no músculo ao qual estiverem presentes, de acordo com a predominância nele.

Fibras musculares do tipo I são chamadas de fibras oxidativas de contração lenta, uma vez que produzem menos força na contração, porém são muito resistentes à fadiga; as fibras do tipo IIA são chamadas de fibras oxidativas de contração rápida, uma vez que produzem mais força, contraem mais rápido do que as do tipo I, e possuem uma leve resistência à fadiga; por último, as fibras do tipo IIB/IIX (apenas camundongos possuem IIB) são chamadas de fibras glicolíticas de contração (muito) rápida, uma vez que geram uma grande força na contração, contraem muito rápido, porém esgotam sua energia igualmente rápido, fadigando com facilidade (SCHIAFFINO, 2011; FRONTERA, 2015; MUKUND, 2020). O fato de existirem diferentes tipos de MyHC possibilita que o sistema muscular esquelético proporcione ao nosso corpo a execução de uma miríade de atividades, desde correr longas distâncias a levantar objetos pesados com rapidez. Vale ressaltar que os diversos músculos do nosso corpo possuem diferentes proporções dessas isoformas da MyHC, de acordo com a sua localização anatômica e, consequentemente, função. A tabela abaixo reúne as informações relevantes sobre cada tipo de fibra (Tabela 1).

	Tipo I (MyHC-I)	Tipo IIA (MyHC-IIA)	Tipo IIB/IIIX MyHC-IIB/IIIX
Contração	Lenta	Moderada/rápida	Muito rápida
Resistência à fadiga	Alta	Moderada	Baixa
Força produzida	Baixa	Média	Alta
Quantidade de mitocôndrias	Alta	Alta	Média
Quantidade de capilares	Alta	Média	Baixa
Capacidade oxidativa	Alta	Alta	Moderada
Capacidade glicolítica	Baixo	Alta	Alta
Área transversa da fibra muscular	Pequena	Média/grande	Grande

Tabela 1- Tabela evidenciando os diferentes tipos de cadeia pesada de miosina (MyHC) e algumas características bioquímicas e morfológicas de cada uma.

O músculo esquelético encerra, assim, os componentes da unidade motora, sendo composta pelos neurônios motores, pela JNM e pela musculatura esquelética. Apesar de os componentes da unidade motora possuírem, alguns mais do que outros, mecanismos de proteção, ainda são passíveis de serem acometidos por patologias, como a Doença do Neurônio Motor (SENDTNER, 2014), patologias que afetam a JNM (RODRÍGUEZ CRUZ, 2020) e patologias que afetam a musculatura esquelética (FRONTERA, 2015). Como o próprio nome já pressupõe, a unidade motora atua como uma unidade, sendo que seus componentes dependem um do outro para um bom funcionamento, sendo comum que alterações ou doenças específicas de um componente afete direta ou indiretamente o outro. Um conjunto de doenças em que isso ocorre são as Síndromes Miastênicas Congênitas (SMCs), que vamos explorar em mais detalhes adiante.

1.5 As Síndromes Miastênicas Congênitas

As SMCs são um conjunto de distúrbios genéticos da transmissão neuromuscular, com início precoce devido a mutações em algumas proteínas envolvidas com as funções da placa motora (SHIEH, 2018; FINSTERER, 2019), sejam elas pré, pós-sinápticas ou na fenda sináptica. Os sintomas clássicos das SMCs são fraqueza, seja ela transitória ou permanente e fadiga acentuada das musculaturas faciais, respiratória, dos membros e extraocular (FINSTERER, 2019; RAMDAS, 2021).

Muito pouco se sabe sobre a epidemiologia das SMCs, por serem um grupo de doenças muito raras, com relatos isolados de casos (IYADURAI, 2020) e por falta de notificação correta. Estima-se que a prevalência das SMCs seja de 25-125/1.000.000 (ABICHT, 2016) ou de 9.2/1.000.000 de crianças e adolescentes abaixo de 18 anos (IYADURAI, 2020). No Brasil, mais especificamente no Paraná, um estudo de 2010 mostrou que a prevalência de SMC é de 0.18/100.000 habitantes (MIHAYLOVA, 2010). O diagnóstico se dá pela identificação dos sintomas clássicos desde o nascimento, fraqueza induzida por esforço, busca negativa por AChR (receptor para acetilcolina) e MuSK (quinase específica do músculo) e resposta baixa no teste de estimulação nervosa repetitiva (SHIEH, 2018). Para um diagnóstico mais específico, são necessários testes genéticos para identificar a causa exata da SMC (SHIEH, 2018; RAMDAS, 2021).

A grande maioria dos casos de SMC são oriundos de alterações pós-sinápticas (ENGEL, 2018; RAMDAS, 2021), estando relacionadas com anormalidades diretamente nos nAChR, seja na sua cinética ou organização (FINSTERER, 2019; RAMDAS, 2021). Já as alterações sinápticas podem estar relacionadas com: I – Alterações da ColQ (*collagen Q*), importante para o funcionamento da AChE; II – Alterações da Agrina, responsável pela organização da JNM e; III – Alterações na Matrix Extracelular, que também é essencial para a manutenção e funcionamento da JNM (RODRÍGUEZ-CRUZ, 2014; WEBSTER, 2018; RAMDAS, 2021).

As mutações pré-sinápticas podem acometer as seguintes proteínas: SLC5A7, CHAT, SLC18A3, SNAP25, VAMP1, SYB1, SYT2, e MUNC13–1, dentre outras (FINSTERER, 2019; RAMDAS, 2021). Esses defeitos pré-sinápticos podem afetar o transporte axonal, a síntese e a reciclagem da ACh e a exocitose da vesícula sináptica (FINSTERER, 2019;

RAMDAS, 2021), sendo que essas proteínas são de suma importância para a neurotransmissão colinérgica (PRADO, 2013).

Diversos modelos animais já foram propostos para se estudar as SMCs, independente qual a sua origem (WEBSTER, 2018). Nos últimos anos, vêm-se estudando, inclusive em pacientes humanos com SMC, alterações em proteínas pré-sinápticas muito importantes para a síntese da ACh. Dentre elas o VACHT merece destaque (O'GRADY, 2016; ARAN, 2017; DELLA-MARINA, 2021). Como mencionado anteriormente, o VACHT tem a função de internalizar a ACh recém-sintetizada dentro de vesículas sinápticas. Já foi demonstrado que mesmo leves alterações na expressão do VACHT são capazes de alterar a quantidade de ACh liberada durante a exocitose das vesículas sinápticas, impedindo a completa ativação dos nAChR (receptores nicotínicos para ACh) (EDWARDS, 2007; PRADO, 2013). Além do mais, como já mencionado, animais *knockout* homozigotos para o VACHT não são compatíveis com a vida, uma vez que eles morrem de falência respiratória (de CASTRO, 2009).

Sabendo-se disto, um modelo com redução parcial (*knockdown*) seria mais interessante para estudar as SMCs de forma longitudinal, uma vez que apresentam redução parcial na expressão do VACHT, sendo compatível com a vida (PRADO, 2006). Esses animais são miastênicos e apresentam alterações neuromusculares (PRADO, 2006; LIMA, 2010; RODRIGUES, 2013; MAGALHÃES-GOMES, 2018; LEITE-SCHETINO, 2019). Entretanto, devido ao amplo papel da ACh, este modelo apresenta alterações sistêmicas e, portanto, disfunções cardiovasculares (LARA, 2010; DURAND, 2015) e renais (SILVA, 2016), além de alterações inflamatórias (LEITE, 2016).

Para contornar este problema, e com o intuito de se estudar as alterações específicas na JNM por deficiência do VACHT, geramos um novo modelo, o mnVACHT-KD, que possui redução na expressão do VACHT apenas em α -motoneurônios (α -MNs) (JOVIANO-SANTOS, 2021). Esse modelo nos permite isolar a alteração da expressão do VACHT apenas aos α -MNs e estudar os efeitos na JNM e na musculatura esquelética, além de testar abordagens terapêuticas. Ademais, não apresentam as alterações comportamentais cognitivas que o modelo VACHT-KD possui (PRADO, 2006).

Por fim, os fármacos utilizados na clínica para se tratar as SMCs são principalmente anticolinesterásicos, com frequência sendo escolhida a piridostigmina, que é um inibidor

reversível da acetilcolinesterase, com meia vida de, aproximadamente, 30 minutos (DODD-BUTERA, 2014). Inicialmente os anticolinesterásicos possuem uma ótima eficácia terapêutica, porém, a médio/longo prazo perdem o seu efeito, sendo que os pacientes pioram substancialmente. A explicação para isto é uma provável alteração na estrutura pós-sináptica gerada por esses fármacos. Alguns estudos dos anos 1970 já mostravam que terapia anticolinesterásica de longa duração poderia exercer efeitos indesejáveis na estrutura pós-sináptica das placas motoras (ENGEL, 1973; GILLIES, 1977). Estudos mais recentes mostram que a piridostigmina é um fármaco capaz de produzir uma perda ou *down-regulation* de nAChR (MORSCH, 2013; RICHTSFELD, 2013). Em um estudo ainda mais recentes, alguns autores demonstraram que o efeito terapêutico da piridostigmina foi reduzido após administração prolongada, em pacientes humanos (VANHAESEBROUCK, 2019; BOBADILLA-QUESADA, 2021).

Devido a isso, vêm-se buscando novas estratégias terapêuticas farmacológicas para o tratamento das SMCs, sendo que os agonistas β_2 -adrenérgicos vêm se tornando um grande candidato.

1.5.1 Agonistas β_2 -adrenérgicos no tratamento das SMCs

Os agonistas β_2 -adrenérgicos são fármacos que desempenham suas ações por meio da ligação a receptores β_2 -adrenérgicos (β_2 -AR), que por sua vez são receptores acoplados à proteína G e ativados endogenamente pela noradrenalina. As proteínas G são compostas por 3 subunidades: $G\alpha$, $G\beta$ e $G\gamma$ (NATURE EDUCATION, 2014), correspondendo a 3% do genoma humano que é responsável pela codificação de aproximadamente 800 receptores acoplados à proteína G diferentes e participando em uma enorme quantidade de funções em nosso organismo (REMAN, 2023). De fato, os β_2 -AR desempenham funções importantes em diversos sistemas, como, por exemplo nos sistemas cardiovascular (MOTIEJUNAITE, 2021), respiratório (MUTLU, 2008) e muscular (SATO, 2011).

A utilização desses fármacos no tratamento de doenças como asma é de suma importância, uma vez que possuem proeminentes efeitos broncodilatadores. Da mesma maneira, a sua utilização no tratamento das SMCs é uma prática comum relatada por médicos na clínica, e os pacientes aderem e respondem bem ao tratamento, com resultados

duradouros, principalmente a longo prazo. Apesar disso, ainda não se sabe exatamente quais são as funções do sistema simpático (e dos β_2 -AR) e dos agonistas β_2 -adrenérgicos na unidade motora, principalmente na JNM e nos MNs, tanto na fisiologia quanto em doenças.

A primeira evidência direta da presença e função do sistema simpático e dos β_2 -AR na JNM foi publicada em 2016, quando os autores demonstraram que a inervação simpática, evidenciada por marcação positiva para TH (tirosina hidroxilase) positiva estava em contato direto com a JNM, assim como a colocalização de β_2 -AR e nAChR (KHAN, 2016). Os autores também demonstraram que a simpatectomia química causava danos morfológicos e funcionais à JNM, e que o tratamento com agonista β_2 -adrenérgico conseguia reverter os prejuízos, demonstrando uma possível importância do sistema simpático e dos β_2 -AR para a JNM (KHAN, 2016). Após esse estudo, outros começaram a demonstrar que agonistas β_2 -adrenérgicos são passíveis de gerar efeitos em componentes da JNM (CLAUSEN, 2018; McMACKEN, 2018) e melhoras em modelos de SMC (McMACKEN, 2019; WEBSTER, 2020) além de amenizar os efeitos adversos da piridostigmina (VANHAESEBROUCK, 2019). Além disso, já sabemos que a adrenalina e noradrenalina exercem função no controle da liberação de ACh (TSENTSEVITSKY, 2019; BUKHARAEVA, 2021; KHUZAKHMETOVA, 2021), além do agonista β_2 -adrenérgico, aparentemente, influenciar na quantidade de ACh liberada na fenda sináptica (GAFUROVA, 2024).

Vários fármacos agonista β_2 -adrenérgicos são utilizados e testados no tratamento para SMC, sendo que ainda não há um consenso de qual é o padrão ouro. Entretanto, um promissor candidato é o formoterol. O formoterol é um agonista β_2 -adrenérgico seletivo de longa duração, de aproximadamente 12 horas, com uma ação de início rápido (FAULDS, 1991; ANDERSON, 1993), características que o colocam como um forte concorrente a uma posição no tratamento das SMCs

Dito isso, para entender a função dos β_2 -AR e seus agonistas, no presente trabalho, inicialmente realizamos a caracterização comportamental e da unidade motora de animais com deleção completa (*knockout*, KO) para β_2 -AR (β_2 -KO). Observamos que, apesar de poucas alterações comportamentais, a deleção dos β_2 -AR foi muito prejudicial para a unidade motora dos animais, gerando efeitos deletérios na musculatura e na JNM.

Posteriormente, partimos para a análise comportamental e tratamento farmacológico do novo modelo de SMC pré-sináptica mnVAcHt-KD. Realizamos um tratamento farmacológico com o fármaco de escolha para o manejo das SMCs, a piridostigmina, para compararmos com os possíveis efeitos do agonista β_2 -adrenérgico formoterol e observamos reversão da fraqueza muscular presente nestes animais. Em seguida, partimos para o tratamento com o formoterol que, apesar de não gerar reversão comportamental motora igual a piridostigmina, promoveu notáveis efeitos benéficos na musculatura esquelética e nos terminais sinápticos da JNM dos animais tratados, inclusive nas JNM que não apresentam liberação de ACh. Em conjunto esses dados demonstram a importância do sistema simpático e dos β_2 -AR tanto na fisiologia quanto no tratamento de doenças neuromusculares como as SMCs, abrindo uma nova janela de possibilidades e investigação.

2 OBJETIVO GERAL

Caracterizar as possíveis alterações motoras geradas pela deleção completa do β_2 -AR em camundongos β_2 -KO e avaliar as propriedades terapêuticas do agonista β_2 -adrenérgico formoterol na unidade motora de animais miastênicos mnVAcHt-KD.

2.1 Objetivos específicos

- 1- Avaliar comportamental motora dos animais β_2 -KO aos três meses de idade;
- 2- Analisar dos componentes da unidade motora dos animais β_2 -KO com três meses de idade no plano ótico através da imunofluorescência;
- 3- Avaliar comportamental motora dos animais miastênicos mnVAcHt-KD com três meses de idade;
- 4- Tratar de animais miastênicos mnVAcHt-KD com o agonista β_2 -adrenérgico formoterol e avaliação dos possíveis benefícios no comportamento motor;
- 5- Analisar dos componentes da unidade motora dos animais miastênicos mnVAcHt-KD após tratamento com formoterol, no plano ótico através da imunofluorescência.

3 METODOLOGIA

3.1 Animais

Utilizamos camundongos fêmeas mnVChT-KD homozigotas, com deleção de, aproximadamente, 70% do VChT em α -MNs (JOVIANO-SANTOS, 2021) e camundongos fêmeas WT, com idade de três meses. Utilizamos também camundongos fêmeas mnVChT-KD heterozigotas (~50% de deleção do VChT) (JOVIANO-SANTOS, 2021) e WT com idade seis meses. Por fim utilizamos animais *knockout* homozigotos para receptores β_2 (β_2 -KO) (ROHRER, 1999) gentilmente cedidos pela Profa. Silvia Guatimosim, já genotipados e os seus respectivos WTs, na idade de três meses. Os animais foram acondicionados em grupos de três a cinco animais por caixa em uma sala com controle de temperatura e ciclo claro-escuro 12:12hrs, comida e água disponibilizadas *ad libitum*. Os animais foram mantidos no biotério do Departamento de Morfologia do ICB/UFMG sob a supervisão da Profa. Cristina Guatimosim. Os animais mnVChT-KD foram genotipados através de PCR seguida por eletroforese em gel de agarose (Figura 6). Os primers utilizados foram os seguintes: FloxGR: 5'-GGC CAC AGT AAG ACC TCC CTT-3'; FloxGF: 5'-GAG AGT ACT TTG CCT GGG AGG-3'; Cre1: 5'-GTC CAA TTT ACT GAC CGT ACA CC-3'; Cre2: 5'-GTT ATT CGG ATC ATC AGC TAC AC-3'; VAT2DET2: 5'-TCA TAG CCC CAA GTG GAG GGA GA-3'; RosaTomato20: 5'-AAG GGA GCT GCA GTG GAG TA3'; RosaTomato21: 5'-CCG AAA ATC TGT GGG AAG TC-3'; RosaTomato103: 5'-GGC ATT AAA GCA GCG TAT CC-3'; RosaTomato105: 5'-CTG TTC CTG TAC GGC ATG G-3'. A Figura 7 evidencia os cruzamentos realizados para gerar os animais mnVChT-KD (JOVIANO-SANTOS, 2021). O projeto foi aprovado pelo CEUA/UFMG sob o protocolo de número 81/2021.

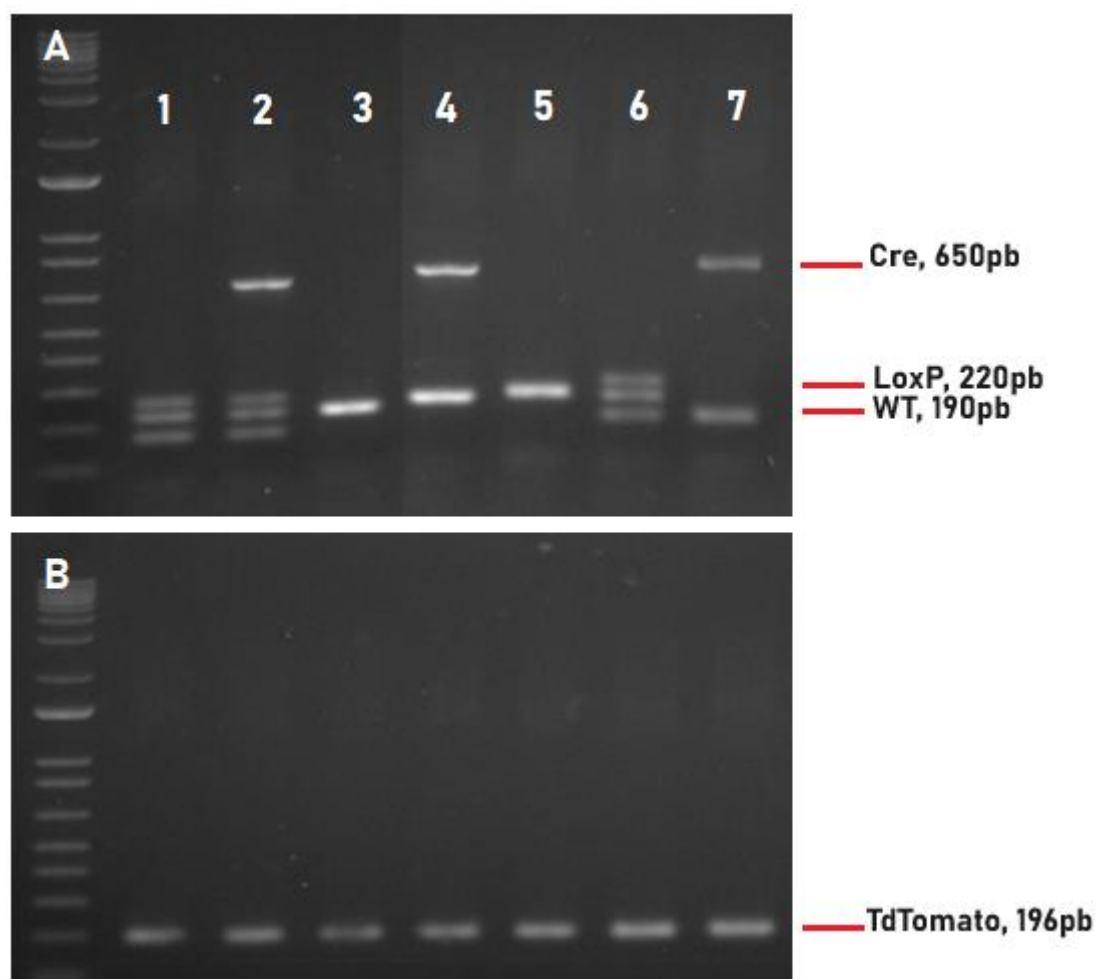


Figura 6 - Gel de genotipagem dos animais mnVACHT-KD. A- Genotipagem para o sistema de edição gênica Cre/LoxP. A genotipagem é realizada para evidenciar a presença/ausência da enzima Cre e dos flaqueadores LoxP. Em 190 pb temos a banda WT, em 220 pb temos a banda LoxP e em 650 pb temos a banda Cre. Os animais heterozigotos apresentam uma banda Cre, uma LoxP e uma WT (canaleta 2). Os animais homozigotos apresentam uma banda Cre e duas LoxP (uma sobreposta à outra) (canaleta 4). Qualquer outra combinação além destas é considerado um animal WT, uma vez que sem a presença da Cre e de ao menos um LoxP não haverá a deleção do VACHT. **B-** Genotipagem para a proteína autofluorescente TdTomato. A proteína TdTomato está presente em 196 pb, sendo que o gel acima evidencia 7 animais homozigotos para o TdTomato. O TdTomato é uma proteína dependente da expressão da Cre, ou seja, apenas será expresso nos animais que também expressam a enzima Cre.

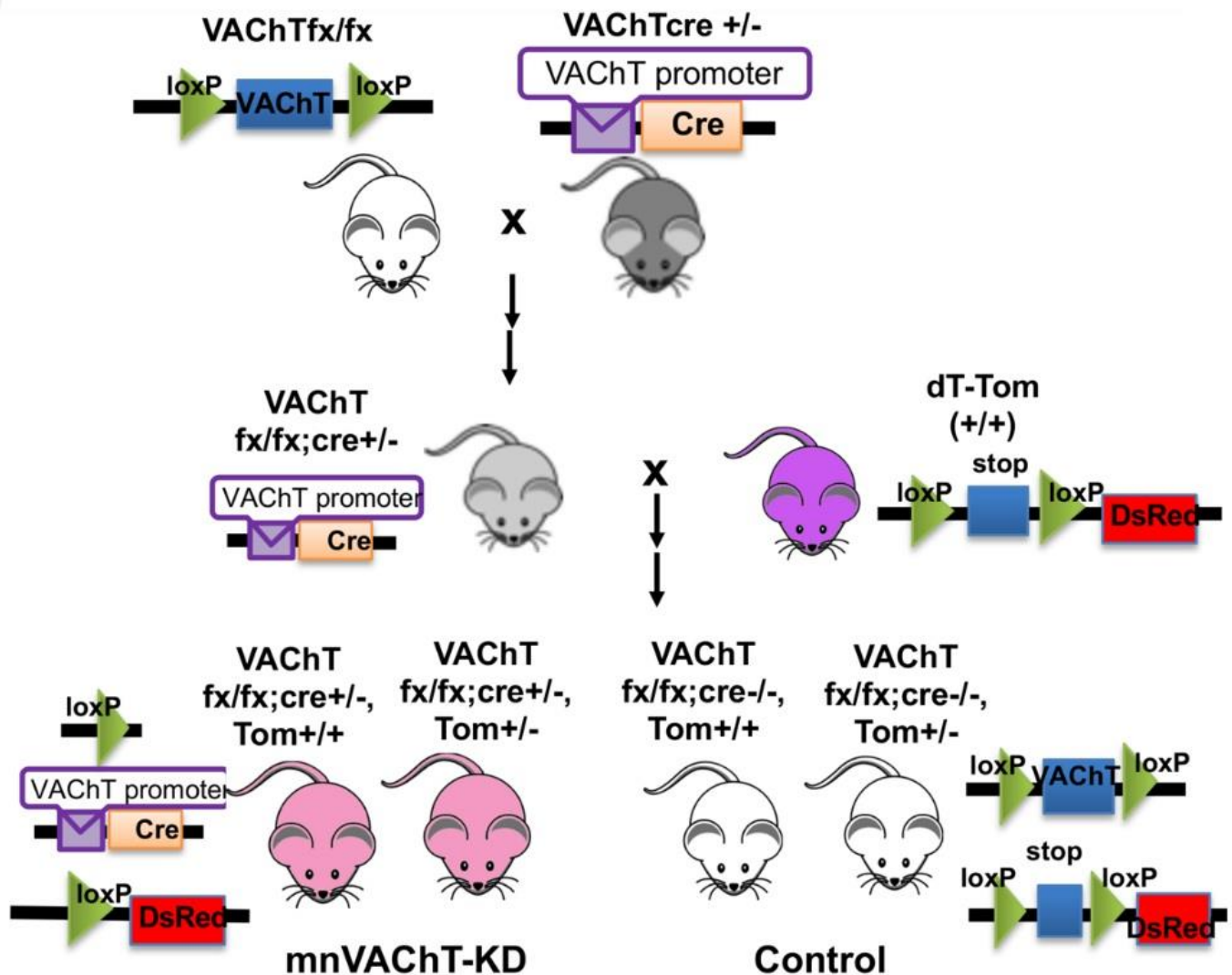


Figura 7 - Esquema ilustrativo demonstrando a geração dos animais mnVACHT-KD. A geração dos animais mnVACHT-KD já foi descrita anteriormente (JOVIANO-SANTOS, 2021). Brevemente, foram cruzados animais VACHT Flox/Flox (gene do VACHT flanqueado pelo loxP) com animais Cre^{+/+}, gerando animais VACHT Flox/Flox Cre^{+/+}. Estes animais possuem a deleção do VACHT, porém ainda não há meios de identificar exatamente em qual motoneurônio está a deleção. Foram então cruzados com animais que expressam a proteína autofluorescente TdTomato^{+/+}, sendo que a expressão desta dependeria da expressão da enzima Cre. Ao final obtivemos animais de genótipo VACHT Flox/Flox Cre^{+/+} TdTomato^{+/+} e animais VACHT Flox/Flox Cre^{+/+} TdTomato^{+/+}, sendo estes considerados animais homozigotos para a deleção do VACHT (outros genótipos não mostrados no esquema ilustrativo). Fonte: JOVIANO-SANTOS, 2021.

3.2 Avaliação comportamental motora e cognitiva

Optamos por avaliar a capacidade motora através de testes padrão ouro utilizados para avaliação motora em roedores. A avaliação cognitiva permite investigar e confirmar a hipótese de que o modelo mnVAcHt-KD não apresenta redução da ACh em regiões cerebrais similares ao modelo predecessor VAcHt-KD. Para investigar o comportamento motor dos camundongos β_2 -KO e mnVAcHt-KD escolhemos os seguintes testes: I- Força da Pegada de duas patas (KOLISNYK, 2013), no qual utilizamos um transdutor de força conectado a um suporte de metal triangular, servindo de apoio para as patas dianteiras do animal. Ao puxarmos levemente o animal para a direção contrária à qual ele está fazendo força (suporte de metal), o aparelho nos dá a leitura da força máxima exercida pelo animal antes de se soltar. Realizamos esta medição três vezes; II- Força da Pegada de quatro patas (KOLISNYK, 2013), no qual consiste, basicamente, na mesma metodologia descrita anteriormente, porém, o animal deve se segurar em um outro suporte, desta vez com apoio para as quatro patas, sendo que o registro de força máxima será de todos os membros ao mesmo tempo. Realizamos esta medição três vezes; III- Teste de Suspensão em Grade (JANICKOVA, 2009), no qual o animal é posicionado em uma grade composta por uma trama de arame de 1cm x 1cm, sendo que ela é invertida logo após o posicionamento do animal. Ele deve, então, se segurar por um máximo de dois minutos, sendo o tempo que ele levou para cair, ou não, contabilizado. Realizamos este experimento três vezes, com um intervalo de descanso de 25 segundos entre cada execução; IV: Teste do Campo Aberto (KLJAKIC., 2006), no qual o animal é posicionado no centro do recipiente e deixado explorar livremente o espaço por um tempo máximo de 30 minutos, sendo que o registro de todo o trajeto e da distância total percorrida é gravado e quantificado automaticamente pelo software do aparelho e; V- Teste do RotaRod (PRADO, 2006), no qual o animal é posicionado em cima de um cilindro rotatório por cinco minutos. Este cilindro aumenta gradativamente sua RPM até um máximo de 25 RPM. Contabilizamos o número total de quedas de cada animal (ao cair o reposicionamos em seu cilindro novamente) e o tempo que ele levou para cair a primeira vez (latência para a queda).

Para os testes cognitivos utilizamos: VI: Teste do Labirinto em Y (KRAEUTER, 2019), no qual o aparato é um “labirinto” composto por três braços organizados no formato de Y. O animal é posicionado sempre no mesmo braço e com a mesma orientação, sendo

deixado explorar todos os braços. Posteriormente, analisamos as filmagens e contabilizamos quantos braços cada animal visitou; VII: Teste *Marble Burying* (ANGOA-PÉREZ, 2013), no qual os animais são posicionados dentro de uma caixa, preenchida pela metade com maravalha e com 20 esferas de vidro distribuídas simetricamente. Deixamos o animal explorar o ambiente e, ao final do experimento, contabilizamos o número de esferas que o animal enterrou na maravalha completamente e; VIII- Teste do Nado Forçado (YANKELEVITCH-YAHAV, 2015), no qual o animal é posicionado em um recipiente contendo água, sendo que ele necessita nadar para se manter flutuando. Uma câmera registra todos os movimentos do animal e, ao final, contabilizamos o tempo de imobilidade total de cada um.

3.3 Tratamento Farmacológico

Optamos por realizar tratamento com o fármaco formoterol, um agonista β_2 -adrenérgico seletivo de longa duração, e utilizamos a dose de 300 μ g/kg (GONÇALVES, 2019), por via subcutânea, sendo o seu veículo DMSO (0.45%) diluído em salina 0.9%.

Optamos por realizar a administração do medicamento por 30 dias corridos. Diariamente registramos os pesos de todos os animais individualmente e, quando necessário, ajustamos o volume de medicamento administrado. Ao final do 30º dia os animais foram sacrificados e os tecidos removidos.

3.4 Avaliação da integridade das placas motoras

Para a avaliação das placas motoras utilizamos o protocolo já descrito anteriormente (VALADÃO, 2018). Os animais foram anestesiados com solução de cetamina (10%) e xilazina (2%) em salina 0.9%, perfundidos por via transcardíaca com 20 mL de PBS 1x, seguido de 20 mL de PFA (paraformaldeído) 4%. Os músculos foram extraídos cirurgicamente e armazenados em PBS 1x a 4°C. Em seguida, realizamos etapa de bloqueio com BSA 3% + Triton X-100 0.5% por 2 horas à temperatura ambiente. Posteriormente, incubamos as amostras em anticorpo primário anti-sinaptotagmina (1:250, DSHB, Znp-1), em solução de bloqueio, overnight a 4°C. No dia seguinte os músculos foram lavados por 10 minutos durante três vezes com PBS 1x. Em seguida adicionamos anticorpo secundário goat anti-mouse AlexaFluor 488 (1:600,

ThermoFisher) e AlexaFluor 555 α -bungarotoxina (1:1000, Invitrogen) ou AlexaFluor 647 α -bungarotoxina (1:1000, Invitrogen), e deixamos incubar por duas horas à temperatura ambiente. Ao final, lavamos as amostras por três vezes de 10 minutos com PBS 1x e montamos as lâminas utilizando o meio de montagem ProLong Gold antifade mountant medium (ThermoFisher).

As imagens foram obtidas utilizando microscópio confocal Zeiss LSM 880, com objetiva de 63x de imersão a óleo (NA 1.4) e linhas de lasers Argon (488 nm) e He-Ne (543 e 633 nm). Foram realizadas seções em série Z para posterior reconstrução tridimensional. As imagens foram analisadas pelo programa ImageJ (FIJI, NIH, USA), utilizando-se a ferramenta *polygon* para se analisar a área dos elementos pré e pós-sinápticos. Para fragmentação, analisamos as terminações de forma individual, através da conversão a um padrão binário e análise das ilhas de fragmentação. Para ambas as análises, consideramos um mínimo de 20 terminações por animal.

3.5 Avaliação da área de secção transversa dos músculos

Após a anestesia descrita no tópico anterior, os animais passaram por perfusão transcardíaca (20 mL de PBS 1x) e tiveram os músculos removidos cirurgicamente. Imediatamente após a remoção dos músculos, foram posicionados em moldes para congelamento e embebidos em O.C.T compound (Tissue Plus, Fisher Health Care) e congelados em isopentano (M32631, Sigma-Aldrich) resfriado por nitrogênio líquido, e posteriormente armazenados em freezer -80 °C. Posteriormente, as amostras congeladas foram seccionadas em fatias de 10 μ m de espessura em corte transversal em relação à orientação das fibras musculares utilizando criostato (Leica CM3050S), e coletadas diretamente em lâminas silanizadas. A marcação foi realizada diretamente sobre as lâminas utilizando o protocolo descrito em ROSSI (2023). Brevemente, realizamos a etapa de bloqueio com BSA 3% + Triton X-100 0.1% por 30 minutos, seguido por incubação com WGA (*Wheat Germ Agglutinin*) Alexa Fluor 647 Conjugate (W32466, Invitrogen) e lavagem com PBX 1x por três vezes de 10 minutos. As lâminas foram montadas utilizando ProLong Gold Antifade Mountant Medium (P36934, Thermo Fisher Scientific) e as imagens foram realizadas utilizando microscópio confocal Zeiss LSM 880 (Zeiss GMBH, Germany) em objetiva de 40x a óleo (NA 1.2) e linha de laser He-Ne (633 nm). Por fim, as imagens foram analisadas utilizando ImageJ e a ferramenta *freehand*.

3.6 Avaliação do tipo de fibras dos músculos

As isoformas MyHC foram identificadas de acordo com o protocolo descrito por MAGALHÃES-GOMES et al. (2018). Brevemente, os camundongos foram sacrificados após anestesia (injeção i.p de cetamina (50 mg/kg) e xilazina (5 mg/kg) seguida de perfusão transcardíaca com PBS 1x. Os músculos *extensor digitorum longus* (EDL), sóleo (SOL) e esternomastoideo (ETM) foram retirados e imediatamente imersos em O.C.T (Tissue Plus, Fisher Health Care), congelados em isopentano (M32631, Sigma-Aldrich) resfriado com nitrogênio líquido e armazenados a -80 °C. Posteriormente, as amostras foram cortadas usando criostato (Leica CM3050S) e fatias transversais de 10 µm foram coletadas em lâminas gelatinizadas. As lâminas contendo as fatias de músculo foram submetidas a uma etapa de bloqueio com 3% de BSA + 0,1% de Triton X-100 por 30 min seguido de 5% de soro de cabra normal + 0,1% de Triton X-100 para 30 min à temperatura ambiente. Foram então incubadas com Aglutinina de Germe de Trigo (WGA) Alexa Fluor 647 (W32466, Invitrogen) e lavadas três vezes com PBS 1x. As lâminas foram montadas usando ProLong Gold Antifade Mountant Medium (P36934, Thermo Fisher Scientific).

Para a análise da composição do tipo de fibra seguidos o protocolo descrito por MAGALHÃES-GOMES et al. (2018). As fatias de músculo (10µm) foram cortadas em criostato (Leica CM3050S) e coletadas em lâminas revestidas com gelatina. Passaram posteriormente por etapa de bloqueio de 30 min à temperatura ambiente (TA) com 3% de BSA (Sigma-Aldrich), 5% de soro de cabra (Sigma-Aldrich) e 0,1% de Triton X-100 (Sigma-Aldrich) diluído em PBS 1x. As lâminas com as fatias foram então incubadas durante a noite a 4 °C com os seguintes anticorpos primários (diluídos em 3% de BSA, 5% de soro de cabra), todos elaborados em camundongos: tipo I (Leica Microsystems Cat# NCL-MHCs Lote# RRID: AB_563898; 1:250); tipo IIA (DSHB Cat# SC-71 Lote# RRID:AB_2147165; 1:100), tipo 2X (DSHB Cat# BF-35 Lote# RRID:AB_2274680, que reconhece todos os tipos de fibras musculares exceto IIX; 1: 100) e tipo 2B (DSHB Cat# BF-F3 Lote# RRID:AB_2266724; 1:100). Anticorpos SC-71, BF-35 e BF-F3. No dia seguinte as lâminas foram lavadas três vezes com PBS 1x e incubadas por 1 h em temperatura ambiente com anticorpos secundários: Alexa 488 cabra anti-IgG1 de camundongo (Thermo Fisher Scientific Cat# A-21121 Lot# RRID:AB_2535764 para anticorpos dos tipos I, IA e IIX e IgM anti-camundongo de cabra Alexa 488 (Thermo Fisher Scientific Cat# A-21042 Lote# RRID: AB_2535711 para anticorpo tipo IIB. As

lâminas foram lavadas 3 vezes novamente com PBS 1x e montadas usando ProLong Gold Antifade Mountant Medium (P36934, Thermo Fisher Scientific). As imagens foram adquiridas usando microscópio confocal Zeiss LSM 880 (Zeiss GMBH, Alemanha), usando objetiva de imersão em óleo 40x (NA 1.2), lasers He-Ne (647 nm) e argônio (488 nm) foram usados para WGA e tipagem de fibra, respectivamente. Cada tipo de fibra foi expresso como uma porcentagem do número total de fibras contadas em uma seção muscular inteira através do ImageJ (NIH).

3.7 Avaliação da integridade dos motoneurônios

O protocolo para marcação dos α -MN foi descrito em ROSSI et al., (2023) Após a eutanásia dos animais e perfusão intracardíaca com PBS 1x e PFA 4%, pequenas seções da medula espinhal cervical e lombar foram removidas e deixadas em PFA 4% por 12 h a 4 °C. No dia seguinte as mesmas amostras foram retiradas das vértebras e imergidas em solução de sacarose 30% com a finalidade de desidratar o tecido para posterior congelamento. Foram posicionadas em moldes para congelamento e embebidas em O.C.T e congeladas em isopentano resfriado por nitrogênio líquido. Novamente utilizamos do criostato para realizar os cortes das seções da medula a 30 μ m de espessura coletadas em lâminas silanizadas. Utilizamos um protocolo de marcação para α -MN através do marcador específico osteopontina (OPN). Também utilizamos DAPI. Rapidamente, os cortes presentes nas lâminas passaram por etapa de bloqueio com BSA 3% + Triton-X 100 0.5% por 30 minutos seguidos por soro de burro a 5% + Triton-X 100 0.5 % por 30 minutos; foram então incubados com anticorpo primário anti-OPN por 12h a 4 °C (goat anti-OPN; 1:100, BDO0615081, RD Systems); no dia seguinte realizamos lavagem com PBX 1x e incubação com anticorpo secundário (IgG2A anti-goat Alexa Fluor 488) e DAPI e as lâminas foram montadas utilizando ProLong Gold (ROSSI, 2023). As imagens foram obtidas utilizando microscópio confocal Zeiss LSM 880, com objetiva de 63x (NA 1.4) e lasers He-Ne (543 nm) e Argon (488 nm) foram usados para TdTomato e OPN, respectivamente) e para evidenciar o núcleo das células (DAPI+). As imagens foram analisadas pelo programa ImageJ e a ferramenta *polygon*. Consideramos para análise apenas os MN que expressavam TdTomato (apenas em animais mnVACHT-KD), OPN e DAPI.

3.8 Análise estatística

Todas as amostras passaram pelos testes de Shapiro-Wilk e Kolmogorov-Smirnov para verificar se possuíam distribuição Gaussiana ou não. Utilizamos os testes t de Student não pareado, two-way ANOVA com pós-teste de Tukey, teste de Mann-Whitney e teste de Kruskal-Wallis com pós-teste de Dunn, dependendo do resultado dos testes de parametricidade. Utilizamos o programa estatístico GraphPad Prism 8 e R 4.0.0 e consideramos como valor significativo $p < 0.05$.

4 RESULTADOS

4.1 A deleção completa dos β_2 -AR compromete parcialmente a capacidade motora de camundongos

Como mencionado anteriormente na introdução, pouco se sabe sobre as funções do sistema simpático e dos β_2 -AR nos MNs e nas suas placas motoras, tanto em condições normais quanto no contexto do tratamento de doenças neuromusculares. Apesar disso, evidências sugerem um importante papel acessório, uma vez que a simpatectomia gera grandes alterações negativas na JNM (KHAN, 2016), e a adrenalina e noradrenalina, aparentemente, possuem funções regulatórias na JNM (TSENTSEVITSKY, 2019, BUKHARAEVA, 2021; KHUZAKHMETOVA, 2021).

Sendo assim, iniciamos a nossa investigação com a seguinte pergunta: se o sistema simpático possui uma função importante na unidade motora, animais β_2 -KO apresentarão alterações no comportamento motor e nos componentes da unidade motora? Partimos, então, para a caracterização comportamental motora destes animais seguida pela investigação das possíveis alterações em MNs, JNM e musculatura esquelética.

Realizamos uma bateria de testes comportamentais compostos pela grade invertida, força da pegada das patas dianteiras e das quatro patas e quantificação da latência para a queda e número total de quedas no Rotarod. Observamos que os animais β_2 -KO apresentaram uma diminuição substancial do tempo de permanência suportando o próprio peso no teste de grade invertida, em comparação com os animais WT (WT: $109,8 \pm 14,59$ s; β_2 -KO: $74,22 \pm 21,03$ s; $p < 0.0005$) (Figura 8 A). Não observamos nenhuma perda na performance motora nos outros testes (Figura 8 B-E).

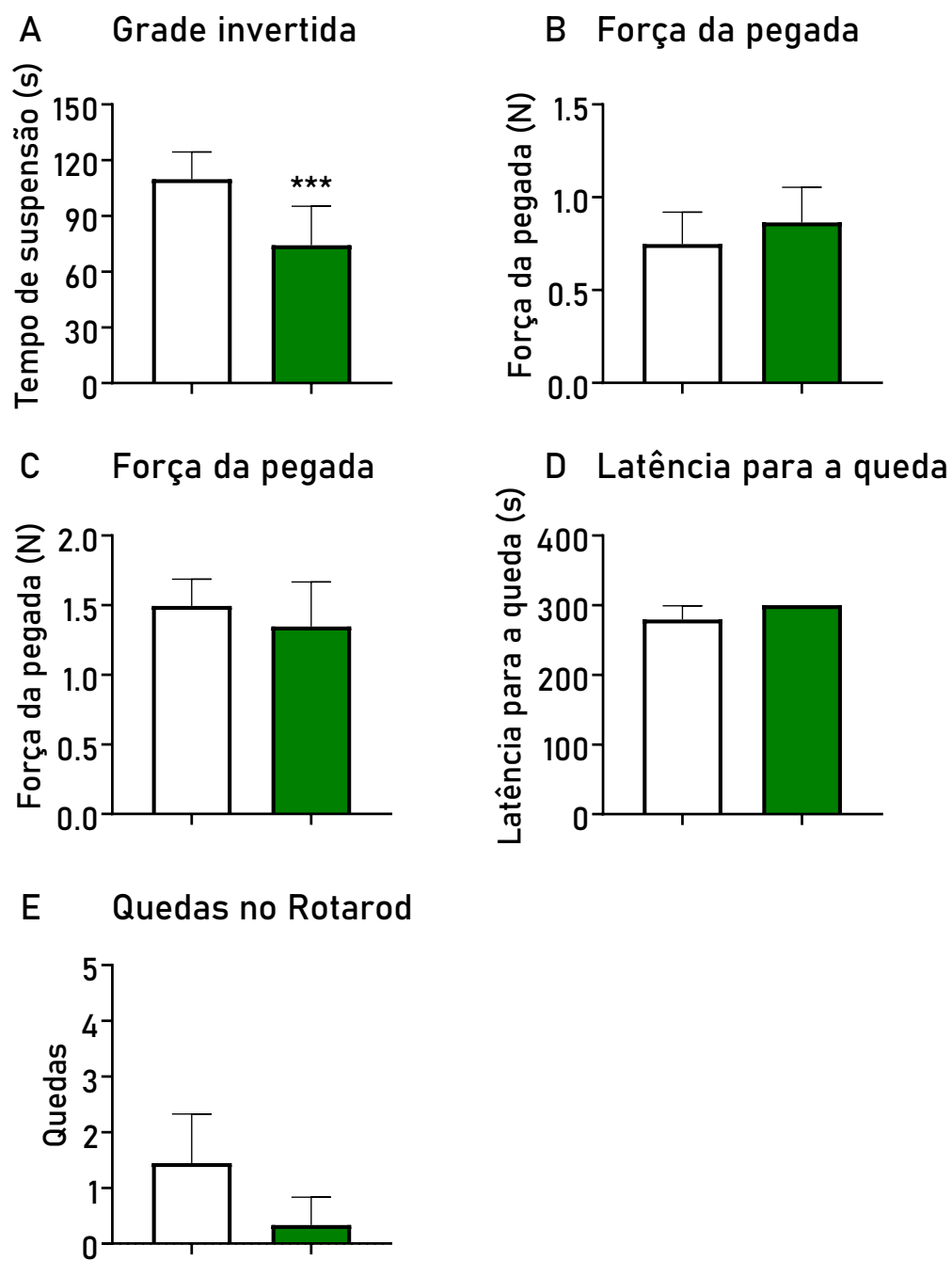


Figura 8 - A deleção completa dos β_2 -AR em camundongos β_2 -comprometeu a força de agarre comparação com animais WT. Animais β_2 -KO de três meses de idade apresentaram alteração comportamental motora na capacidade de sustentar o próprio peso no **A**- Teste de grade invertida; apesar de nenhuma diferença ter sido observada em **B** e **C**- Força da pegada das patas dianteiras e das quatro patas, respectivamente, e em **D** e **E**- Latência para a queda em segundos e número total de quedas no Rotarod, respectivamente. *** $p < 0.0005$, teste t de Student não pareado, resultados representam a média $\pm$ DP. $n = 11$ WT e 9 β_2 -KO.

4.2 Os motoneurônios de camundongos *knockout* para o β_2 -AR são semelhantes aos de animais WT

Uma vez que identificamos alterações motoras nos animais β_2 -KO, nos questionamos se os componentes da sua unidade motora poderiam ser afetados pela falta de sinalização adrenérgica, influenciando nas alterações motoras. Decidimos iniciar nossa investigação pelos MNs, mais especificamente pelos α -MNs, depois seguir para a placa motora e, por fim, a musculatura esquelética. Escolhemos realizar a análise dos α -MNs presentes nas regiões cervical e lombar, uma vez que são eles que inervam a musculatura relacionada com a execução dos testes comportamentais realizados anteriormente. Quando analisamos os α -MNs dos animais β_2 -KO comparados a animais controle, não observamos nenhuma diferença no tamanho entre os grupos (Figura 9). Tanto na porção cervical quanto na porção lombar, os α -MNs de ambos os grupos possuem a mesma área, como podemos observar no gráfico que quantifica a área dos MNs da porção cervical e lombar, respectivamente (Figura 9 A e B) e nas imagens representativas evidenciando os MNs da cervical de animais WT e β_2 -KO, respectivamente (Figura 9 A' e A''), assim como nas imagens representativas que evidenciam os MNs da região lombar de animais WT e β_2 -KO, respectivamente (Figura 9 B' e B'').

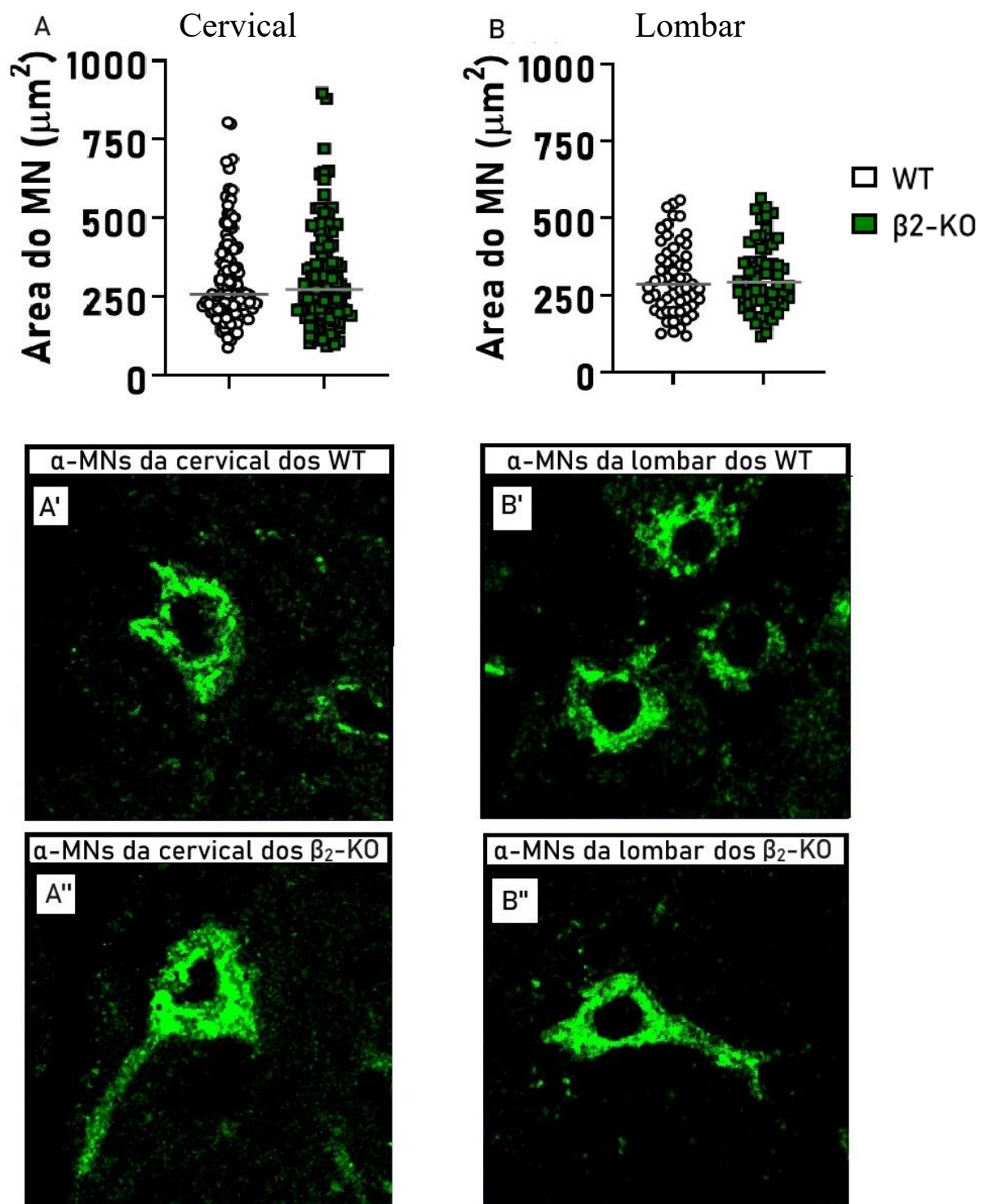


Figura 9 - Mesmo com a deleção completa de β_2 -AR o tamanho dos α -MNs de animais β_2 -KO é similar ao de animais WT. A quantificação da área dos α -MNs presentes na **A- região cervical e na **B**- região lombar mostra que a deleção dos receptores β_2 -adrenérgicos não foi capaz de promover alterações de tamanho entre os α -MNs de animais *knockout* e WTs. Teste de Mann-Whitney, resultados representam a mediana. n=**

4 animais por grupo, 3 cortes por região para cada animal. Foram consideradas células positivas para marcação de OPN e para DAPI.

4.3 A ausência dos β_2 -AR promove profundas alterações na morfologia da JNM de diferentes músculos dos animais β_2 -KO

Após constatararmos que a deleção dos receptores β_2 -adrenérgicos não alterou a área dos α -MNs, seguimos com a análise dos outros componentes da unidade motora, mais especificamente a JNM. Observamos que a deleção completa do receptor β_2 -adrenérgico nestes animais foi capaz de produzir profundas alterações na JNM, já com três meses de idade. A primeira e mais significativa alteração observada foi a na área dos terminais pré e pós-sinápticos, a qual estava muito diminuída nos animais com deleção nos músculos EDL (pré-sináptico: WT: 361,9 μm^2 , β_2 -KO: 333,1 μm^2 ; pós-sináptico: WT: 384,6 μm^2 , β_2 -KO: 348,3 μm^2 , $p < 0.05$ (Figura 10 A e B, Figura 11 A e A'), no SOL (pré-sináptico: WT: 394,4 μm^2 , β_2 -KO: 266,6 μm^2 ; pós-sináptico: WT: 415,7 μm^2 , β_2 -KO: 267,3 μm^2 ; $p < 0.0001$ (Figura 10 C e D, Figura 11 B e B') e no ETM (pré-sináptico: WT: 439,0 μm^2 , β_2 -KO: 350,6 μm^2 ; pós-sináptico: WT: 461,5 μm^2 , β_2 -KO: 351,2 μm^2 ; $p < 0.0005$) (Figura 10 E e F, Figura 11 C e C').

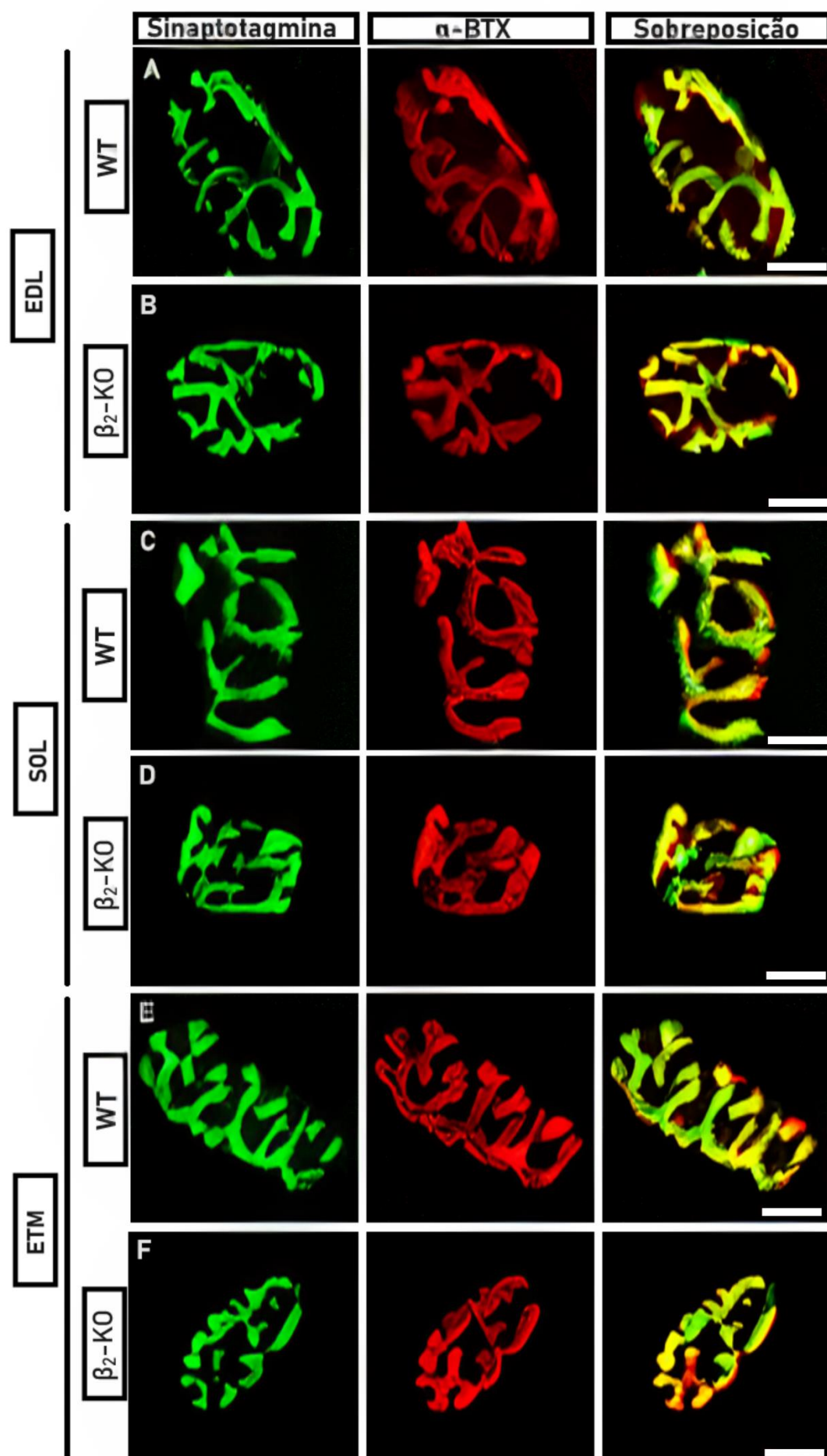


Figura 10 - Painel representativo evidenciando que os músculos EDL, SOL e ETM dos animais β_2 -KO apresentam drástica redução de tamanho de seus elementos sinápticos em relação aos animais WT. A e B- Imagens representativas mostrando que a deleção dos β_2 -AR gerou uma redução da área dos elementos sinápticos do músculo EDL nos animais β_2 -KO; de maneira similar, observamos a mesma diferença nos músculos SOL em C e D, e ETM, em E e F. Pré- (sinaptotagmina em verde) e pós-sinápticos (α -bungarotoxina em vermelho). Barra de escala: 50 μ m.

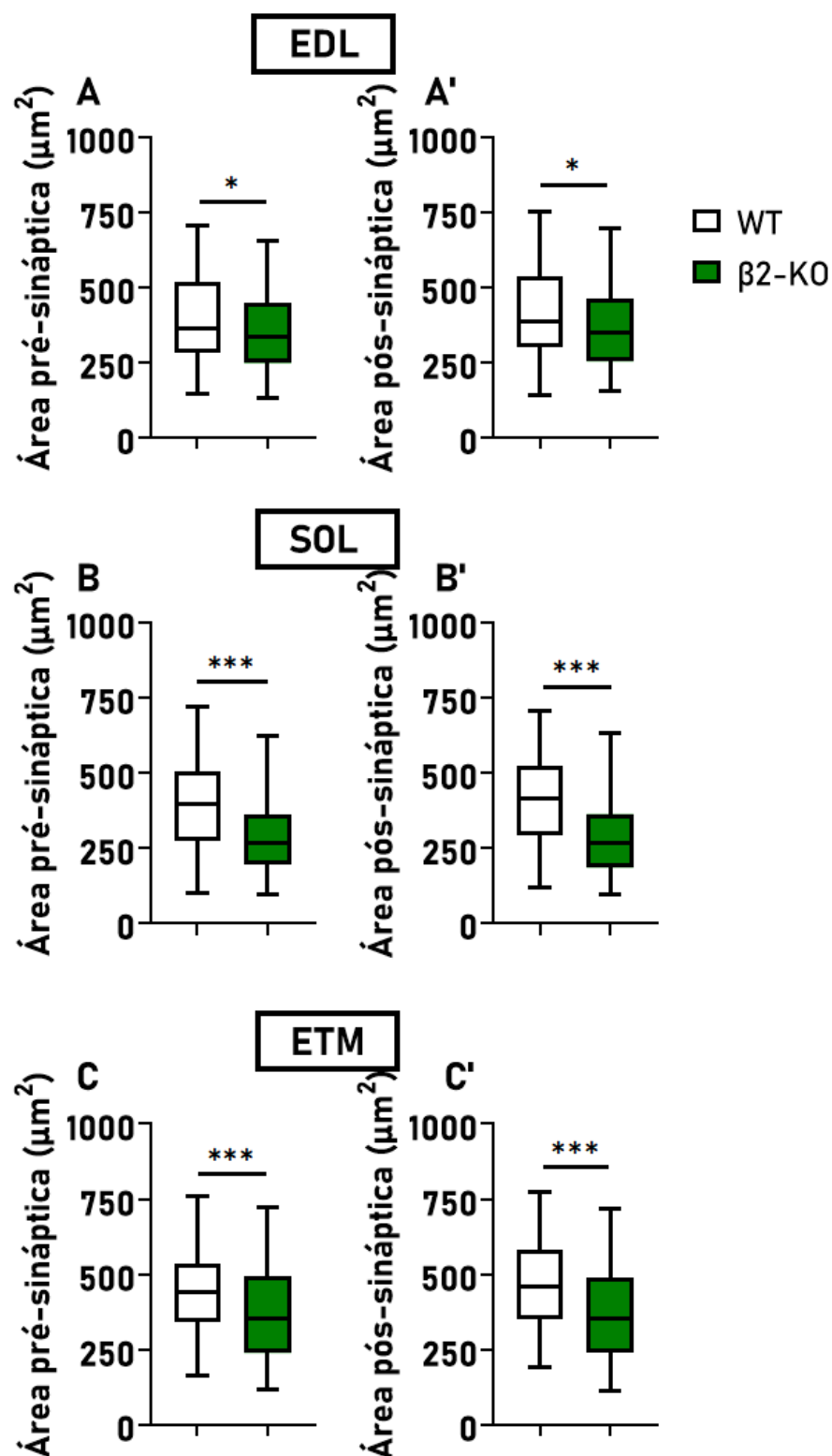


Figura 11 - A quantificação da área dos elementos pré- e pós-sináptico dos animais WT e β_2 -KO demonstra a diminuição da área das JNMs em todos os músculos. A e A'- A quantificação da área dos elementos pré- e pós-sinápticos do músculo EDL, respectivamente, confirma a diminuição na área da JNM dos animais β_2 -KO em

comparação com os animais WT; de forma ainda mais evidente, em **B** e **B'**, observamos a diminuição da área dos elementos pré- e pós-sinápticos do músculo SOL, respectivamente, e em **C** e **C'** a diminuição da área dos elementos pré- e pós-sinápticos do músculo ETM, respectivamente. * $p < 0.05$, *** $p < 0.0005$. Teste de Mann-Whitney, resultados representam a mediana. $n = 4$ animais por grupo, 20 JNM analisadas por animal.

Após observarmos as alterações no tamanho da JNM, partimos para as análises de fragmentação e colocalização dos terminais sinápticos. Ao quantificarmos o índice de fragmentação do elemento pós-sináptico nos animais β_2 -KO, observamos que a JNM do EDL se manteve preservada (Figura 12 A e B e Figura 13 A), entretanto, de forma contrária, houve um aumento na quantidade de ilhas de fragmentação tanto no músculo SOL (WT: $13,93 \pm 5,05$ %, β_2 -KO: $48,21 \pm 17,66$ %, $p < 0.005$) (Figura 12 C e D e Figura 13 B) quanto no ETM (WT: $13,01 \pm 5,64$ %, β_2 -KO: $56,55 \pm 5,48$ %, $p < 0.0001$) (Figura 12 E e F, Figura 13 C). Apesar disso, não observamos alteração no índice de colocalização dos terminais sinápticos entre os animais WT e β_2 -KO para nenhum dos músculos analisados (Figura 13 D-F).

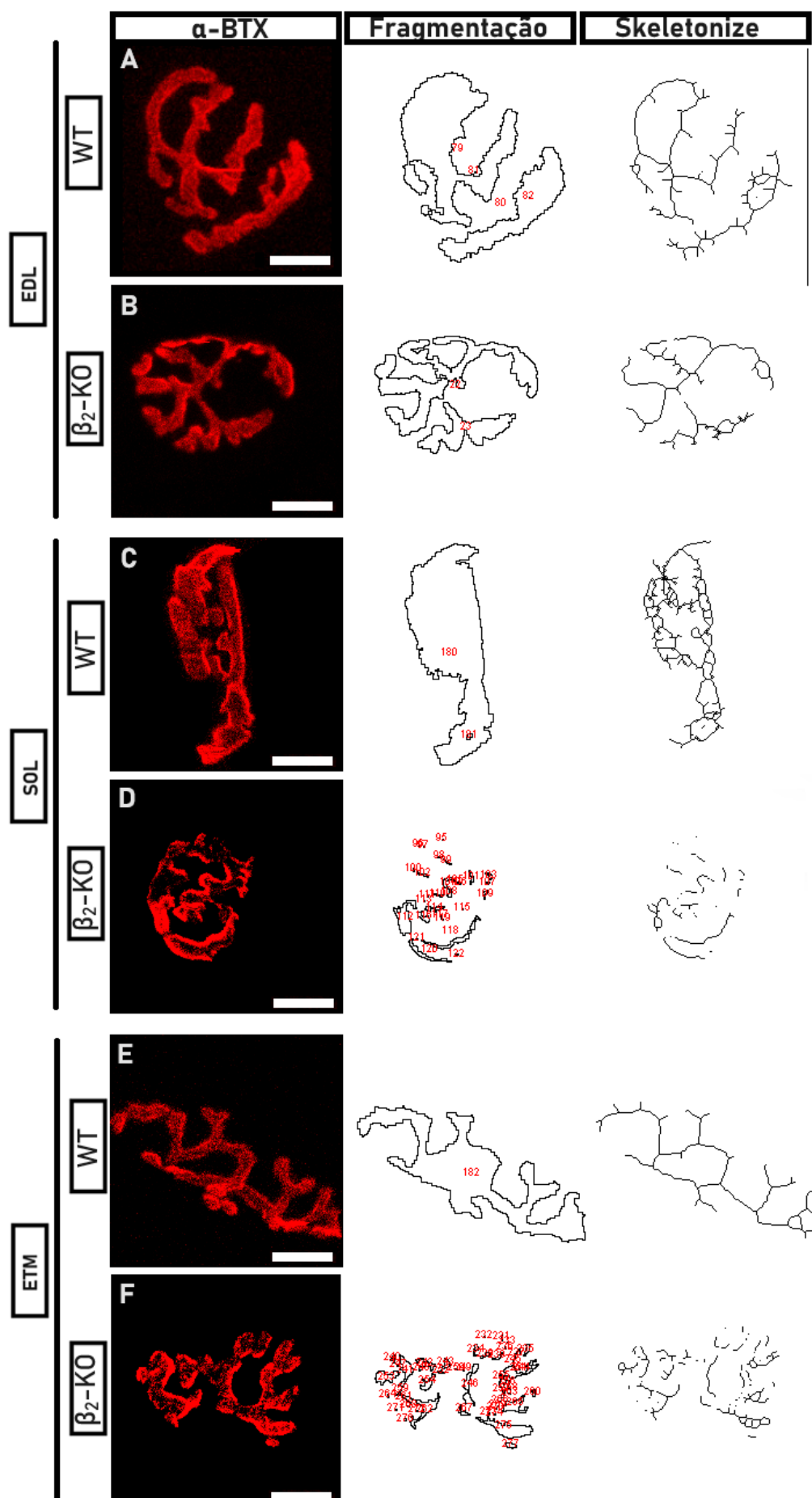


Figura 12 - Paineis representativos demonstrando o aumento da fragmentação do elemento pós-sinápticos (α -bungarotoxina em vermelho) dos músculos EDL, SOL e ETM nos animais β_2 -KO. A e B- As micrografias dos elementos pós-sinápticos do músculo EDL dos animais WT e β_2 -KO, respectivamente, demonstra que não há diferenças em relação à fragmentação; **C e D-** De modo oposto, as micrografias pós-sinápticas do músculo SOL demonstram uma clara fragmentação do elemento pós-sináptico dos animais e β_2 -KO; o que também se repetiu em **E e F-** Evidenciando a fragmentação presente no elemento pós-sináptico do músculo ETM dos animais WT e β_2 -KO, respectivamente. Pós-sinápticos (α -bungarotoxina em vermelho). Barra de escala: 50 μ m.

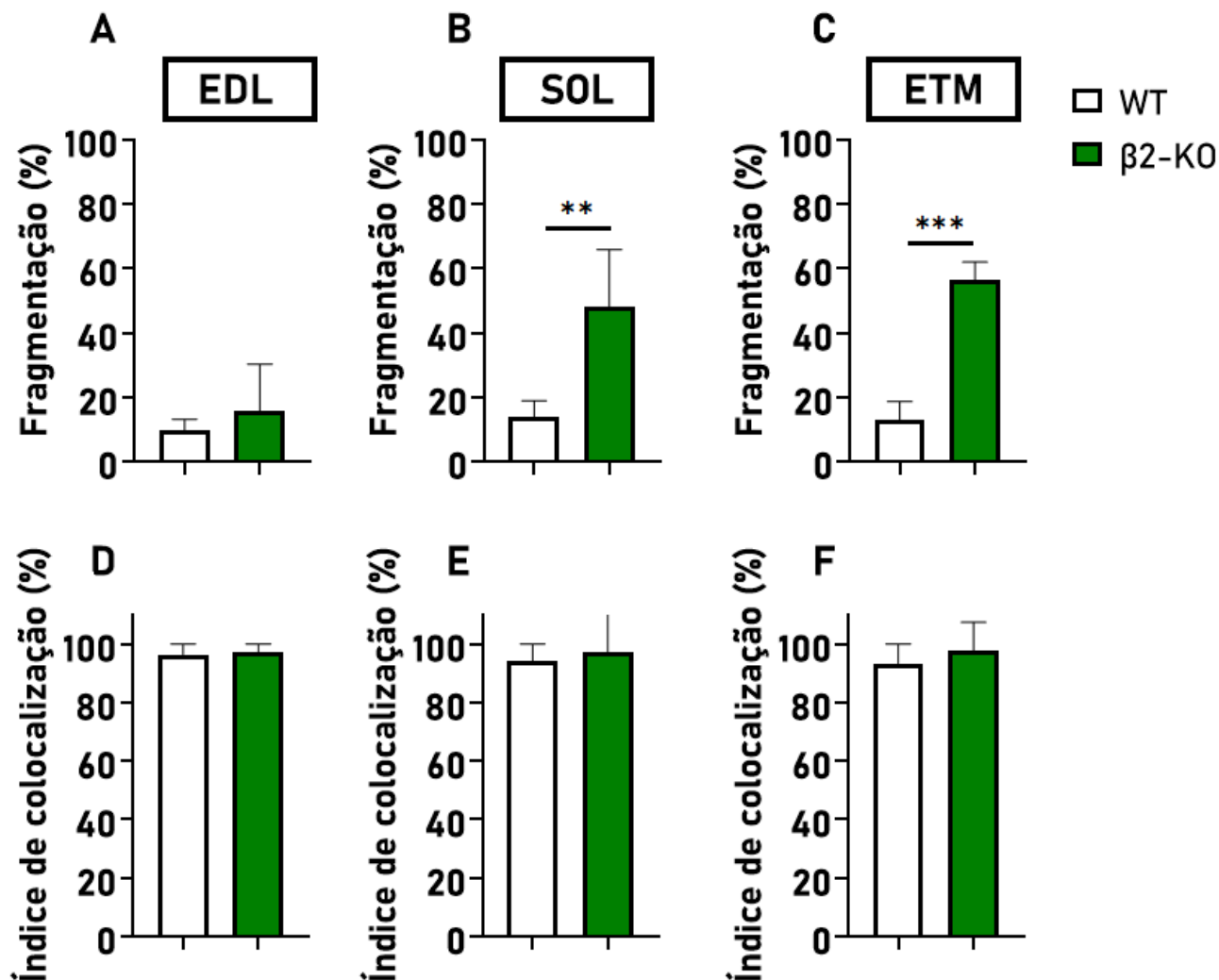


Figura 13 - A análise da fragmentação e colocalização da JNM dos animais WT e β_2 -KO confirma o observado nas micrografias representativas, uma desestabilização da JNM nos animais β_2 -KO. A-C- Ao quantificar os índices de fragmentação pós-sináptica dos músculos EDL, SOL e ETM, respectivamente, observamos que os músculos SOL e ETM são afetados bruscamente pela deleção dos β_2 -AR; D-F- Em contrapartida, apesar de apresentarem elevado índice de fragmentação, nenhum músculo demonstrou alteração na colocalização de seus elementos sinápticos. **p<0.005, *p<0.0001. Teste de Mann-Whitney, resultados representam a mediana, para a colocalização. Teste t de Student não pareado, resultados representam a média $\pm$ DP, para a fragmentação. n= 4 animais por grupo, 20 JNM analisadas por animal.**

4.4 A deleção completa do β_2 -AR gera atrofia em músculo de contração lenta

O último componente da unidade motora analisado foi a musculatura esquelética, mais especificamente os músculos de contração rápida e lenta das patas, EDL e SOL, respectivamente, e o músculo rápido-híbrido do pescoço, ETM. Já é bem descrito na literatura que os β_2 -AR possuem funções importantes na musculatura esquelética (SATO, 2011), sendo que alterações são esperadas com a deleção desses receptores. Entretanto, ao contrário do que observamos a nível de JNM, a deleção do β_2 -AR promoveu alterações apenas na musculatura de contração lenta do músculo SOL, que atrofiou em relação ao seu controle WT (WT: $1462 \mu\text{m}^2$, β_2 -KO: $1117 \mu\text{m}^2$, $p < 0.0001$) (Figura 14 C-D). É importante mencionar que músculos com perfil oxidativo, ou seja, de contração lenta, apresentam maior expressão de receptores β_2 adrenérgicos do que o restante (JOASSARD, 2013).

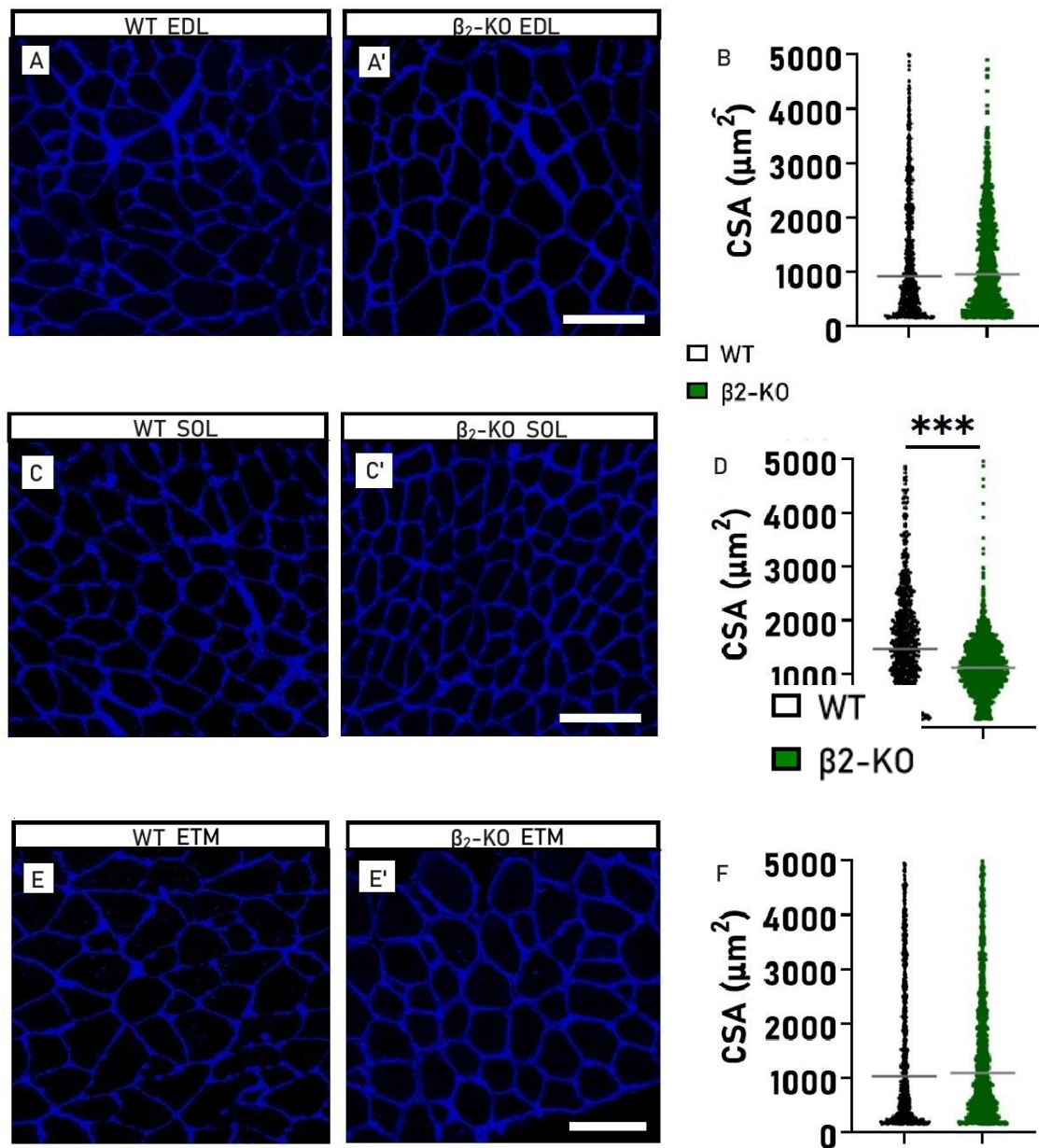


Figura 14 - A deleção dos β_2 -AR causa atrofia das fibras de músculo de contração lenta, mas não de músculos com perfil de contração rápida. A e A'- Imagem representativa da musculatura EDL dos animais WT e β_2 -KO, respectivamente; B- A área de secção transversa do músculo puramente rápido EDL não sofreu alteração em animais β_2 -KO; C e C'- Imagem representativa da musculatura SOL dos animais WT e β_2 -KO, respectivamente; D- Entretanto, a área do músculo puramente lento SOL foi afetada drasticamente, com os animais β_2 -KO diminuindo a área das fibras em relação aos animais WT; E e E'- Imagem representativa da musculatura ETM dos animais WT e β_2 -KO, respectivamente; F- Já a musculatura do ETM, híbrido-rápido, também não foi afetada. *p<0.0001. Teste de Mann-Whitney, resultados representam a mediana. n= 4**

animais por grupo, 1 corte transversal na porção média à longitudinal do músculo por animal foi utilizado para análise, para cada músculo, por animal.

Com o conjunto de resultados mostrados até aqui, mostramos que a deleção dos β_2 -AR é capaz de promover profundas alterações na unidade motora dos animais e refletir no comportamento. Dessa forma, demonstramos que o Sistema Nervoso Simpático, através dos β_2 -AR possui importância na unidade motora, gerando subsídios para a utilização de agonistas β_2 -adrenérgicos para o tratamento das SMCs. Sendo assim, partimos para a caracterização das alterações comportamentais motoras dos animais mnVACHT-KD e a investigação de como o tratamento de animais mnVACHT-KD miastênicos com o agonista β_2 -adrenérgico formoterol pode afetar positivamente esse modelo de SMC.

4.5 Camundongos mnVACHT-KD homozigotos fêmeas possuem fenótipo miastênico

O nosso grupo de pesquisa, durante a caracterização inicial desse novo modelo, mostrou que os camundongos mnVACHT-KD fêmeas homozigotas apresentavam déficits motores a partir de 2 meses de idade, com fenótipo mais pronunciado aos 6 meses (JOVIANO-SANTOS., 2021). No entanto, restava a dúvida se animais fêmeas mnVACHT-KD heterozigotas apresentavam também alterações motoras. Logo, a primeira pergunta que fizemos nesse projeto foi se os animais heterozigotos e homozigotos fêmeas apresentavam o mesmo fenótipo quando avaliados por testes comportamentais motores. Escolhemos investigar as fêmeas, inicialmente, uma vez que os machos, por motivo ainda desconhecido sobrevivem somente até os dois meses e meio de vida, ao passo que as fêmeas possuem uma sobrevida maior (aproximadamente 7 meses). Para o grupo experimental heterozigoto utilizamos animais mnVACHT-KD fêmeas e WT, de 6 meses de idade. Realizamos três testes comportamentais motores: força da pegada de duas patas, força da pegada de quatro patas e o teste de grade invertida. Em nenhum deles foi observada diferença entre os grupos WT e mnVACHT-KD heterozigotos (Figura 15). Como eles não apresentaram deficiência motora nesses testes, optamos por não aplicar nenhum outro.

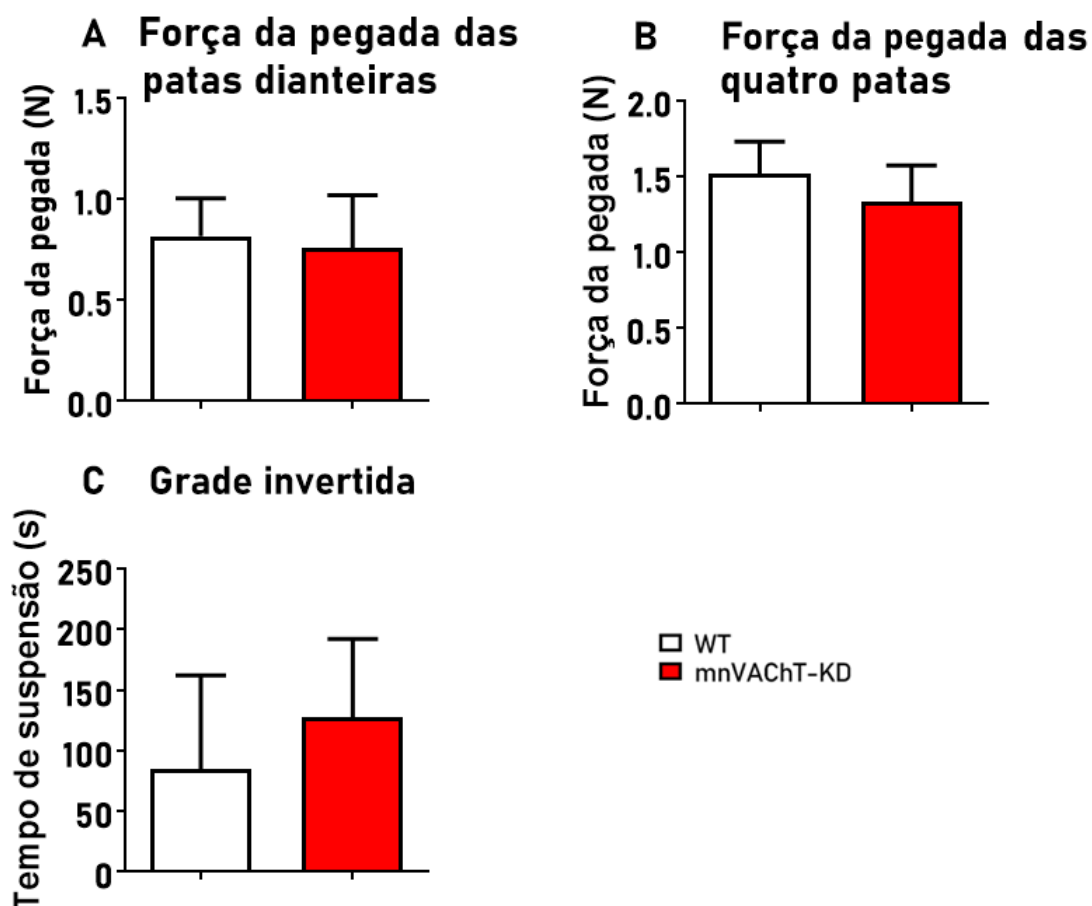


Figura 15 - Animais fêmeas mnVAcHt-KD heterozigotas não apresentam fenótipo miastênico em comparação com animais WT. A e B- Os animais fêmeas mnVAcHt-KD heterozigotas não apresentaram redução na força da pegada das patas dianteiras e nem das quatro patas, respectivamente; **C-** Similarmente, nenhuma alteração foi observada no tempo sustentando o próprio peso no teste de grade invertida. Teste t de Student não pareado, resultados representam a média $\pm$ DP. n= 7 por grupo.

Uma vez que os animais heterozigotos possuem fenótipo similar aos animais WT, partimos para a investigação da capacidade motora dos mnVAcHt-KD fêmeas homozigotas e WTs, aos 3 meses de idade. Realizamos os mesmos testes de anteriormente e adicionamos os testes do RotaRod e Campo Aberto. Observamos que as fêmeas mnVAcHt-KD homozigotas, na idade de três meses, apresentaram uma diminuição drástica na força da pegada de duas (WT: $0,9116 \pm 0,1041$ N; mnVAcHt-KD: $0,6690 \pm 0,1679$ N, $p < 0.005$) (Figura 16 A) e quatro patas (WT: $1,619 \pm 0,1893$ N; mnVAcHt-KD: $1,2884 \pm 0,2028$ N, $p < 0.005$) (Figura 16 B) comparado com os animais WT. Da

mesma forma, observamos que os animais mnVACHT-KD conseguiam sustentar o seu peso por menos tempo no teste de grade invertida comparado com os animais WT (WT: $116,9 \pm 7,075$ s; mnVACHT-KD: $51,51 \pm 30,09$ s) (Figura 16 C). Já nos testes Rotarod e campo aberto não observamos diferenças entre os grupos (Figura 16 D-F).

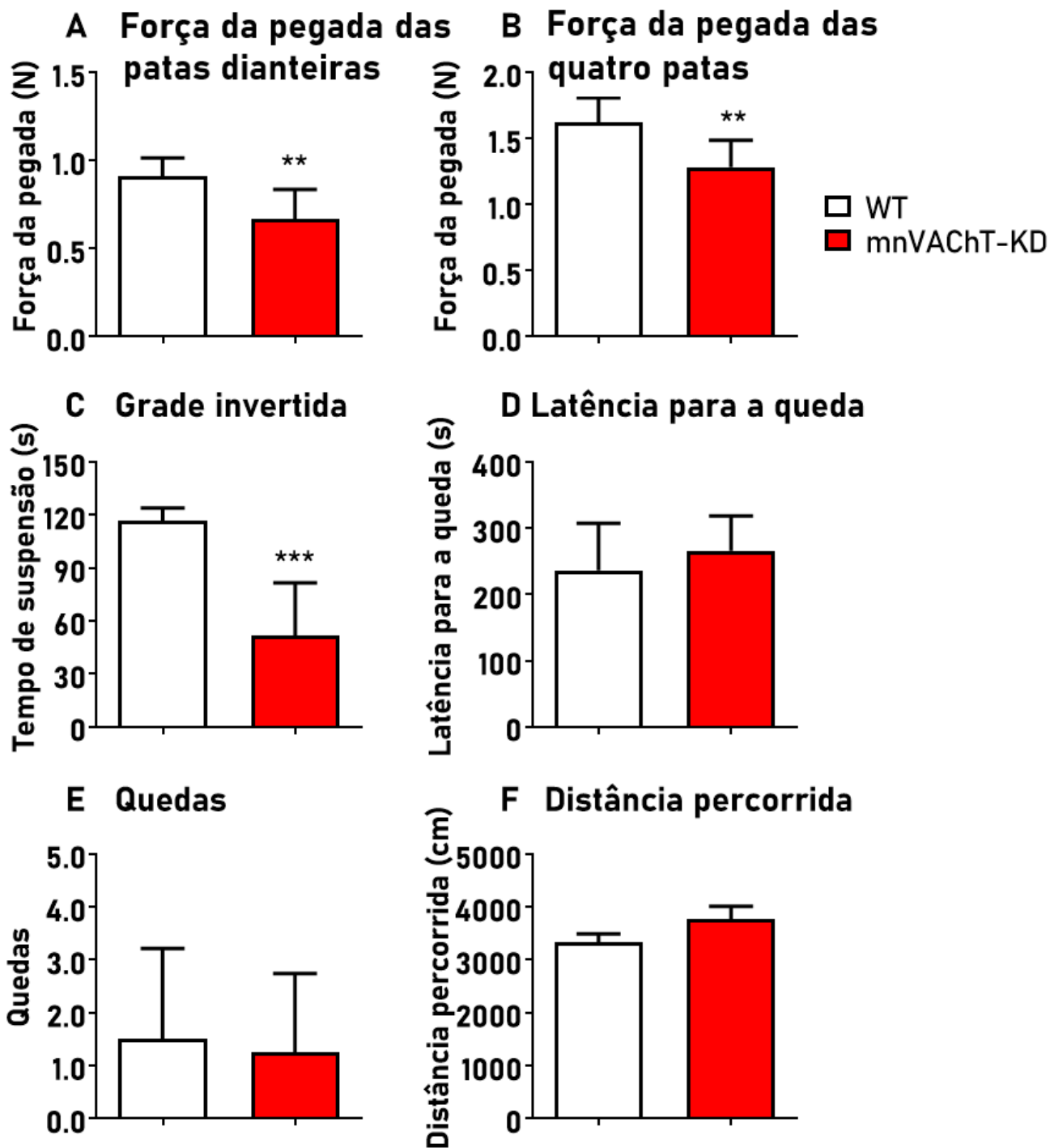


Figura 16 - Animais fêmeas mnVACHT-KD homozigotas apresentam fenótipo miastênico em comparação com animais WT a três meses de idade. A e B- Em

oposição aos animais heterozigotos, notamos que os mnVACHT-KD homozigotos apresentam uma redução significativa na força da pegada das patas dianteiras e das quatro patas, respectivamente; **C**- De forma semelhante, também observamos diminuição no tempo sustentando o próprio peso no teste de grade invertida; **D** e **E**- Apesar dos déficits motores nos testes anteriores, não observamos perdas nos parâmetros de latência para a queda e número total de quedas no Rotarod, respectivamente, assim como não observamos nenhuma redução na **F**- Distância total percorrida no teste de campo aberto. ** $p < 0.005$, *** $p < 0.0005$. Teste t de Student não pareado, resultados representam a média $\pm$ DP. n= 9 por grupo.

O modelo estabelecido previamente pelo grupo dos Professores Vânia e Marco Prado, o VACHT-KD, apesar de miastênico, também possui alterações cognitivas, como déficit de memória (PRADO, 2006). O mnVACHT-KD apresenta a deleção do VACHT restrita apenas a α -MNs, portanto, acreditamos que isso não implicaria em alterações comportamentais não-motoras. Sendo assim, optamos por realizar três testes comportamentais cognitivos com a finalidade de confirmar que esse novo modelo não possui alterações cognitivas, como, comportamento compulsivo, déficit de memória e sinais depressivos através dos testes de *Marble Burying*, labirinto em Y e nado forçado respectivamente. Em nenhum dos testes mencionados observamos alterações (Figura 17).

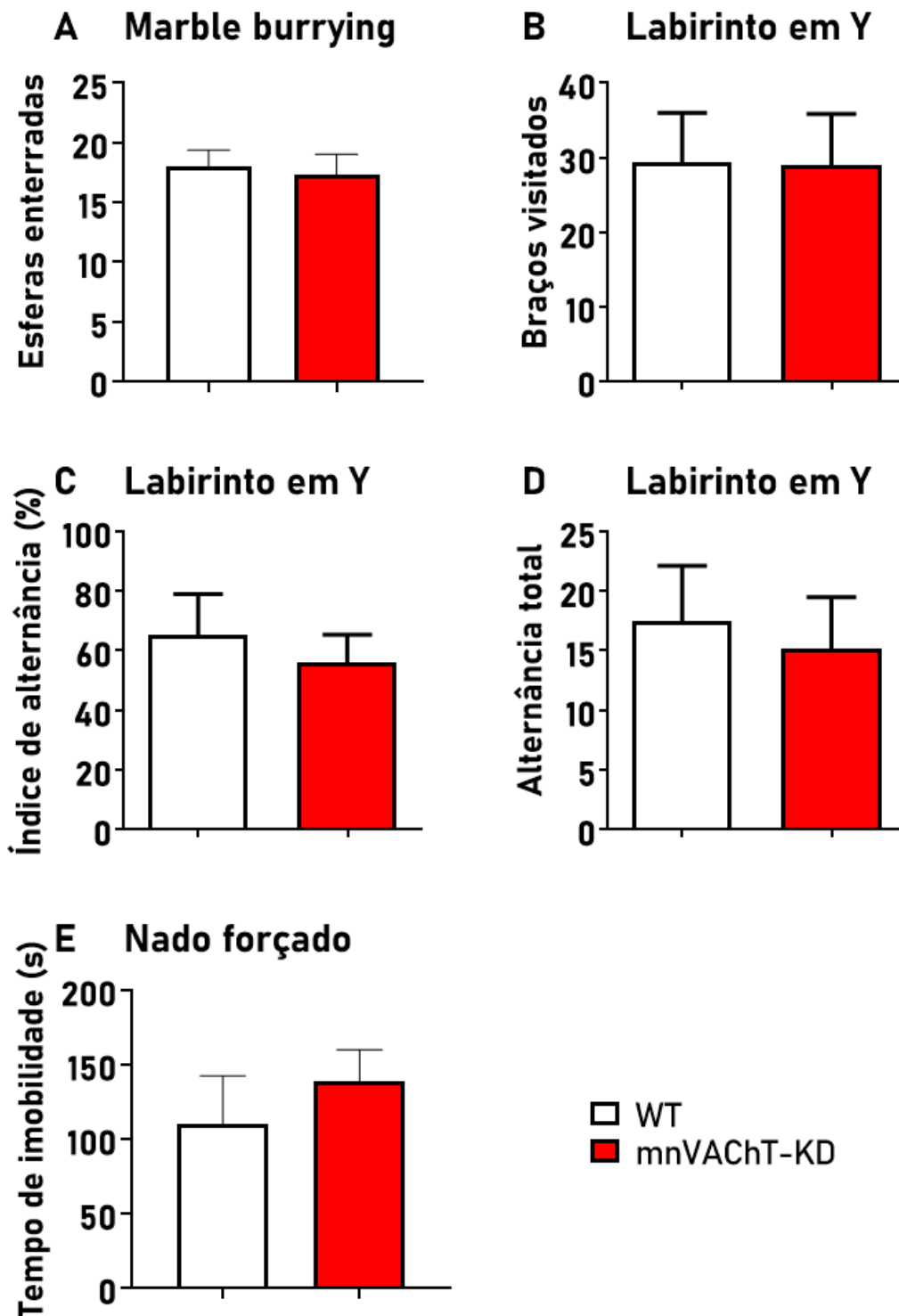


Figura 17 - Animais fêmeas mnVACHT-KD homozigotas não apresentam alterações cognitivas quando comparadas a animais WT. Assim como esperado, os animais mnVACHT-KD não apresentam alterações cognitivas nos testes de **A - *Marble Burying***, número de esferas enterradas, no teste de labirinto em Y em **B, C e D**- Número de braços visitados, alternância entre os braços e total de alternância entre braços, respectivamente,

e no E- Tempo de imobilidade no teste de nado forçado. Teste t de Student não pareado, resultados representam a média $\pm$ DP. n= 9 por grupo.

De posse dos resultados descritos acima, decidimos realizar todos os experimentos seguintes utilizando-se camundongos mnVAcHt-KD homozigotos. Iniciamos, então, o tratamento farmacológico dos mnVAcHt-KD homozigotos com o formoterol na tentativa de minimizar os seus déficits motores e entender como a unidade motora responderia.

4.6 O tratamento de camundongos fêmeas mnVAcHt-KD de três meses com formoterol não foi capaz de melhorar o fenótipo miastênico

Anteriormente a qualquer intervenção farmacológica, realizamos os testes comportamentais e observamos as alterações descritas anteriormente. Também certificamos de que os grupos fossem divididos aleatoriamente e de forma que nenhum deles contivesse alguma tendência a uma pior ou melhor performance do que outro (dados não mostrados). Durante 30 dias corridos realizamos injeções s.c de formoterol a 300 μ g/kg e, ao final do tratamento, uma nova bateria de testes comportamentais para comparar se houve alguma evolução em relação às alterações presentes antes da intervenção. Em nenhum dos testes comportamentais escolhidos observamos melhora do grupo mnVAcHt-KD^{for} em relação ao seu análogo veículo (Figura 18). Na Figura 18 E, no resultado da distância total percorrida no Teste do Campo Aberto, todos os grupos elevaram a distância percorrida, eliminando um possível efeito do formoterol.

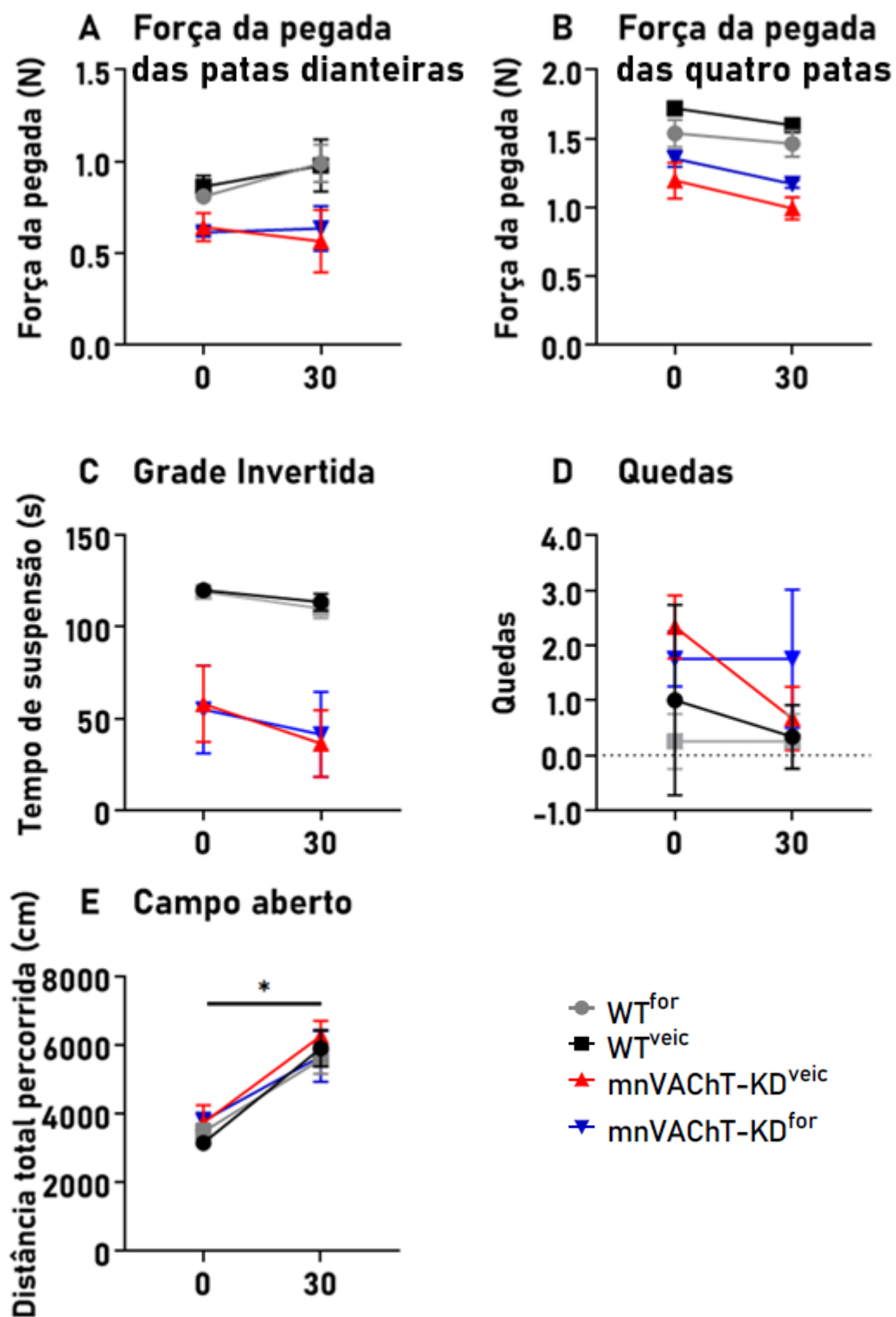


Figura 18 - Curva de comportamentos motores antes (0), e ao final (30) do tratamento com formoterol. O formoterol não foi capaz de reverter os déficits motores dos animais mnVAcHT-KD. A e B- O teste de força da pegada de duas e quatro patas, respectivamente, evidencia que a administração de formoterol não foi capaz de melhorar as alterações motoras observadas anteriormente nos animais mnVAcHT-KD;

C- De forma semelhante, nenhuma melhora foi observada nos testes de suspensão em grade; e **D-** Quedas no teste de Rotarod, e; **E-** No teste do campo aberto todos os animais aumentaram a distância percorrida. Teste two-way ANOVA e pós-teste de Tukey. Resultados representam a média $\pm$ DP. $n=5$ para todos os grupos.

Apesar de constatararmos que a administração de formoterol não resultou em melhorias na capacidade motora das fêmeas mnVAcHt-KD, decidimos investigar se ele seria capaz de promover algum tipo de alteração nos componentes da unidade motora. O primeiro a ser analisado foram os α -MN das porções cervical e lombar da medula espinhal.

4.7 Camundongos fêmeas mnVAcHt-KD tratadas com formoterol exibem alterações nos α -MN

Optamos, então, por iniciar investigando a unidade motora pelos α -MN, seguindo pela sua comunicação com a musculatura esquelética, a JNM e por fim, a musculatura esquelética. Ao final do tratamento com formoterol, realizamos a retirada e o seccionamento da medula espinhal em suas porções cervical e lombar, e realizamos o protocolo de imunomarcção dos α -MN e DAPI, em todos os grupos (WT^{veic}, WT^{for}, mnVAcHt-KD^{veic} e mnVAcHt-KD^{for}). É importante salientar que o modelo mnVAcHt-KD nos permite identificar quais α -MNs (e consequentemente quais JNMs) possuem a deleção do VAcHt, uma vez que ele apresenta a expressão da proteína fluorescente vermelha TdTomato. Sendo assim, toda vez que observarmos um corpo neuronal ou JNM com fluorescência no comprimento de onda 555 nm (vermelho) podemos afirmar que o VAcHt está deletado.

Inicialmente analisamos todos os α -MNs, independente da presença do TdTomato. O formoterol foi capaz de promover uma diminuição no tamanho dos α -MN presentes na cervical tanto dos animais WT (WT^{veic}: 520,6 μm^2 , WT^{for}: 306,8 μm^2 , $p<0.0001$) quanto dos animais mnVAcHt-KD (mnVAcHt-KD^{veic}: 627,0 μm^2 , mnVAcHt-KD^{for}: 474,4 μm^2 , $p<0.0001$) (Figura 19 A-E). Porém, não promoveu alterações nos α -MN presentes na região lombar de nenhum grupo (Figura 19 F).

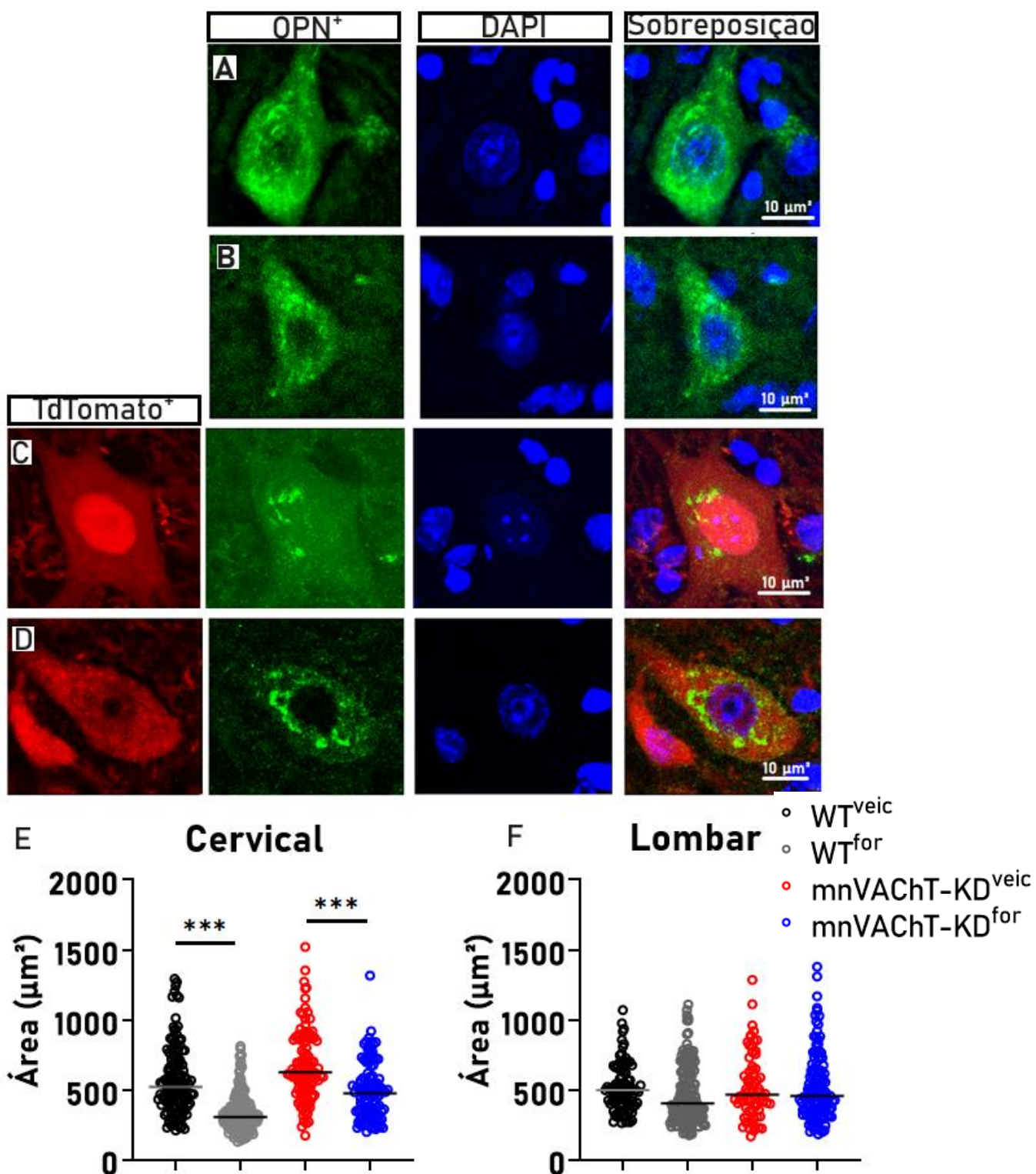


Figura 19 - O formoterol causou redução na área dos motoneurônios do segmento cervical de camundongos WT e mnVACHT-KD. A, B, C e D- Painel de imagens representativas evidenciando os α -MN da região cervical dos animais WT^{veic}, WT^{for}, mnVACHT-KD^{veic} e mnVACHT-KD^{for}, respectivamente. Podemos observar que os animais que receberam formoterol, nos grupos WT^{for} e mnVACHT-KD^{for} apresentaram

redução na área dos α -MN, em comparação com seus respectivos controles. **E e F-** A quantificação da área dos α -MN presentes na região cervical confirma que o formoterol induziu uma diminuição da área dos α -MN dos animais tratados. *** $p < 0.0001$, teste de Kruskal-Wallis e pós teste de Dunn, resultados representam a mediana. $n = 5$ animais por grupo, 3 cortes por região para cada animal. Foram consideradas células positivas para marcação de OPN (verde), para DAPI (azul) e, para animais mnVChT-KD, foram incluídas as TdTomato⁺ (vermelho).

Como a expressão do TdTomato permite a avaliação individual das consequências da deleção do VChT para o α -MN e comparar com um α -MN sem deleção no mesmo animal, realizamos a comparação dos α -MN que não expressam o VChT e os que expressam. Observamos que apesar da deleção do VChT, apenas os α -MN da cervical dos animais que receberam formoterol possuíam diferença de tamanho entre si (Figura 20 B). É importante lembrar que, para esta análise, consideramos apenas os α -MN que possuíam marcação TdTomato⁺ (apenas nos mnVChT-KD), OPN⁺ e DAPI⁺, e que os animais mnVChT-KD possuem aproximadamente 70% de deleção do VChT em α -MN, ou seja, a população de neurônios deletados é consideravelmente maior do que de neurônios sem deleção do VChT.

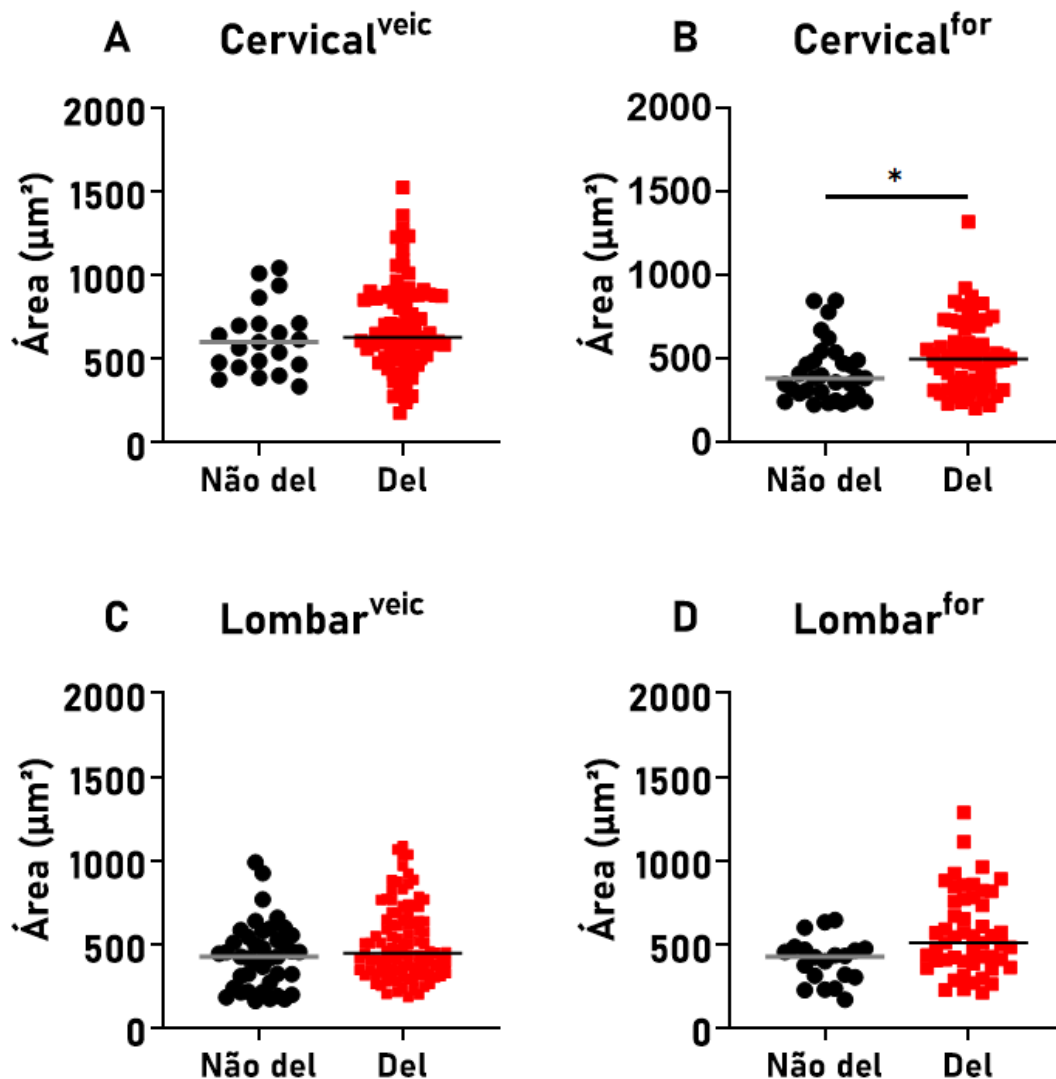


Figura 20 - Comparação da área dos motoneurônios sem deleção e com deleção do VACHT em animais mnVACHT-KD. A- A quantificação da área dos α -MN presentes na região cervical de animais mnVACHT-KD que receberam veículo mostrou que não há diferença entre os α -MN com deleção do VACHT e os sem deleção; B- Entretanto, quando realizamos a quantificação da área dos α -MN presentes na região cervical de animais mnVACHT-KD que receberam formoterol, notamos que os α -MN com deleção do VACHT possuem um tamanho ligeiramente maior do que os sem a deleção; C- Não observamos alteração na área dos α -MN presentes na região cervical de animais mnVACHT-KD que receberam veículo; D- E nem na área dos α -MN presentes na região lombar de animais mnVACHT-KD que receberam formoterol. * $p<0.05$, teste de Mann-Whitney, resultados representam a mediana. $n=5$ animais por grupo, 3 cortes por região para cada animal. Foram consideradas células positivas para marcação de OPN (verde), DAPI (azul) e TdTomato (vermelho).

4.8 O tratamento de camundongos fêmeas mnVAcHT-KD de três meses com formoterol promoveu alterações na JNM

O próximo componente da unidade motora analisado foi a JNM. Quando partimos para a sua análise observamos que o formoterol foi capaz de induzir um aumento na área dos terminais pré- (WT^{veic}: 373,4 μm^2 , WT^{for}: 475,5 μm^2 , mnVAcHT-KD^{veic}: 252,6 μm^2 , mnVAcHT-KD^{for}: 429,5 μm^2 , $p < 0.0001$) e pós-sinápticos (WT^{veic}: 389,2 μm^2 , WT^{for}: 500,3 μm^2 , mnVAcHT-KD^{veic}: 258,6 μm^2 , mnVAcHT-KD^{for}: 468,8 μm^2 , $p < 0.0001$ dos músculos EDL (Figura 21 A e 22 A e B), SOL (pré: WT^{veic}: 290,9 μm^2 , WT^{for}: 471,9 μm^2 , mnVAcHT-KD^{veic}: 358,6 μm^2 , mnVAcHT-KD^{for}: 530,7 μm^2 , $p < 0.0001$; pós: WT^{veic}: 306,2 μm^2 , WT^{for}: 483,7 μm^2 , mnVAcHT-KD^{veic}: 372,0 μm^2 , mnVAcHT-KD^{for}: 535,0 μm^2 , $p < 0.0001$) (Figura 21 B e 22 C e D) e no músculo ETM (pré: WT^{veic}: 304,0 μm^2 , WT^{for}: 459,3 μm^2 , mnVAcHT-KD^{veic}: 286,0 μm^2 , mnVAcHT-KD^{for}: 318,0 μm^2 , $p < 0.0001$, pós: WT^{veic}: 339,4 μm^2 , WT^{for}: 499,9 μm^2 , mnVAcHT-KD^{veic}: 309,7 μm^2 , mnVAcHT-KD^{for}: 304,2 μm^2 , $p < 0.0001$) (Figura 21 C e 22 E e F).

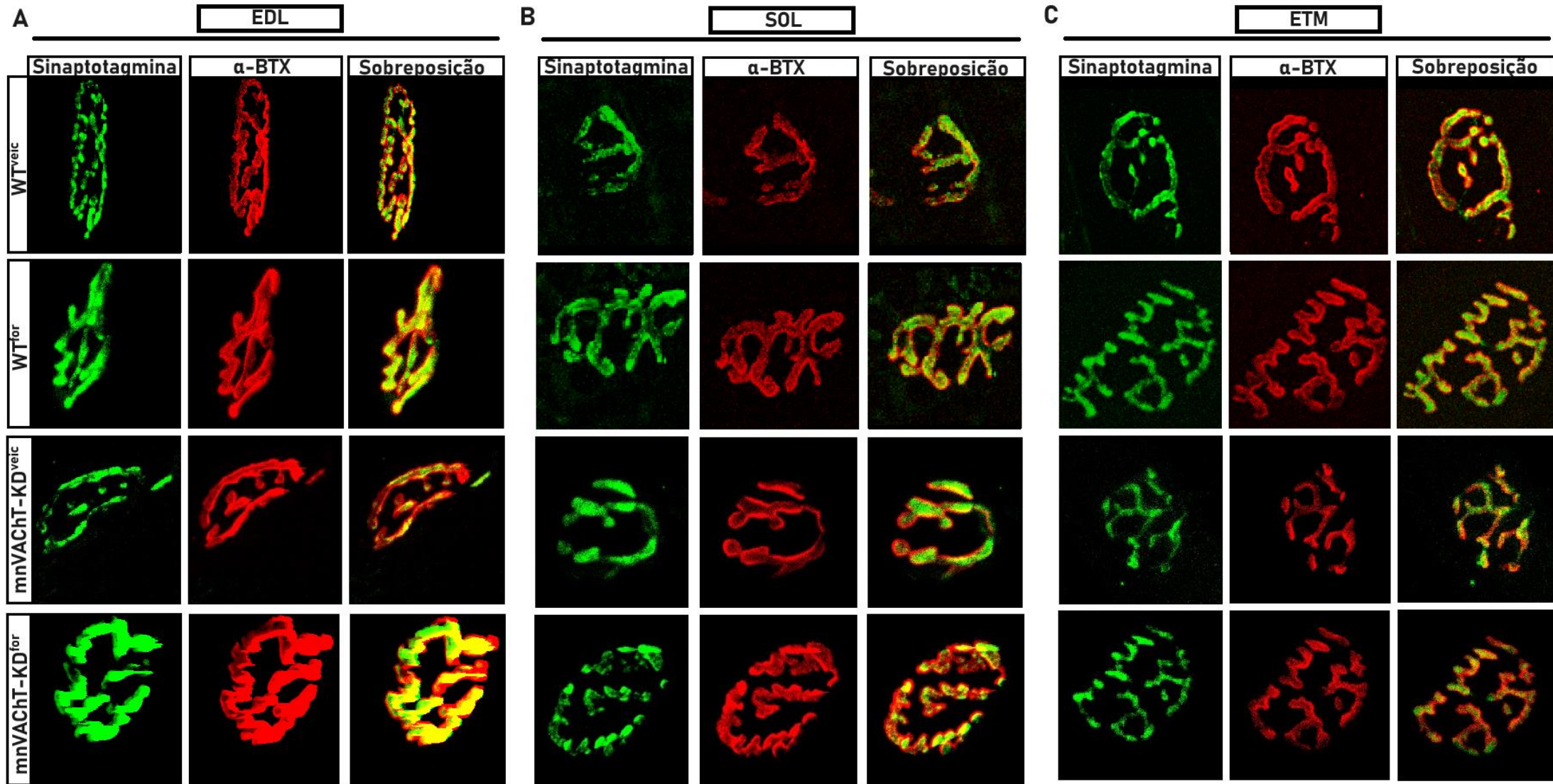


Figura 21 - Painele contendo micrografias representativas evidenciando o aumento da área dos elementos pré e pós-sináptico dos animais *mnVACHT-KD* em comparação com animais WT. Observamos que em **A**- há um aumento na área dos elementos sinápticos da musculatura EDL nos animais *mnVACHT-KD*^{for} em comparação com o *mnVACHT-KD*^{veic}, apesar de nenhuma alteração entre animais WT. Em **B**- podemos observar que tanto o grupo WT^{for} quanto o grupo *mnVACHT-KD*^{for} apresentaram notável incremento da área dos elementos sinápticos quando comparados aos seus respectivos controles que receberam veículo. Em **C**- observamos um aumento da área dos elementos sinápticos apenas nos animais WT^{for} em comparação com os WT^{veic}. Pré- (sinaptotagmina em verde) e pós-sinápticos (α -bungarotoxina em vermelho).

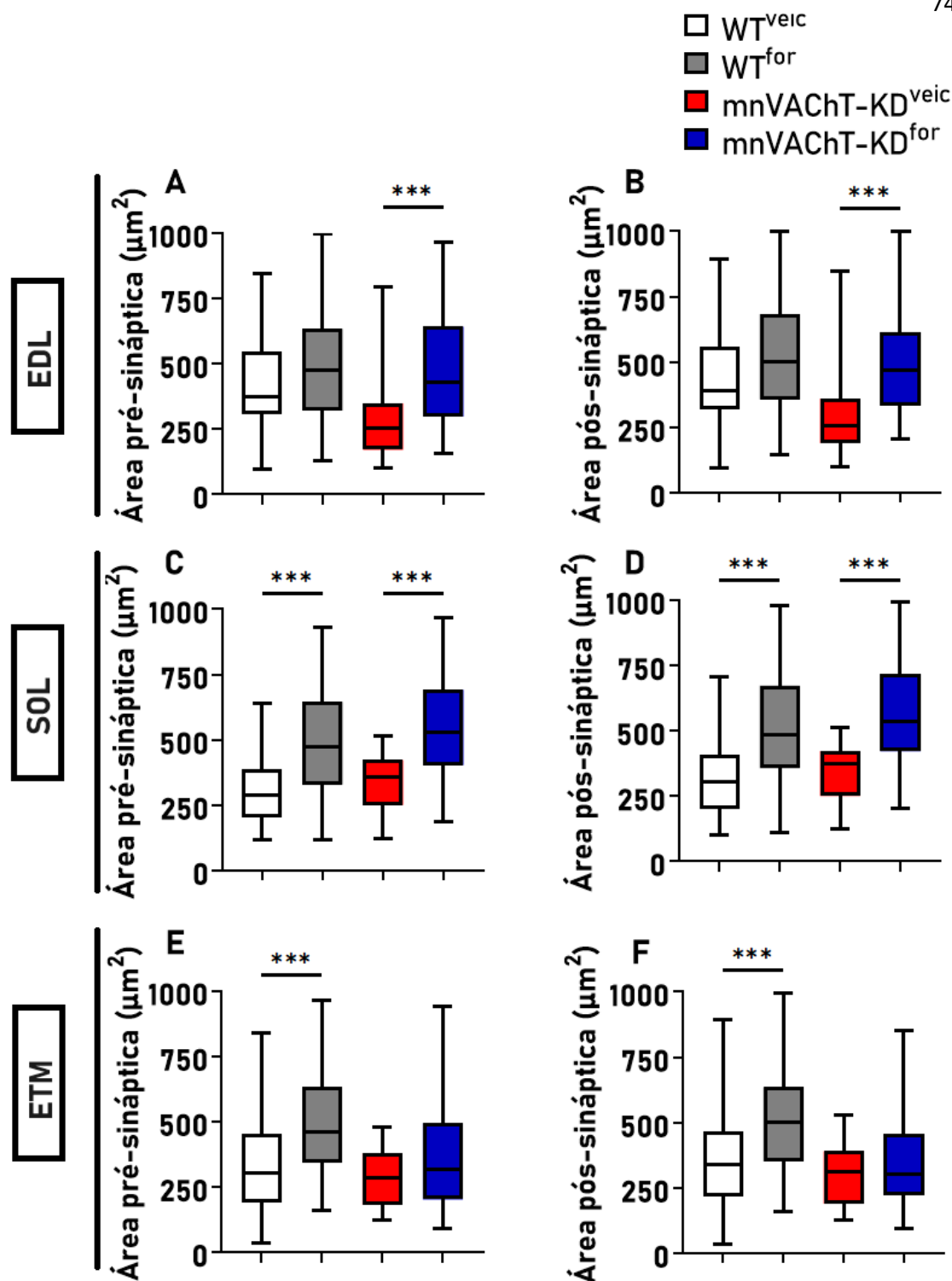


Figura 22 - A quantificação da área dos elementos pré- e pós-sináptico dos animais WT e mnVACHT-KD mostrou que o formoterol foi capaz de hipertrofiar a JNM de animais WT e mnVACHT-KD. **A** e **A'**- A área dos elementos pré- e pós-sinápticos do músculo EDL, respectivamente, sofreu hipertrofia após o tratamento com formoterol, em mnVACHT-KD; **B** e **B'**- Já na JNM do músculo SOL, os animais WTs também apresentaram elevação da área dos elementos pré- e pós-sinápticos, respectivamente; **C** e **C'**- Entretanto, no músculo misto ETM, observamos apenas aumento na área dos

elementos pré- e pós-sinápticos dos animais WT, respectivamente. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$. Kruskal-Wallis, resultados representam a mediana. $n = 5$ animais por grupo, 20 JNM analisadas por animal.

Fica bem evidente que o formoterol é capaz de gerar alterações no plano da JNM. Entretanto, os resultados anteriores representam a análise de todas as JNM dos animais mnVChT-KD, ou seja, JNM sem deleção do VChT (TdTomato⁻) e JNM com deleção do VChT (TdTomato⁺). Mas, será que existe alguma diferença entre as JNM sem deleção (TdTomato⁻) e as com deleção (TdTomato⁺)? Partimos então para a análise dos dois tipos de JNM dentro do mesmo animal. É importante lembrar que, este tipo de análise só é possível ser realizada com animais mnVChT-KD, uma vez que os animais WT não possuem JNM com deleção do VChT.

A primeira observação que realizamos foi que a deleção do VChT afeta de forma diferente o músculo rápido EDL e o lento SOL. No EDL a JNM sem a presença do VChT diminui de tamanho em relação à JNM com o VChT (área pré-sináptica da JNM TdTomato⁻: $369,9 \mu\text{m}^2$, área pré-sináptica da JNM TdTomato⁺: $245,3 \mu\text{m}^2$, $p < 0,0001$; área pós-sináptica da JNM TdTomato⁻: $349,7 \mu\text{m}^2$, área pós-sináptica da JNM TdTomato⁺: $255,7 \mu\text{m}^2$, $p < 0,0001$) (Figura 23 A e B) e o contrário ocorre com o SOL, aumentando sua área (área pré-sináptica da JNM TdTomato⁻: $255,0 \mu\text{m}^2$, área pré-sináptica da JNM TdTomato⁺: $358,6 \mu\text{m}^2$, $p < 0,0001$; área pós-sináptica da JNM TdTomato⁻: $273,9 \mu\text{m}^2$, área pós-sináptica da JNM TdTomato⁺: $372,0 \mu\text{m}^2$, $p < 0,0001$) (Figura 23 C e D).

Entretanto, quando adicionamos formoterol, há um aumento na área da JNM deletada em comparação com a deletada que recebeu veículo em ambos os músculos (EDL: área pré-sináptica da JNM TdTomato⁻: $331,6 \mu\text{m}^2$, área pré-sináptica da JNM TdTomato⁺: $385,5 \mu\text{m}^2$, $p < 0,0005$; área pós-sináptica da JNM TdTomato⁻: $394,1 \mu\text{m}^2$, área pós-sináptica da JNM TdTomato⁺: $417,7 \mu\text{m}^2$, $p < 0,0001$, Figura 23 A e B); (SOL: área pré-sináptica da JNM TdTomato⁻: $408,4 \mu\text{m}^2$, área pré-sináptica da JNM TdTomato⁺: $468,2 \mu\text{m}^2$, $p < 0,005$; área pós-sináptica da JNM TdTomato⁻: $453,8 \mu\text{m}^2$, área pós-sináptica da JNM TdTomato⁺: $472,9 \mu\text{m}^2$, $p < 0,005$, Figura 23 C e D). Constatamos, também, que as JNMs

não deletadas dos animais mnVACHT-KD são semelhantes em tamanho às JNMs provenientes de animais WT (dados não mostrados).

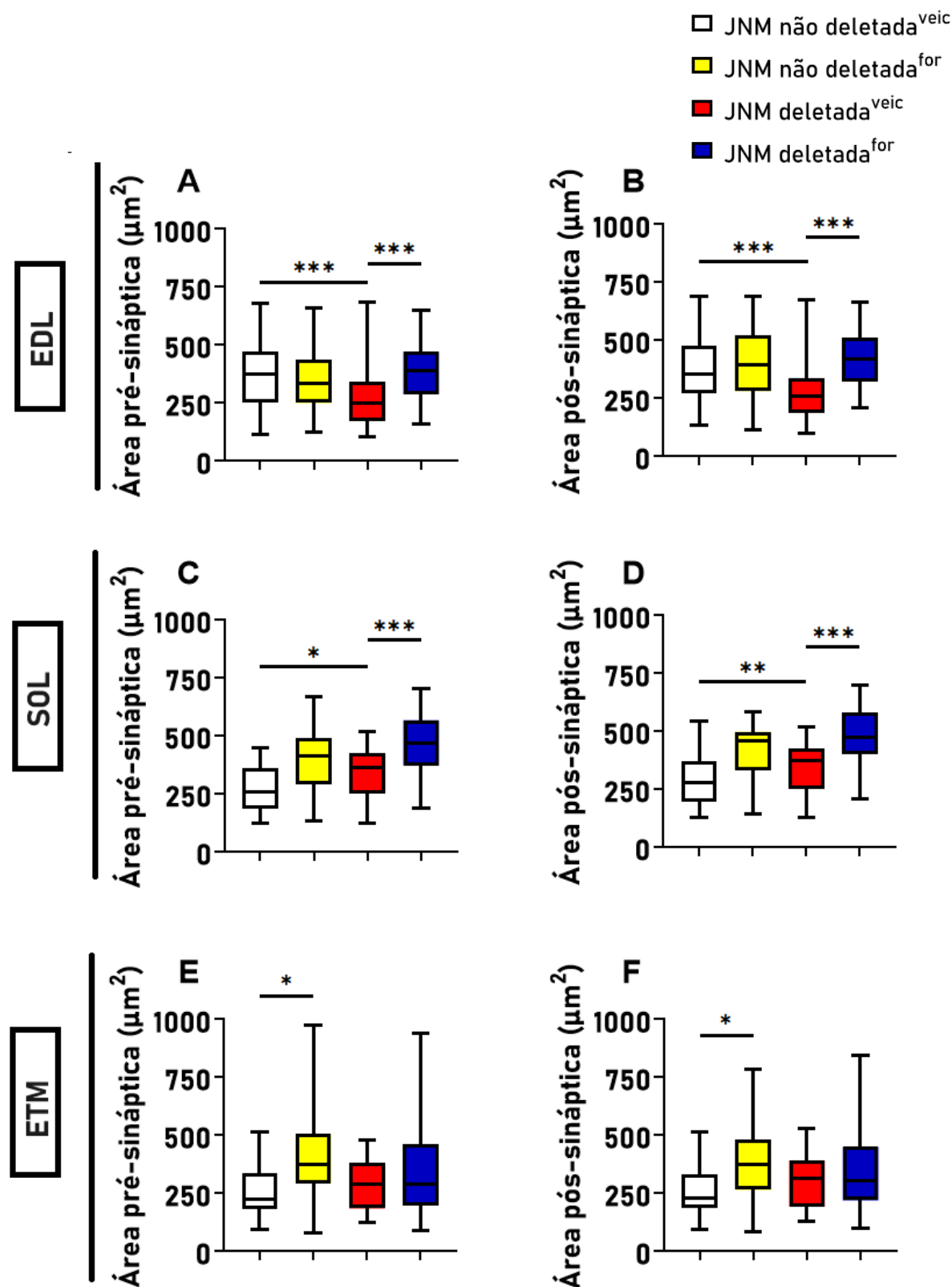


Figura 23 - A análise da área dos elementos pré- e pós-sinápticos mostrou que a deleção do VACHT afeta a morfologia da JNM de forma distinta de acordo com a

musculatura e o formoterol foi capaz de hipertrofiar as JNMs deletadas. A e B- A quantificação da área da JNM mostrou que a ausência do VACHT induz atrofia dos elementos sinápticos no EDL, entretanto, ao realizarmos o tratamento com formoterol, a JNM deletada aumentou de tamanho em comparação com seu controle que recebeu veículo; **C e D-** De forma contrária, o a ausência do VACHT induziu aumento na área dos elementos sinápticos do SOL. Já ao administrarmos formoterol, a JNM deletada também aumentou de tamanho em comparação ao grupo veículo; **E e F-** Todavia, apenas os animais WT sofreram alterações na área dos elementos sinápticos do músculo ETM. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$. Kruskal-Wallis, resultados representam a mediana. $n = 5$ animais por grupo, 20 JNM analisadas por animal.

Após observarmos alterações no tamanho das JNMs, decidimos analisar um parâmetro muito importante utilizado para avaliar a integridade e função da JNM, a colocalização entre os terminais sinápticos. Uma vez que os terminais pré- e pós-sinápticos precisam se colocalizar para que a neurotransmissão ocorra de forma efetiva, a análise de colocalização nos fornece informações importantes a respeito da integridade da comunicação neuromuscular. Quando realizamos essa análise, observamos uma leve diminuição da colocalização dos terminais no músculo SOL (Figura 24 B). Entretanto, como observamos uma hipertrofia nos resultados anteriores dos elementos sinápticos no SOL, é possível que essa leve diminuição na colocalização seja transitória.

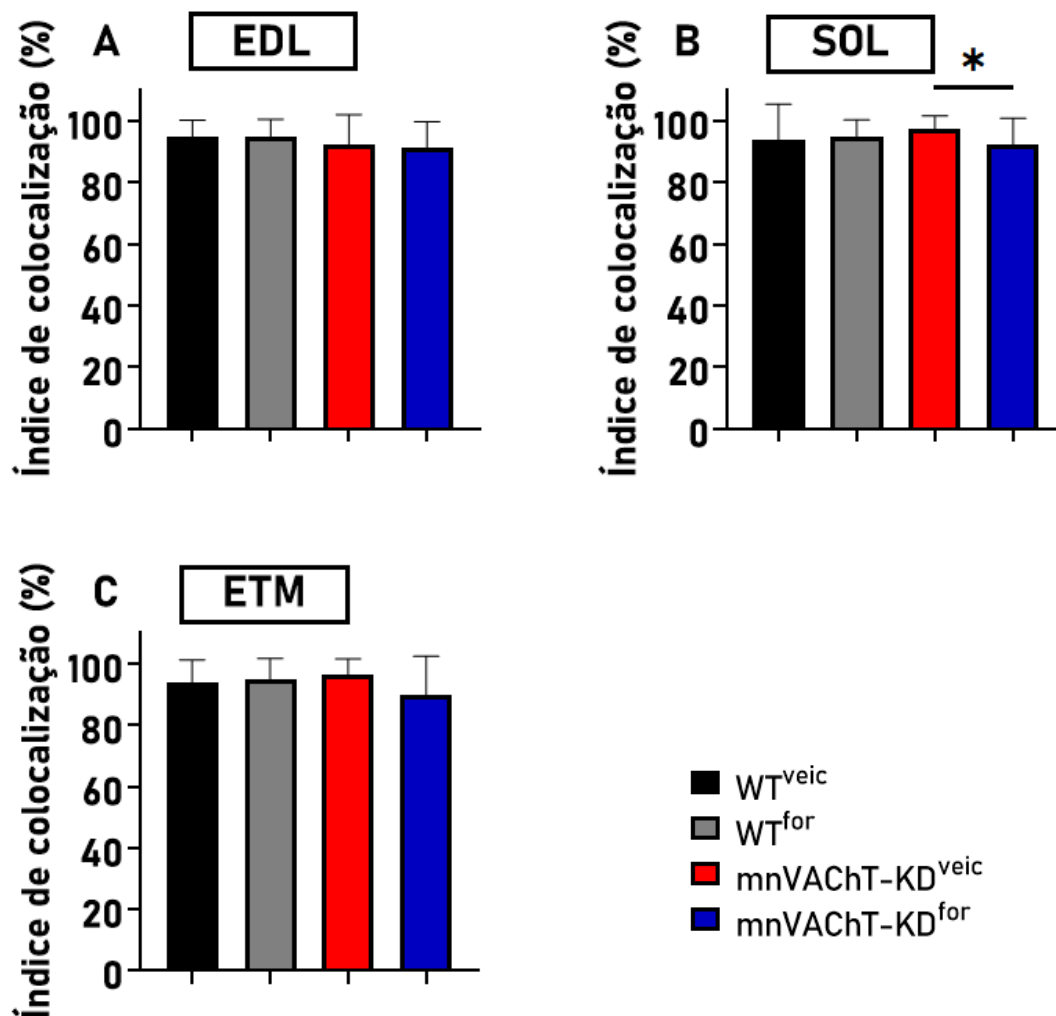


Figura 24 - Análise da colocalização dos elementos sinápticos dos animais WT e β_2 -KO. A- A quantificação da porcentagem de colocalização dos elementos sinápticos do músculo EDL mostrou que não há comprometimento em nenhum grupo; B- Entretanto, observamos uma leve alteração no índice de colocalização dos elementos sinápticos do músculo SOL; C- E similar ao primeiro resultado, não observamos alterações no índice de colocalização dos elementos sinápticos do músculo ETM. Teste de Kruskal-Wallis, resultados representam a mediana. n= 5 animais por grupo, 20 JNM analisadas por animal. *p<0.05.

Assim como a colocalização, a análise da fragmentação também nos fornece informações importantes a respeito da estrutura e função da JNM. A fragmentação elevada é um achado comum em JNM que estão sofrendo algum insulto ou degeneração e, níveis mais

baixos de fragmentação são observáveis em JNMs saudáveis, uma vez que a JNM é uma estrutura plástica, em constante adaptação. Quando realizamos a quantificação da porcentagem de fragmentação do terminal pós-sináptico do EDL, SOL e ETM, observamos que nenhum grupo apresentou níveis elevados de fragmentação (Figura 25).

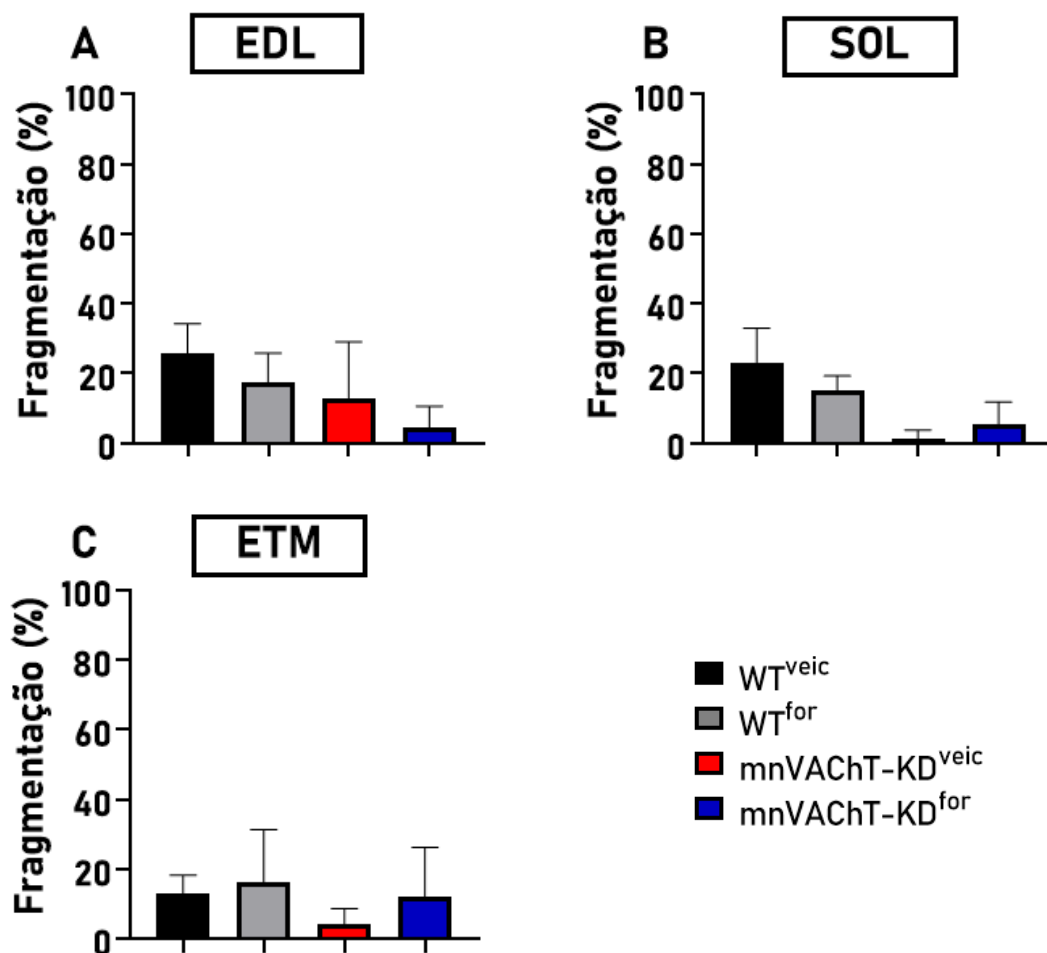


Figura 25 - Animais mnVAcHt-KD não apresentam fragmentação do terminal pós-sináptico dos músculos EDL, SOL e ETM. A- A quantificação da porcentagem de fragmentação dos elementos pós-sináptico do músculo EDL não está alterada em nenhum grupo; B- Observação que também se repetiu no músculo SOL, com nenhum grupo com JNM fragmentada e; C- Similar aos outros resultados, não observamos alterações na porcentagem de fragmentação dos elementos pós-sináptico do músculo ETM. Teste de Kruskal-Wallis, resultados representam a mediana. n= 5 animais por grupo, 20 JNM analisadas por animal.

4.9 O tratamento de camundongos fêmeas mnVACHT-KD de três meses com formoterol foi capaz de hipertrofiar a musculatura esquelética

No último componente da unidade motora, o músculo esquelético, analisamos primeiro a área de secção transversa da fibra muscular esquelética. Já é bem descrito que o sistema adrenérgico possui papel muito importante no músculo esquelético (LYNCH, 2008). Os β_2 -AR estão envolvidos diretamente na hipertrofia muscular (LYNCH, 2008; JOASSARD, 2013) e são capazes de melhorar a degeneração muscular, assim como melhorar sua função durante a senescência (RYALL, 2003), durante o estresse oxidativo (HIGASHIHARA, 2021) e em casos de SMCs (BURKE, 2013; LEE, 2018; VANHAESEBROUCK, 2019). Sendo assim, é esperado que encontremos alterações na musculatura esquelética, tanto de animais WT quanto mnVACHT-KD.

De fato, observamos que o formoterol induziu hipertrofia das fibras musculares tanto nos animais WT quanto nos animais mnVACHT-KD. Entretanto, antes de falamos sobre os efeitos do formoterol, é interessante notar que a deleção do VACHT por si já é capaz de promover alterações na fibra muscular esquelética. Apesar da deleção do VACHT não ter sido capaz de alterar a fibra muscular do EDL (Figura 26 A e A'), nos músculos SOL e ETM observamos que as fibras musculares dos animais mnVACHT-KD que receberam veículo aumentaram a área de secção transversa em relação aos animais WT^{veic} (SOL: WT^{veic}: 1040 μm^2 , mnVACHT-KD^{veic}: 1629 μm^2 , $p < 0.0001$) (Figura 26 B e B') (ETM: WT^{veic}: 1298 μm^2 , mnVACHT-KD^{veic}: 1944 μm^2 , $p < 0.0001$) (Figura 26 C e C').

Quando partimos para a análise dos efeitos promovidos pela administração do formoterol, observamos que, assim como esperado, o formoterol promoveu hipertrofia da musculatura esquelética em todos os músculos analisados dos animais WT^{for} em comparação com os WT^{veic} (EDL: WT^{veic}: 1034 μm^2 , WT^{for}: 1262 μm^2 , $p < 0.0001$; SOL: WT^{veic}: 1040 μm^2 , WT^{for}: 1391 μm^2 , $p < 0.0001$; ETM: WT^{veic}: 1298 μm^2 , WT^{for}: 1685 μm^2 , $p < 0.0001$) (Figura 26 A-A', B-B' e C-C', respectivamente). Já nos animais mnVACHT-KD, os efeitos foram observados nos músculos rápido EDL (mnVACHT-KD^{veic}: 1103 μm^2 , mnVACHT-KD^{for}: 1246 μm^2 , $p < 0.0001$) (Figura 26 A e A') e híbrido-rápido ETM (mnVACHT-KD^{veic}: 1096 μm^2 , mnVACHT-KD^{for}: 1679 μm^2 , $p < 0.0001$) (Figura 26 C e C').

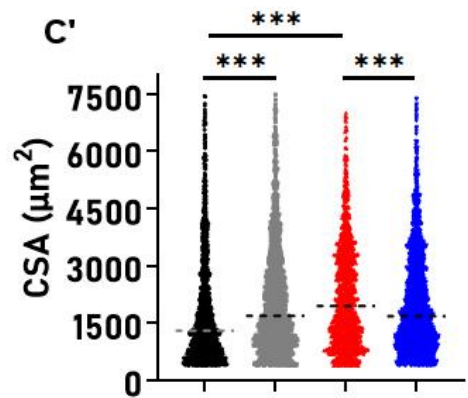
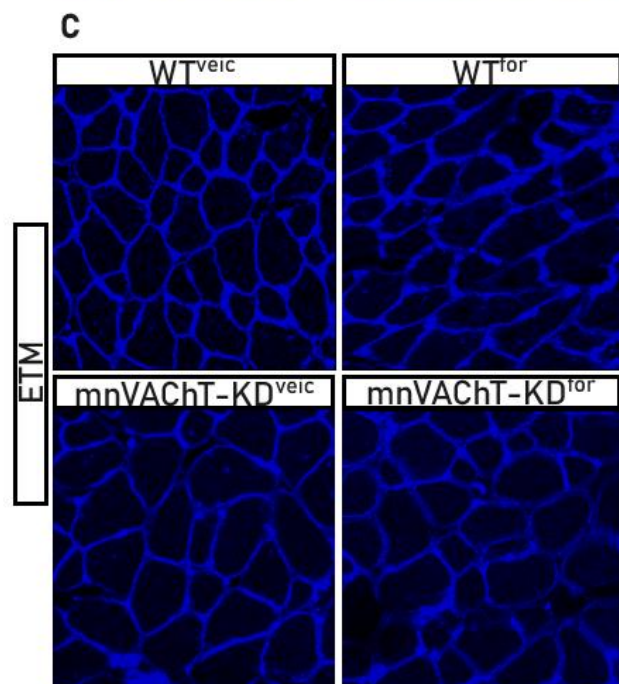
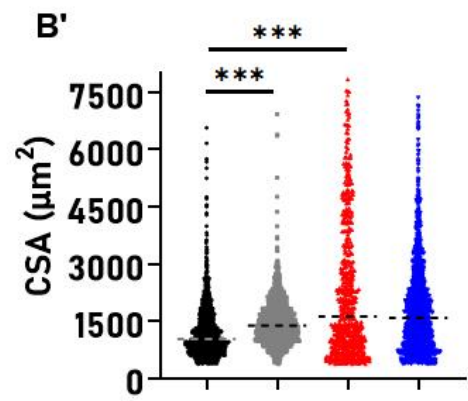
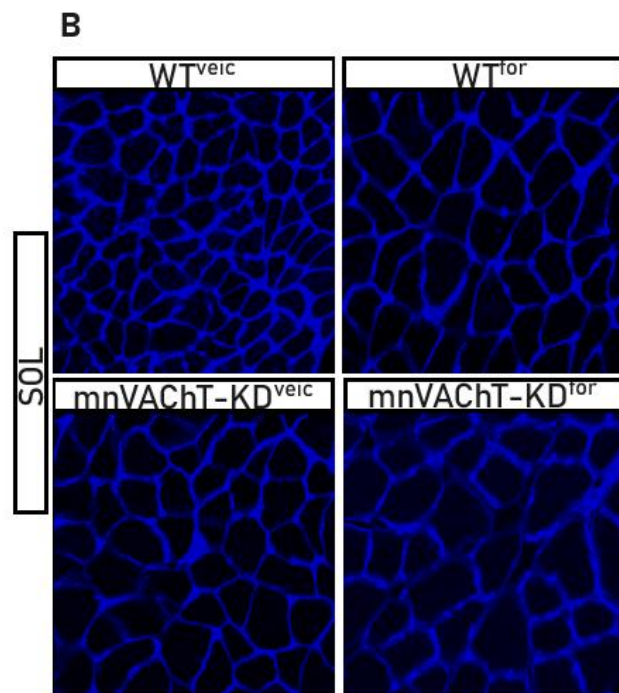
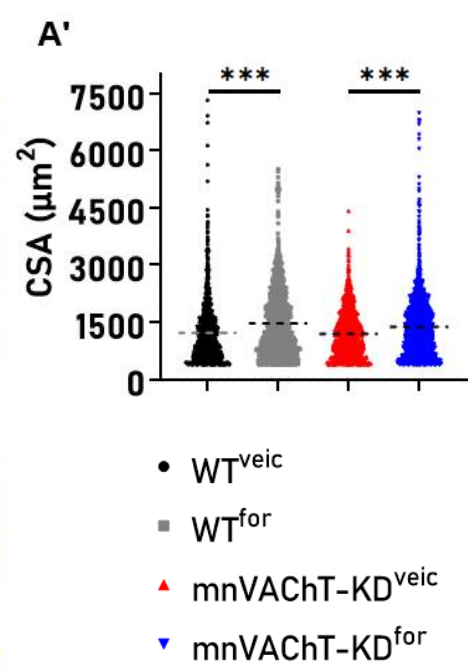
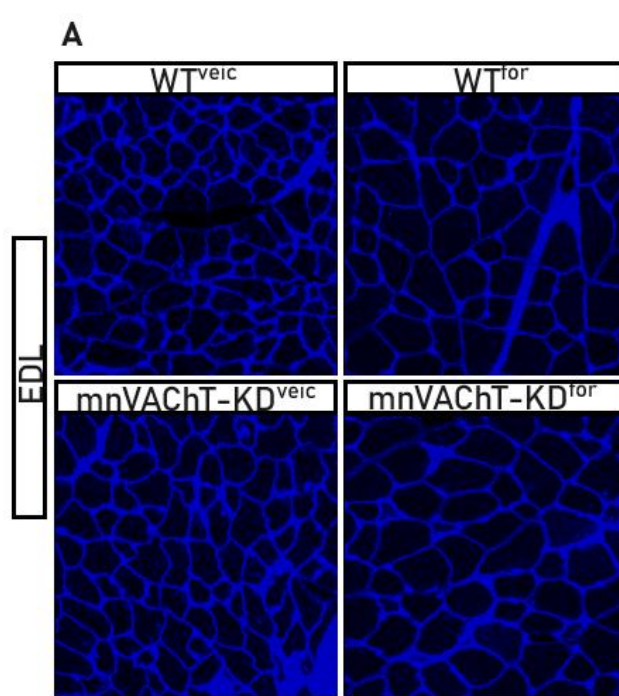


Figura 26 - Análise da área de secção transversa dos músculos EDL, SOL e ETM dos animais WT e mnVACHT-KD mostra que o formoterol foi capaz de promover hipertrofia na musculatura esquelética. **A-** Paineis representativos contendo micrografias evidenciando o aumento da CSA ocasionada pelo formoterol no EDL. **A'-** A quantificação da CSA mostra que o formoterol foi capaz de aumentar a área de secção transversa do EDL tanto em animais WT quanto em animais mnVACHT-KD; **B-** Paineis representativos contendo micrografias evidenciando o aumento da CSA ocasionada pelo formoterol apenas nos animais WT no músculo SOL; **B'-** A quantificação da CSA do SOL mostra que a deleção do VACHT induziu aumento da área da fibra muscular nos animais mnVACHT-KD^{veic}. Quando administramos formoterol, observamos um aumento na área das fibras dos animais WT, apesar de não ter induzido o mesmo efeito nos animais mnVACHT-KD; **C-** Paineis representativos contendo micrografias evidenciando o aumento da CSA ocasionada pelo formoterol nos animais WT e diminuição nos mnVACHT-KD^{for} no músculo ETM; **C'-** A quantificação da CSA mostra que, novamente, a deleção do VACHT afetou o ETM da mesma forma que o SOL anteriormente, aumentando a área da fibra muscular dos mnVACHT-KD^{veic}. Quando o formoterol foi administrado, notamos hipertrofia das fibras dos animais WT, porém reduziu de tamanho a área de secção transversa nos animais mnVACHT-KD^{veic}. ** $p < 0.005$, *** $p < 0.0005$. Teste de Kruskal-Wallis, resultados representam a mediana. $n = 5$ animais por grupo, 1 corte transversal na porção média à longitudinal do músculo por animal foi utilizado para análise, para cada músculo, por animal.

4.10 O tratamento de camundongos fêmeas mnVACHT-KD de três meses com formoterol alterou a proporção de fibras do músculo esquelético

Para finalizar as análises da unidade motora, investigamos se administração de formoterol era capaz de promover alterações na proporção de tipos de fibras em cada músculo analisado. A deleção do VACHT foi capaz de promover um aumento da expressão de fibras do tipo IIB no SOL, em comparação com animal WT (% IIB WT^{veic}: 0,0 %, mnVACHT-KD^{veic}: 25,89 %, $p < 0.05$) (Figura 27 B). Já a administração de formoterol em animais WT não promoveu nenhuma alteração no perfil das fibras em nenhum músculo (Figura 27 D-F). Em animais mnVACHT-KD, o formoterol induziu diminuição da porcentagem de fibras do tipo IIA no EDL (% IIA mnVACHT-KD^{veic}: $31,23 \pm 3,66$ %,

mnVACHT-KD^{for}: $5,52 \pm 3,16$ %, $p < 0.0001$) (Figura 27 G) e de IIB no ETM (% IIB mnVACHT-KD^{veic}: $59,96 \pm 13,42$ %, mnVACHT-KD^{for}: $31,66 \pm 7,38$ %, $p < 0.05$) (Figura 27 I).

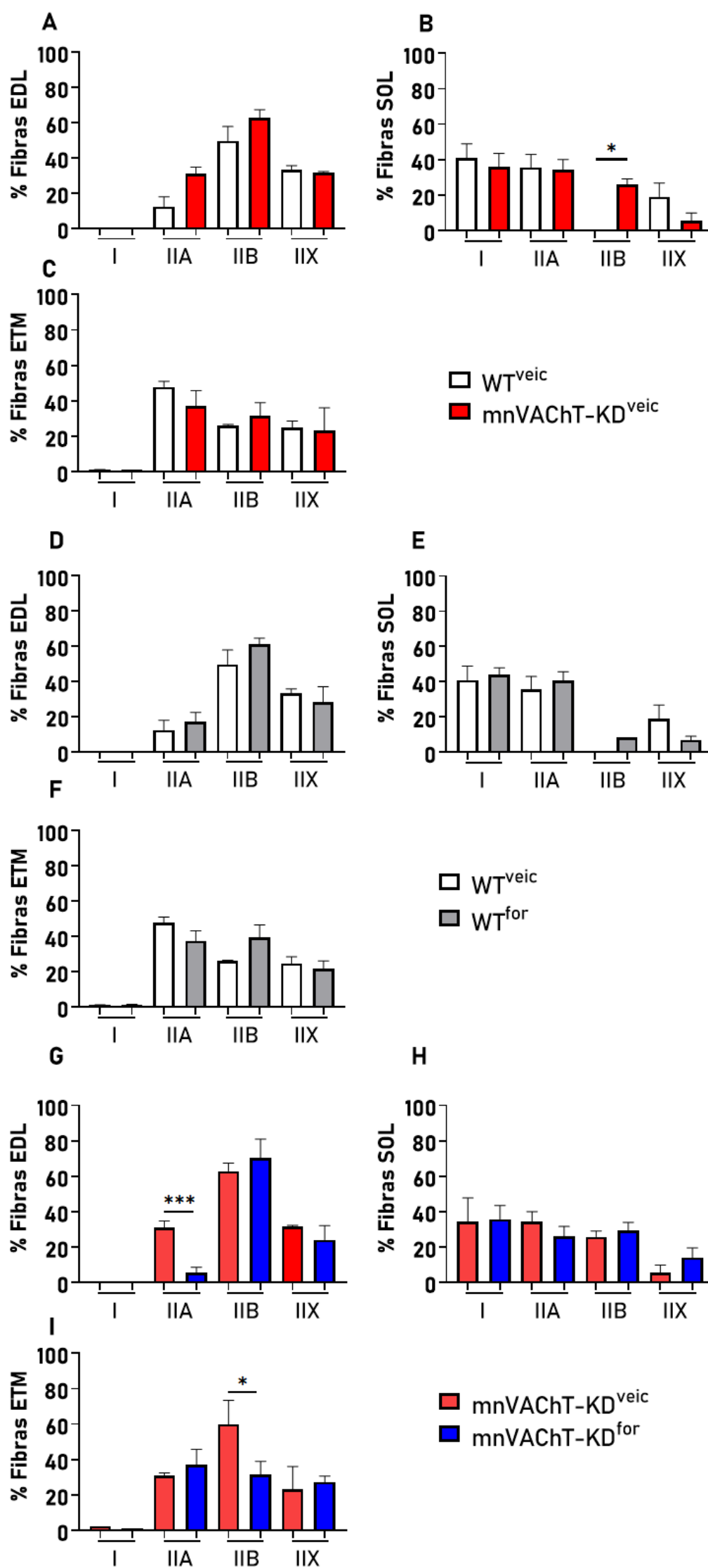


Figura 27 - Quantificação do perfil do tipo de fibras dos músculos EDL, SOL e ETM dos animais WT e mnVACHT-KD. **A-C-** Perfil do tipo de fibras dos músculos EDL, SOL e ETM nos animais WT^{veic} e mnVACHT-KD^{veic}, respectivamente. Podemos notar que a deleção do VACHT induziu um aumento atípico de fibras IIB no músculo SOL de animais mnVACHT-KD; **D-F-** A administração de formoterol não foi capaz de induzir nenhuma alteração no perfil de tipos de fibras de animais WT. **G-I-** Já nos animais mnVACHT-KD, o formoterol induziu uma diminuição do percentual das fibras do tipo IIA no músculo EDL, e uma diminuição do tipo de fibra IIB no músculo ETM. * $p < 0.05$, ** $p < 0.005$. Teste de one-way ANOVA, resultados representam a média $\pm$ DP. $n = 5$ animais por grupo, 1 corte transversal na porção média à longitudinal do músculo por animal foi utilizado para análise, para cada músculo e tipo de fibra, por animal.

5 DISCUSSÃO

Neste trabalho avaliamos a importância dos β_2 -AR para a neurotransmissão na JNM e demonstramos que nos animais β_2 -KO, que não expressam esses receptores, há alteração comportamental motora e, principalmente, na JNM e na musculatura esquelética. Isso demonstra a importância do sistema simpático e dos β_2 -AR para a manutenção e regulação da transmissão neuromuscular. Em uma segunda etapa, partimos para o tratamento de animais mnVAcHT-KD com o agonista β_2 -adrenérgico formoterol. Esses animais consistem em uma inovadora forma de se estudar SMCs de origem pré-sináptica pela deleção específica do VAcHT exclusivamente em α -MNs (JOVIANO-SANTOS, 2021). Demonstramos por meio do tratamento que o formoterol foi capaz de produzir diversas alterações benéficas nos componentes da unidade motora, apesar de não reverterem o quadro miastênico.

As SMCs são um conjunto raro de doenças genéticas que afetam a transmissão sináptica na JNM, podendo ter origem pré, pós-sináptica ou sináptica/membrana basal, sendo que até hoje, mais de 30 alterações genéticas já foram identificadas (RAMDAS, 2021). As mutações pós-sinápticas são as mais comuns, entretanto, as de origem pré-sináptica estão ganhando espaço e atraindo cada vez mais atenção, especialmente alterações no gene SLC18A3, que codifica o VAcHT (O'GRADY, 2016; HAKONEN, 2019; LAMOND, 2021; DELLA-MARINA, 2021).

O VAcHT é uma proteína de suma importância para a neurotransmissão colinérgica, sendo que pequenos distúrbios na sua expressão já são capazes de alterar a quantidade de neurotransmissor liberado na fenda sináptica, impossibilitando a saturação dos AChR (EDWARDS, 2007; PRADO, 2013). O *knockout* do VAcHT em modelos animais se mostrou incompatível com a vida, evidenciando a importância deste transportador (de CASTRO, 2009). O modelo VAcHT-KD é robusto para se estudar SMCs e que possui uma redução parcial da expressão do VAcHT (PRADO, 2006; LIMA, 2010; RODRIGUES, 2013). Entretanto, as alterações na expressão do VAcHT não se limitam apenas à unidade motora, se estendendo por todo o organismo do camundongo, sendo que eles apresentam alterações centrais (PRADO, 2006), cardiovasculares (LARA, 2010; DURAND, 2015), renais (SILVA, 2016) e inflamatórias (LEITE, 2016).

Apesar do modelo VACHT-KD (PRADO, 2006) ser bem estudado e robusto, ainda não possuíamos um modelo em que fosse possível estudar especificamente uma JNM com ausência do VACHT e, conseqüentemente, de ACh. Desenvolvemos então, em parceria com os professores Marco e Vânia Prado um novo modelo animal para estudá-la, o mnVACHT-KD, o qual possui deleção parcial do VACHT apenas em um grupo específico de MNs, os α -MNs (JOVIANO-SANTOS, 2021).

Como mencionado anteriormente na introdução, o tratamento usual para as SMCs é a administração de fármacos anticolinesterásicos, especialmente a piridostigmina. Esses fármacos possuem como mecanismo de ação, já bem conhecido, o bloqueio temporário da enzima AChE. Dessa forma a ACh não é degradada rapidamente, da forma como seria sem a presença do fármaco, e vai permanecer na fenda sináptica por mais tempo, estimulando diversas vezes os receptores para ACh (na placa motora, os nAChR são os mais importantes). Entretanto, fármacos anticolinesterásicos são conhecidos por perderem o efeito após certo tempo de uso, uma vez que a estimulação repetida dos receptores nicotínicos na placa motora pode promover internalização e *downregulation* (MORSCH, 2013; RICHTSFELD, 2013).

Outros fármacos já foram sugeridos para o tratamento das SMCs, e dentre eles, a classe de agonistas β_2 -adrenérgicos é a que se destaca. Relatos médicos demonstraram que esses fármacos possuíam uma boa aceitação pelo paciente, além de promoverem efeitos benéficos que perduravam por longo prazo, com baixos efeitos colaterais. Até 2016 ainda não sabíamos explicar o porquê esses fármacos funcionavam para tratar essa doença (e outras doenças neuromusculares), e nem o mecanismo de ação deles na placa motora, até que Khan et al., (2016) demonstrou pela primeira vez que o sistema simpático está em íntima associação com a placa motora. Nesse trabalho pioneiro, os autores mostraram que axônio marcado para tirosina hidroxilase (marcador de precursor de catecolaminas) inervava JNMs e que continham β_2 -AR. De maneira interessante, foi observada colocalização entre β_2 -AR com outros componentes da JNM. Essa foi a primeira vez que foi comprovado que a JNM, antes exclusivamente uma sinapse colinérgica, agora possui um elemento novo, o sistema simpático (adrenérgico). Após esse trabalho diversos outros começaram a surgir e mostrar diversos efeitos benéficos dos agonistas β_2 -adrenérgicos em modelos de SMCs (McMACKEN, 2018; McMACKEN, 2019; WEBSTER, 2020; VANHAESEBROUCK, 2019). No contexto fisiológico, fora do escopo de doenças que

afetam a neurotransmissão na placa motora, outros trabalhos também começaram a surgir demonstrando a função da adrenalina e noradrenalina na regulação de processos na JNM, como na exocitose e no recrutamento de vesículas sinápticas (TSENTSEVITSKY, 2019, BUKHARAEVA, 2021; KHUZAKHMETOVA, 2021; GAFUROVA, 2024). Além disso, agonistas β_2 -adrenérgicos têm demonstrado serem eficazes no tratamento de outras doenças neurodegenerativas, como a Esclerose Lateral Amiotrófica (ELA) (BARTUS, 2016).

Com tudo isso em mente, nos perguntamos então: se o sistema simpático e os β_2 -AR possuem importante função no sistema neuromuscular, o que aconteceria com a unidade motora e qual fenótipo motor seria gerado se os β_2 -AR fossem deletados completamente de um animal? Para responder essa pergunta, utilizamos animais β_2 -KO e partimos para a caracterização do comportamento motor e da unidade motora desses animais. Realizamos diversos testes comportamentais e observamos que apenas no teste da grade invertida os animais β_2 -KO apresentavam déficit motor em comparação com os animais WTs (Figura 8 A). Apesar disso, sabemos que vários fatores devem acontecer antes de detectarmos uma alteração fenotípica motora e, que mesmo sem déficits graves evidentes no comportamento, a unidade motora pode sofrer alterações. Dessa forma, partimos para analisar a unidade motora e iniciamos pelos α -MNs, tanto da porção cervical quanto da lombar, e não observamos diferença no tamanho dos α -MNs analisados entre WT e β_2 -KO (Figura 9). Por outro lado, encontramos grandes alterações nas JNMs de todos os músculos: EDL, SOL e ETM, tanto no quesito área dos terminais sinápticos (Figuras 10 e 11) quando fragmentação do terminal pós-sináptico (Figuras 12 e 13), indicando uma possível perda na neurotransmissão muscular nesses músculos. Por último, a musculatura esquelética mostrou estar alterada no músculo de contração lenta SOL (Figura 14) e não no restante.

Em conjunto, estes resultados reforçam a hipótese de que os β_2 -AR exercem funções importantes na unidade motora. Apesar de não conseguirmos afirmar exatamente quais, a deleção completa desses receptores promoveu alterações importantes nas JNMs e na musculatura esquelética. De fato, sabemos que os β_2 -AR se localizam com os nAChR na placa motora (KHAN, 2016). Neste trabalho, quando os autores realizaram a simpatectomia química utilizando o composto *6-hydroxydopamine*, observou-se que a JNM perdeu tanto sua complexidade quanto atrofiou, resultado semelhante ao obtido em

nosso trabalho. Quando eles adicionaram o simpatomimético clenbuterol, esses efeitos foram revertidos, demonstrando que os resultados obtidos foram devido à diminuição da atividade simpática, e não a uma possível toxicidade do composto *6-hydroxydopamine*.

Entretanto, o que explica a diminuição de tamanho e de complexidade das JNM quando não recebem sinalização simpática? Apesar de, aparentemente, outros receptores do sistema simpático também estarem envolvidos com a regulação da liberação de ACh, os β_2 -AR estão envolvidos na regulação de eventos espontâneos (mEPPs) e na sincronização da liberação evocada (EPPs) (TSENTSEVITSKY, 2019), além da quantidade (quanta) de ACh liberada durante eventos evocados (TSENTSEVITSKY, 2020, GAFUROVA, 2024). Apesar de importantes efeitos no elemento pré-sináptico, como evidentes elevações na liberação de ACh (GAFUROVA, 2024), os β_2 -AR aparentam estarem muito mais envolvidos com o elemento pós-sináptico, interagindo com nAChR e também com a musculatura esquelética. O agonista adrenérgico salbutamol é capaz de induzir aumento da área da JNM, aumentar a densidade de receptores pós-sinápticos e induzir aumento de mEPPs (McMACKEN, 2019). Outros estudos mostram que a mesma droga é capaz de induzir incremento nos clusters de nAChR (CLAUSEN, 2018; RODRIGUES, 2019) além de regular a estabilidade desses receptores (RODRIGUES, 2019). Dessa forma, alterações na expressão dos β_2 -AR podem causar danos à JNM, como os observados em nosso trabalho.

Além da existência de β_2 -AR na JNM, já é reconhecido que esses receptores desempenham papel muito importante diretamente na musculatura esquelética, tanto fisiologicamente quanto na doença (RYAL, 2008; JOASSARD 2013). Nós encontramos redução da área de secção transversa apenas no músculo sóleo, e não no EDL e ETM. Músculos com perfil contrátil igual ao sóleo, ou seja, músculos oxidativos lentos, possuem maior proporção de β_2 -AR do que músculos rápidos (RYALL, 2002; RYALL, 2003; SATO, 2011), sendo passíveis de serem mais afetados do que os músculos rápidos EDL e híbrido-rápido ETM. Interessantemente, apesar disso, em tratamentos os músculos rápidos geralmente são mais afetados por agonistas β_2 -adrenérgicos do que os músculos lentos (LYNCH, 2008).

No que diz respeito aos testes comportamentais, observamos apenas diminuição da performance no teste da grade invertida, e não do restante. Especificamente, o teste da

grade invertida consiste em um teste de resistência muscular, no qual o animal suporta seu próprio peso por 2 minutos, repetindo o processo três vezes com descasos de 25 segundos entre as baterias. Já o teste de força de agarre consiste em medições da força máxima exercida pelo animal sobre o aparelho. Esses dois testes são exemplos clássicos de recrutamento de diferentes unidades motoras, com o primeiro recrutando unidades lentas e o segundo recrutando unidades rápidas. Somando-se o fato de termos encontrado alterações na JNM e na musculatura do SOL, que é um músculo lento, recrutado no teste da grade invertida, temos uma linearidade na explicação dos resultados.

Apesar de o EDL não ter apresentado alteração na área de secção transversa da musculatura, explicado pela menor presença de β_2 -AR (RYALL, 2002; RYALL, 2003; SATO, 2011), o mesmo apresentou atrofia da JNM. Músculos rápidos, em específico o EDL, possuem uma margem de segurança maior do que músculos lentos, como o SOL (WOOD, 1997; WOOD, 2001). Isso possibilita que suas alterações sejam menores, ou pelo menos demorem mais a aparecerem, tanto a nível de JNM, musculo esquelético e comportamento, explicando o resultado dos testes que mensuram a força máxima do animal (força da pegada) não apresentarem redução entre os grupos. Neste teste o EDL é muito utilizado. O ETM é um músculo híbrido com predominância de fibras rápidas, o que explica a ausência de alterações nas fibras musculares, assim como o EDL.

Uma vez que demonstramos que os β_2 -AR possuem papel na unidade motora seguimos para testar quais são os benefícios de um tratamento com agonista β_2 -adrenérgico (formoterol) em modelo de SMC, o mnVACHT-KD. Já sabíamos que os déficits motores desses animais se iniciam com 2 meses de idade (JOVIANO-SANTOS, 2021), por isso escolhemos utilizar animais com três meses de idade, onde, teoricamente, os déficits seriam ainda maiores. Utilizamos fêmeas devido ao fato, ainda não explicado, de os machos apresentarem sobrevida reduzida de, no máximo, 2,5 meses. Antes de a iniciar qualquer tratamento, decidimos investigar se os animais heterozigotos não possuíam alterações comportamentais e, de fato não apresentaram nenhuma alteração, mesmo com idade de 6 meses (Figura 15). Já os animais homozigotos, com aproximadamente 75% de deleção do VACHT em α -MNs, apresentaram robusta redução de performance nos testes de força da pegada de duas e quatro patas e no teste de grade invertida (Figura 16). Esses resultados estão de acordo com resultados já encontrados no modelo anterior, o VACHT-

KD (PRADO, 2006) e com os encontrados na primeira caracterização dos mnVAcHT-KD (JOVIANO-SANTOS, 2021).

Apesar de as alterações motoras serem semelhantes entre os modelos, o VAcHT-KD apresentava redução da expressão do VAcHT no organismo todo, resultando em diversas outras alterações, dentre elas cognitivas. Para demonstrar que os mnVAcHT-KD possuem redução do VAcHT apenas em α -MNs, e não apresentam alterações cognitivas, realizamos uma bateria de testes comportamentais cognitivos, obtendo o resultado negativo para alterações cognitivas (Figura 17), como esperado.

Sendo assim, o mnVAcHT-KD é um modelo que mimetiza de forma mais robusta as alterações induzidas pela deleção do VAcHT no elemento pré-sináptico, possibilitando estudar de forma mais precisa doenças neuromusculares e a neurotransmissão colinérgica. Podemos agora dar seguimento para a investigação da utilização de agonista β_2 -adrenérgico para o tratamento de SMCs.

Partimos para o tratamento com formoterol na dose de 300 $\mu\text{g/kg}$, por 30 dias, via s.c. Iniciamos a investigação pela análise comportamental motora, repetindo todos os testes motores realizados anteriormente ao final do tratamento, no 30º dia. Observamos que o formoterol não foi capaz de reverter o grave quadro miastênico evidenciado previamente pelos testes comportamentais motores.

Quando se utiliza agonistas β_2 -adrenérgicos, a dose e o tempo de tratamento são fatores determinantes para os efeitos. Em nosso trabalho utilizamos dose de 300 $\mu\text{g/kg}$ de formoterol, via s.c. Muitos trabalhos na literatura utilizam doses muito mais altas, juntamente com um tratamento prolongado e às vezes combinado com outro fármaco. McMacken et al., (2019), utilizou salbutamol e albuterol por 7 semanas, enquanto o nosso tratamento durou 4 e utilizamos apenas formoterol. Já Vanhaesebrouck et al., (2019) realizou o tratamento por 12 semanas, sendo que administrou o salbutamol através da água sempre disponível para os animais, e também utilizou previamente uma dosagem muito alta de piridostigmina, de 14 mg/kg. Tudo indica que os agonistas β_2 -adrenérgicos possuem uma eficácia terapêutica a longo prazo e talvez, em doses um pouco mais elevadas. Isso é uma possível explicação para nossos resultados comportamentais (Figura 18).

Apesar disso, partimos para a análise da unidade motora iniciando pelos α -MNs, seguindo para a JNM e, por fim a musculatura esquelética. Observamos que, curiosamente, o formoterol produziu alterações nos α -MNs, mais especificamente nos α -MNs da cervical (Figura 19). Alguns estudos já demonstraram a existência de β_2 -AR na medula espinhal (ATLAS, 1978; PATTERSON, 1987; MIZUKAMI, 2004). Sabemos que o formoterol é capaz de passar pela barreira hematoencefálica (SASAKI, 1982), sendo possível que ele induza efeitos diretamente sobre os α -MNs. De fato, a ativação de β_2 -AR neuronais está relacionada, na maioria dos casos, com síntese proteica de fatores homeostáticos (GELINAS, 2005; ZHOU, 2013) e de vias de sobrevivência relacionadas ao BDNF (COUNTS, 2010), e formoterol já é conhecido por estimular vias anti-proteolíticas (GONÇALVES, 2019). Por fim, aparentemente existe um gradiente de sensibilidade a alterações na medula espinhal, com neurônios nas porções superiores da cervical sendo mais afetados do que os mais profundos na região lombar (AZZARITO, 2020). Apesar de diversos efeitos benéficos já terem sido descritos, ainda não sabemos o mecanismo pelo qual o formoterol promoveu o efeito de diminuição da área do neurônio, tanto em animais WTs quanto em mnVChT-KD tratados, no segmento cervical da medula espinhal.

Partimos, então, para a análise da JNM. Encontramos um significativo aumento na área dos terminais pré e pós-sinápticos das musculaturas EDL, SOL e ETM (Figura 21 e 22). Alguns trabalhos mostram que agonistas β_2 -adrenérgicos são capazes de promoverem alterações na placa motora (McMACKEN, 2018; McMACKEN, 2019; VANHAESEBROUCK, 2019; WEBSTER, 2020). Por exemplo, já se sabe que agonistas β_2 -adrenérgicos podem modular mudanças nos terminais sinápticos, e que a administração de salbutamol promove incremento na área do terminal pós-sináptico tanto do músculo EDL (VANHAESEBROUCK, 2019) quanto no lumbrical (músculo 100% rápido da pata do camundongo) bem como no músculo SOL (McMACKEN, 2019). É importante ressaltar que, em ambos os trabalhos, não foram realizadas medidas diretas do terminal pré-sináptico, ao contrário do nosso, onde medimos ambos o pré e o pós-sináptico. Além disto, ambos os estudos utilizaram agonistas β_2 -adrenérgicos com meia vida muito inferior à do formoterol, que é de 12 horas. Outros trabalhos também mostram que agonistas β_2 -adrenérgicos promovem melhorias na organização dos clusters de receptores colinérgicos (CLAUSEN, 2018). Nossos dados estão de acordo com os dados da literatura.

Entretanto, essa última análise foi realizada juntando-se JNMs com deleção do VACHT (TdTomato⁺) com JNMs sem deleção do VACHT (TdTomato⁻), ou seja, é um resultado considerando o animal como um todo. A seguir nos perguntamos: como será que a deleção do VACHT afeta as JNMs de forma individual, e há diferença entre tratar JNM deletada e não deletada com formoterol? Na Figura 23 observamos que a deleção do VACHT afeta de forma diferente o músculo rápido EDL (Figura 23 A e B) e o lento SOL (Figura 23 C e D). No EDL a JNM sem a presença do VACHT diminui de tamanho em relação à JNM com o VACHT, e o contrário ocorre com o SOL. Entretanto, quando adicionamos formoterol, há um aumento na área da JNM deletada em comparação com a JNM deletada controle, que recebeu veículo em ambos os músculos. Esse é um resultado muito interessante e intrigante, pois mostra que mesmo em uma JNM com ausência do VACHT, e consequentemente, sem liberação de ACh, a estimulação simpática via β_2 -AR é capaz de promover uma modificação profunda e direta na estrutura da JNM. Sabemos que agonistas β_2 -adrenérgicos estão envolvidos com o recrutamento e aumento da exocitose de vesículas sinápticas e liberação de ACh (GAFUROVA, 2024), mas em condições normais. Podemos especular que o mesmo pode estar ocorrendo também nas JNMs com ausência de neurotransmissor. Experimentos de eletrofisiologia serão necessários para responder essa pergunta.

Posteriormente medimos a fragmentação e colocalização da JNM e não observamos mudanças entre os grupos, exceto no músculo sóleo, em que o grupo mnVACHT-KD^{for} apresentou uma leve diminuição de colocalização em relação ao grupo mnVACHT-KD^{veic} (Figura 24 para colocalização e Figura 25 para fragmentação). Alguns modelos, como os camundongos com mutação no gene que codifica a Agrina, também não possuem alterações na fragmentação dos terminais pós-sinápticos (SPENDIFF, 2020). A alteração do nosso modelo está presente no terminal pré-sináptico e, talvez nesta idade, as alterações em relação à fragmentação pós-sináptica ainda não se manifestaram.

Por fim, analisamos a musculatura esquelética, que faz parte do último componente da unidade motora. Observamos, novamente, que a deleção do VACHT afeta de forma diferente os músculos, sendo que não ocasionou nenhuma alteração no EDL, mas hipertrofia no SOL e no ETM (Figura 26). Em trabalho de 2018, nosso grupo de pesquisa já havia mostrado que em animais VACHT-KD, a deleção do VACHT afeta de forma diferente os músculos, com o rápido EDL atrofiando e o lento SOL hipertrofiando

(MAGALHÃES-GOMES, 2018). Uma possibilidade é a hipertrofia de fibras do tipo I devido a genes importantes para a miogênese estarem aumentados na ausência do VACHT (MAGALHÃES-GOMES, 2018). Isso também explica o porquê do ETM ter aumentado de tamanho, uma vez que é um músculo híbrido, contendo também fibras lentas do tipo I em sua composição.

Quando o formoterol foi adicionado, observamos hipertrofia nos animais WT^{for} em todos os músculos. Esse resultado era esperado, uma vez que agonistas β_2 -adrenérgicos são conhecidos por induzirem hipertrofia muscular. O clenbutetol, por exemplo, é capaz de, além de promover, além da hipertrofia muscular (HINKLE, 2002, SATO, 2013), induzir efeitos anti-atrofia em camundongos (HINKLE, 2002). O formoterol também induz hipertrofia muscular e outras ações anabólicas e protetoras (BUSQUETS, 2012; JOASSARD, 2013; GÓMEZ-SanMIGUEL, 2016; HIGASHIHARA, 2021). Juntamente com a hipertrofia, também observamos incremento de peso nos animais que receberam formoterol (dado não mostrado). É recorrente o achado de ganho de massa corporal em tratamentos com agonistas β_2 -adrenérgicos (VANHAESEBROUCK, 2019; WEBSTER, 2020; HIGASHIHARA, 2021) e isto inclui o formoterol (GÓMEZ-SanMIGUEL, 2016; MARTÍN, 2018; SALAZAR-DEGRACIA, 2018). Em respeito aos animais mnVACHT-KD tratados com formoterol, mais uma vez os efeitos foram diferentes entre os músculos, sendo que no EDL observamos hipertrofia, no SOL nenhum efeito foi observado, e no ETM a área de secção transversa do músculo retornou a níveis de WT^{for} .

Em relação à proporção dos diferentes tipos de fibras em cada músculo, observamos que a deleção do VACHT alterou fibras do tipo IIB no SOL, aumentando-as em comparação com o animal WT^{veic} (Figura 27). Fibras do tipo IIB são fibras presentes em músculos rápidos, como no EDL, e são atípicas em músculos lentos como o sóleo (BLOEMBERG, 2012), evidenciando um possível mecanismo compensatório ocorrendo na ausência do VACHT. O tratamento com formoterol não induziu trocas nas proporções de fibras entre animais WT, entretanto induziu diminuição de fibras do tipo IIA no EDL e IIB no ETM. Dados da literatura sugerem que, no geral, agonistas β_2 -adrenérgicos promovem alterações nas proporções das fibras no sentido de mais fibras rápidas glicolíticas (LYNCH, 2008). Apesar disso, não encontramos essa alteração. Entretanto, alguns trabalhos utilizando o formoterol a baixas doses (100 $\mu\text{g/kg}$) também não observaram nenhuma alteração na composição de fibras (GEHRIG, 2010). Possuímos duas

explicações para isso, não excludentes: talvez a mudanças de fibras esteja acontecendo, e não houve tempo para ser quantificada; ou a dose e o tempo de tratamento não foram suficientes para promover os estímulos necessários.

Finalmente, nossos dados evidenciam e fortalecem a hipótese de que o sistema simpático e os β_2 -AR possuem importante função acessória na unidade motora, especialmente na JNM. Fornecemos, também, dados inéditos mostrando que mesmo na ausência de ACh a estimulação dos β_2 -AR é capaz de produzir efeitos benéficos na JNM. Todavia, novos estudos são necessários para esclarecer quais são os mecanismos de ação envolvidos nesses efeitos. Apesar de não fazerem parte desta tese, possuímos dados ainda em fase de experimentação que comprovam que tratamento com agonistas β_2 -adrenérgicos são eficazes quando utilizados a longo prazo. Em um desses experimentos realizamos tratamento de animais mnVAcT-KD machos homozigotos e observamos que eles apresentaram melhora no quadro miastênico em relação aos seus análogos que receberam veículo, além de terem prolongado a sobrevida substancialmente, que sem o formoterol é de 2,5 meses.

6 CONCLUSÃO

Em conjunto, os dados desse trabalho demonstram que o sistema simpático possui importante função na unidade motora e ajuda a elucidar e apoiar a utilização de agonistas β_2 -adrenérgicos no tratamento de SMCs e outras doenças neuromusculares. Demonstramos que na ausência de β_2 -AR grandes mudanças são ocasionadas na unidade motora, principalmente na JNM e na musculatura esquelética, refletindo no comportamento motor. Através da utilização do novo modelo para se estudar SMCs, o mnVACHT-KD, demonstramos que a utilização de fármacos agonistas β_2 -adrenérgicos, como o formoterol, estão se tornando importantes estratégias para o tratamento de SMCs, e de outras que acometam a unidade motora, uma vez que observamos diversas alterações benéficas em componentes da unidade motora dos animais mnVACHT-KD. Apesar de a curto prazo e em dosagem baixa não observarmos melhora motora, estamos conduzindo estudos contendo tratamentos de longo prazo e contendo combinação de drogas para propormos a melhor estratégia terapêutica.

7 REFERÊNCIAS

- ABICHT A, MÜLLER J S, LOCHMÜLLER H. Congenital Myasthenic Syndromes. 2003 May 9 [Updated 2016 Jul 14]. In: ADAM MP, ARDINGER HH, PAGON RA, et al., editors. **GeneReviews®** [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2019.
- ADRIAN, E. D.; BRONK, D. W. The discharge of impulses in motor nerve fibres. *The Journal of Physiology*, v. 67, n. 2, p. 9–151, 20 mar. 1929.
- ANDERSON, G. P. Formoterol: Pharmacology, molecular basis of agonism, and mechanism of long duration of a highly potent and selective β_2 -adrenoceptor agonist bronchodilator. *Life Sciences*, v. 52, n. 26, p. 2145–2160, jan. 1993.
- ANGOA-PÉREZ, M. *et al.* Marble Burying and Nestlet Shredding as Tests of Repetitive, Compulsive-like Behaviors in Mice. *Journal of Visualized Experiments*, n. 82, p. 1–7, 24 dez. 2013.
- ARAN, A. *et al.* Vesicular acetylcholine transporter defect underlies devastating congenital myasthenia syndrome. *Neurology*, v. 88, n. 11, p. 1021–1028, 2017.
- ATLAS, D.; MELAMED, E. Direct mapping of β -adrenergic receptors in the rat central nervous system by a novel fluorescent β -blocker. *Brain Research*, v. 150, n. 2, p. 377–385, jul. 1978.
- AZZARITO, M. *et al.* Tracking the neurodegenerative gradient after spinal cord injury. *NeuroImage: Clinical*, v. 26, n. February, p. 102221, 2020.
- BETTS, J.G. *Anatomy and Physiology*. 2^a ed. Houston: OpenStax, 2022.
- BLOEMBERG, D.; QUADRILATERO, J. Rapid Determination of Myosin Heavy Chain Expression in Rat, Mouse, and Human Skeletal Muscle Using Multicolor Immunofluorescence Analysis. *PLoS ONE*, v. 7, n. 4, p. e35273, 18 abr. 2012.

BOBADILLA-QUESADA, E. J. *et al.* Early and long-term effect of the treatment with pyridostigmine in patients with GMPPB-related congenital myasthenic syndrome. *Neuromuscular Disorders*, v. 30, n. 9, p. 719–726, set. 2020.

BUKHARAEVA, E. *et al.* Adrenoceptors modulate cholinergic synaptic transmission at the neuromuscular junction. *International Journal of Molecular Sciences*, v. 22, n. 9, 2021.

BURKE, G. *et al.* Salbutamol benefits children with congenital myasthenic syndrome due to DOK7 mutations. *Neuromuscular Disorders*, v. 23, n. 2, p. 170–175, 2013.

BUSQUETS, S. *et al.* Formoterol treatment downregulates the myostatin system in skeletal muscle of cachectic tumour-bearing rats. *Oncology Letters*, v. 3, n. 1, p. 185–189, 2012.

CLAUSEN, L.; COSSINS, J.; BEESON, D. Beta-2 Adrenergic Receptor Agonists Enhance AChR Clustering in C2C12 Myotubes: Implications for Therapy of Myasthenic Disorders. *Journal of Neuromuscular Diseases*, v. 5, n. 2, p. 231–240, 29 maio 2018.

COUNTS, S. E.; MUFSON, E. J. Noradrenaline activation of neurotrophic pathways protects against neuronal amyloid toxicity. *Journal of Neurochemistry*, v. 113, n. 3, p. 649–660, 6 maio 2010.

DE CASTRO, B. M. *et al.* Reduced expression of the vesicular acetylcholine transporter causes learning deficits in mice. *Genes, Brain and Behavior*, v. 8, n. 1, p. 23–35, 2009.

DE CASTRO, B. M. *et al.* The Vesicular Acetylcholine Transporter Is Required for Neuromuscular Development and Function. *Molecular and Cellular Biology*, v. 29, n. 19, p. 5238–5250, 1 out. 2009.

DE JAEGER, X. *et al.* Decreased acetylcholine release delays the consolidation of object recognition memory. *Behavioural Brain Research*, v. 238, n. 1, p. 62–68, 2013.

DELLA-MARINA, A. *et al.* Phenotypical and Myopathological Consequences of Compound Heterozygous Missense and Nonsense Variants in SLC18A3. *Cells*, v. 10, n. 12, p. 3481, 9 dez. 2021.

DODD-BUTERA, T.; BRODERICK, M. Pyridostigmine. *Encyclopedia of Toxicology*. [S.l.]: Elsevier, 2014. v. 3. p. 1162–1164.

DOYÈRE, L.M.F. Memoire sur les Tardigrades. *Ann. des Sci. Nat. Zool. S II* 1840, 14, 269–362.

DUCHATEAU, J.; ENOKA, R. M. Distribution of motor unit properties across human muscles. *Journal of Applied Physiology*, v. 132, n. 1, p. 1–13, 1 jan. 2022.

DURAND, M. T. *et al.* Autonomic cardiocirculatory control in mice with reduced expression of the vesicular acetylcholine transporter. *American Journal of Physiology-Heart and Circulatory Physiology*, v. 309, n. 4, p. H655–H662, 15 ago. 2015.

LIDELL, C.S. Recruitment and some other features of reflex inhibition. *Proceedings of the Royal Society of London. Series B, Containing Papers of a Biological Character*, v. 97, n. 686, p. 488–518, abr. 1925.

EDWARDS, R. H. The Neurotransmitter Cycle and Quantal Size. *Neuron*, v. 55, n. 6, p. 835–858, set. 2007.

ENGEL AG, LAMBERT EH, SANTA T. Study of long-term anticholinesterase therapy - effects on neuromuscular-transmission and on motor end- plate fine-structure. *Neurology* 1973; 23: 1273–81.

ENGEL, A. G. Congenital Myasthenic Syndromes in 2018. *Current Neurology and Neuroscience Reports*, v. 18, n. 8, p. 46, 12 ago. 2018.

ENGEL, Andrew G; SHEN, Xin-ming; SELCEN, Duygu; *et al.* Congenital myasthenic syndromes: pathogenesis, diagnosis, and treatment. **The Lancet Neurology**, v. 14, n. 4, p. 420–434, 2015.

FAULDS, DIANA. HOLLINGSHEAD, LISA M. GOA, K. L. Formoterol A Review of its Pharmacological Properties and Therapeutic Potential in Reversible Obstructive Airways Disease. *Drugs*, v. 42, n. 5, p. 895–912, nov. 1991.

FINSTERER, J. Congenital myasthenic syndromes. *Orphanet Journal of Rare Diseases*, v. 14, n. 1, p. 57, 26 dez. 2019.

FLOETER, Mary Kay. Structure and function of muscles fibers and motor units. In: KARPATI, N. Disorders of voluntary muscle. 8. Ed. Montreal: Cambridge University Press, 2010. Cap. 1, p. 1-10.

FRIESE, A. *et al.* Gamma and alpha motor neurons distinguished by expression of transcription factor Err3. *Proceedings of the National Academy of Sciences*, v. 106, n. 32, p. 13588–13593, 11 ago. 2009.

FRONTERA, W. R.; OCHALA, J. Skeletal Muscle: A Brief Review of Structure and Function. *Calcified Tissue International*, v. 96, n. 3, p. 183–195, 8 mar. 2015.

GAFUROVA, C. R. *et al.* β_2 - Adrenergic Regulation of the Neuromuscular Transmission and Its Lipid - Dependent Switch. *Molecular Neurobiology*, n. 0123456789, 2024.

GEHRIG, S. M. *et al.* Making Fast-Twitch Dystrophic Muscles Bigger Protects Them from Contraction Injury and Attenuates the Dystrophic Pathology. *The American Journal of Pathology*, v. 176, n. 1, p. 29–33, jan. 2010.

GELINAS, J. N.; NGUYEN, P. V. β -Adrenergic Receptor Activation Facilitates Induction of a Protein Synthesis-Dependent Late Phase of Long-Term Potentiation. *The Journal of Neuroscience*, v. 25, n. 13, p. 3294–3303, 30 mar. 2005.

GILLIES JD, Allen J. Effects of neostigmine and pyridostigmine at the neuromuscular junction. *Clin Exp Neurol* 1977; 14: 271–9.

GÓMEZ-SANMIGUEL, A. B. *et al.* Formoterol decreases muscle wasting as well as inflammation in the rat model of rheumatoid arthritis. *American Journal of Physiology-Endocrinology and Metabolism*, v. 310, n. 11, p. E925–E937, 1 jun. 2016.

GONÇALVES, D. A. *et al.* Insulin/IGF1 signalling mediates the effects of β 2 -adrenergic agonist on muscle proteostasis and growth. *Journal of Cachexia, Sarcopenia and Muscle*, v. 10, n. 2, p. 455–475, abr. 2019.

GPCR. Scitable by nature education, 2014. Disponível em: <https://www.nature.com/scitable/topicpage/gpcr-14047471/>.

HAKONEN, A. H. *et al.* SLC18A3 variants lead to fetal akinesia deformation sequence early in pregnancy. *American Journal of Medical Genetics Part A*, v. 179, n. 7, p. ajmg.a.61186, 6 maio 2019.

HIGASHIHARA, T. *et al.* β 2-adrenergic receptor agonist counteracts skeletal muscle atrophy and oxidative stress in uremic mice. *Scientific Reports*, v. 11, n. 1, p. 9130, 28 abr. 2021.

HINKLE, R. T. *et al.* Skeletal muscle hypertrophy and anti-atrophy effects of clenbuterol are mediated by the β 2-adrenergic receptor. *Muscle & Nerve*, v. 25, n. 5, p. 729–734, 28 maio 2002.

IYADURAI, S. J. P. Congenital Myasthenic Syndromes. *Neurologic Clinics*, v. 38, n. 3, p. 541–552, 2020.

JANICKOVA, H. *et al.* Deletion of the vesicular acetylcholine transporter from pedunculopontine/laterodorsal tegmental neurons modifies gait. *Journal of Neurochemistry*, v. 140, n. 5, p. 787–798, 2017.

JOASSARD, O. R.; DURIEUX, A.-C.; FREYSSINET, D. G. β 2-Adrenergic agonists and the treatment of skeletal muscle wasting disorders. *The International Journal of Biochemistry & Cell Biology*, v. 45, n. 10, p. 2309–2321, out. 2013.

JOVIANO-SANTOS, J. V. *et al.* Motoneuron-specific loss of VAcHT mimics neuromuscular defects seen in congenital myasthenic syndrome. *The FEBS Journal*, v. 288, n. 18, p. 5331–5349, 25 set. 2021.

KHAN, M. M. *et al.* Sympathetic innervation controls homeostasis of neuromuscular junctions in health and disease. *Proceedings of the National Academy of Sciences*, v. 113, n. 3, p. 746–750, 19 jan. 2016.

KHUZAKHMETOVA, V.; BUKHARAEVA, E. Adrenaline Facilitates Synaptic Transmission by Synchronizing Release of Acetylcholine Quanta from Motor Nerve Endings. *Cellular and Molecular Neurobiology*, v. 41, n. 2, p. 395–401, 9 mar. 2021.

KLJAKIC, O. *et al.* Chemogenetic activation of VGLUT3-expressing neurons decreases movement. *European Journal of Pharmacology*, v. 935, n. August, p. 175298, 2022.

KOLISNYK, B., Guzman, M.S., Raulic, S., Fan, J., Magalhaes, A.C., Feng, G., Gros, R., Prado, V.F., Prado, M.A.M., 2013. ChAT-ChR2-EYFP Mice Have Enhanced Motor Endurance But Show Deficits in Attention and Several Additional Cognitive Domains. *J. Neurosci.* 33, 10427–10438.

KRAEMER, W.J. Fisiologia do exercício. Teoria e prática. 2ª ed. Rio de Janeiro: GuanabaraKoogan, 27 de Junho de 2017.

KRAEUTER, A.; GUEST, P. C.; SARNYAI, Z. The Y-Maze for Assessment of Spatial Working and Reference Memory in Mice. *Methods in Molecular Biology*. [S.l: s.n.], 2019. v. 1916. p. 105–111.

LAMOND, A.; BUCKLEY, D.; O'DEA, J.; TURNER, L. Variants of SLC18A3 leading to congenital myasthenic syndrome in two children with varying presentations. *BMJ Case Rep.* 2021, 14, e237799.

LARA, A. *et al.* Dysautonomia Due to Reduced Cholinergic Neurotransmission Causes Cardiac Remodeling and Heart Failure. *Molecular and Cellular Biology*, v. 30, n. 7, p. 1746–1756, 1 abr. 2010.

LEE, M.; BEESON, D.; PALACE, J. Therapeutic strategies for congenital myasthenic syndromes. *Annals of the New York Academy of Sciences*, v. 1412, n. 1, p. 129–136, 2018.

LEGAY, C.; MEI, L. Moving forward with the neuromuscular junction. *Journal of Neurochemistry*, v. 142, p. 59–63, ago. 2017.

LEITE, H. R. *et al.* Vesicular acetylcholine transporter knock down-mice are more susceptible to inflammation, c- Fos expression and sickness behavior induced by lipopolysaccharide. *Brain, Behavior, and Immunity*, v. 57, p. 282–292, out. 2016.

LEITE-SCHETINO, L. P. *et al.* Evaluation of the neuromuscular junction in a middle-aged mouse model of congenital myasthenic syndrome. *Muscle & Nerve*, v. 60, n. 6, p. 790–800, 23 dez. 2019.

LEPORE, E. *et al.* Neuromuscular Junction as an Entity of Nerve-Muscle Communication. *Cells*, v. 8, n. 8, p. 906, 16 ago. 2019.

LI, L.; XIONG, W.-C.; MEI, L. Neuromuscular Junction Formation, Aging, and Disorders. *Annual Review of Physiology*, v. 80, n. 1, p. 159–188, 10 fev. 2018.

LIMA, R. DE F. *et al.* Quantal release of acetylcholine in mice with reduced levels of the vesicular acetylcholine transporter. *Journal of Neurochemistry*, v. 113, n. 4, p. 943–951, maio 2010.

LYNCH, G. S.; RYALL, J. G. Role of β -Adrenoceptor Signaling in Skeletal Muscle: Implications for Muscle Wasting and Disease. *Physiological Reviews*, v. 88, n. 2, p. 729–767, abr. 2008.

MAGALHÃES-GOMES, M. P. S. *et al.* Fast and slow-twitching muscles are differentially affected by reduced cholinergic transmission in mice deficient for VACHT: A mouse model for congenital myasthenia. *Neurochemistry International*, v. 120, n. February, p. 1–12, nov. 2018.

MARQUES, M. J.; CONCHELLO, J.-A.; LICHTMAN, J. W. From Plaque to Pretzel: Fold Formation and Acetylcholine Receptor Loss at the Developing Neuromuscular Junction. *The Journal of Neuroscience*, v. 20, n. 10, p. 3663–3675, 15 maio 2000.

MARTÍN, A. I. *et al.* Formoterol treatment prevents the effects of endotoxin on muscle TNF/NF- κ B, Akt/mTOR, and proteolytic pathways in a rat model. Role of IGF-I and miRNA 29b. *American Journal of Physiology - Endocrinology and Metabolism*, v. 315, n. 4, p. E705–E714, 2018.

MCARDLE, W.D. Fisiologia do exercício – Nutrição, Energia E Desempenho Humano. 8ª ed. Rio de Janeiro: GuanabaraKoogan, 24 de Abril de 2013.

MCMACKEN, G. *et al.* The beta-adrenergic agonist salbutamol modulates neuromuscular junction formation in zebrafish models of human myasthenic syndromes. *Human Molecular Genetics*, v. 27, n. 9, p. 1556–1564, 1 maio 2018.

MCMACKEN, G. M. *et al.* Salbutamol modifies the neuromuscular junction in a mouse model of ColQ myasthenic syndrome. *Human Molecular Genetics*, v. 28, n. 14, p. 2339–2351, 15 jul. 2019.

MIHAYLOVA, V. *et al.* Molecular characterisation of congenital myasthenic syndromes in Southern Brazil. *Journal of Neurology, Neurosurgery and Psychiatry*, v. 81, n. 9, p. 973–977, 2010.

MIZUKAMI, T. Immunocytochemical Localization of β 2-Adrenergic Receptors in the Rat Spinal Cord and Their Spatial Relationships to Tyrosine Hydroxylase-immunoreactive Terminals. *The Kurume Medical Journal*, v. 51, n. 3/4, p. 175–183, 2004.

MORSCH, M. *et al.* Pyridostigmine but not 3,4-diaminopyridine exacerbates ACh receptor loss and myasthenia induced in mice by muscle-specific kinase autoantibody. *The Journal of Physiology*, v. 591, n. 10, p. 2747–2762, maio 2013.

MOTIEJUNAITE, J.; AMAR, L.; VIDAL-PETIOT, E. Adrenergic receptors and cardiovascular effects of catecholamines. *Annales d'Endocrinologie*, v. 82, n. 3–4, p. 193–197, 2021.

MUKUND, K.; SUBRAMANIAM, S. Skeletal muscle: A review of molecular structure and function, in health and disease. *WIREs Systems Biology and Medicine*, v. 12, n. 1, p. 1–46, 13 jan. 2020.

MUTLU, G. M.; FACTOR, P. Alveolar Epithelial β 2 -Adrenergic Receptors. *American Journal of Respiratory Cell and Molecular Biology*, v. 38, n. 2, p. 127–134, fev. 2008.

NGUYEN, M. L.; COX, G. D.; PARSONS, S. M. Kinetic Parameters for the Vesicular Acetylcholine Transporter: Two Protons Are Exchanged for One Acetylcholine. *Biochemistry*, v. 37, n. 38, p. 13400–13410, 1 set. 1998.

O'GRADY, G. L. . *et al.* Variants in SLC18A3 , vesicular acetylcholine transporter , cause congenital myasthenic syndrome. *Neurology*, v. 87, 2016.

PATTERSON, S. I.; HANLEY, M. R. *Autoradiographic evidence for fl-adrenergic receptors on capsaicin-sensitive primary afferent terminals in rat spinal cord.* *Neuroscience Letters*. [S.l: s.n.], 1987.

PRADO, V. F. *et al.* Mice Deficient for the Vesicular Acetylcholine Transporter Are Myasthenic and Have Deficits in Object and Social Recognition. *Neuron*, v. 51, n. 5, p. 601–612, set. 2006.

PRADO, V. F. *et al.* Regulation of cholinergic activity by the vesicular acetylcholine transporter. *Biochemical Journal*, v. 450, n. 2, p. 265–274, 1 mar. 2013.

QUATREFAGES DE BRÉAU, J.L.A. De Mémoire sur l'Eolidine paradoxale (Eolidina paradoxum Nob.). *Ann. Sci. Nat. Zool. S. II* 1843, 19, 274–312.

RAMDAS, S.; BEESON, D. Congenital myasthenic syndromes : where do we go from here ? *Neuromuscular Disorders*, v. 31, n. 10, p. 943–954, 2021.

REMAN, Saba. Biochemistry, G Protein Coupled Receptors. National Libraru of Medicine, 2023.

RICHTSFELD, M. *et al.* Prolonged Administration of Pyridostigmine Impairs Neuromuscular Function with and without Down-regulation of Acetylcholine Receptors. *Anesthesiology*, v. 119, n. 2, p. 412–421, 1 ago. 2013.

RODRIGUES, A. C. Z. *et al.* The sympathetic nervous system regulates skeletal muscle motor innervation and acetylcholine receptor stability. *Acta Physiologica*, v. 225, n. 3, p. e13195, 22 mar. 2019.

RODRIGUES, H. A. *et al.* Reduced Expression of the Vesicular Acetylcholine Transporter and Neurotransmitter Content Affects Synaptic Vesicle Distribution and Shape in Mouse Neuromuscular Junction. *PLoS ONE*, v. 8, n. 11, p. e78342, 8 nov. 2013.

RODRÍGUEZ CRUZ, P. M. *et al.* The Neuromuscular Junction in Health and Disease: Molecular Mechanisms Governing Synaptic Formation and Homeostasis. *Frontiers in Molecular Neuroscience*, v. 13, n. December, p. 1–22, 3 dez. 2020.

RODRÍGUEZ-CRUZ, Pedro M.; PALACE, Jacqueline; BEESON, David. Inherited disorders of the neuromuscular junction: an update. **Journal of Neurology**, v. 261, n. 11, p. 2234–2243, 2014.

ROHRER, D. K. *et al.* Cardiovascular and Metabolic Alterations in Mice Lacking Both b1- and b2-Adrenergic Receptors. *Journal of Biological Chemistry*, v. 274, n. 24, p. 16701–16708, 1999.

ROSSI, L. *et al.* Neuromuscular defects after infection with a beta coronavirus in mice. *Neurochemistry International*, v. 169, n. June, p. 105567, out. 2023.

RUDOLF, R.; KHAN, M. M.; WITZEMANN, V. Motor Endplate—Anatomical, Functional, and Molecular Concepts in the Historical Perspective. *Cells*, v. 8, n. 5, p. 387, 27 abr. 2019.

RYALL, J. G. *et al.* β 2 -Agonist administration reverses muscle wasting and improves muscle function in aged rats. *The Journal of Physiology*, v. 555, n. 1, p. 175–188, 15 fev. 2003.

RYALL, J. G. *et al.* β 2 -Agonist fenoterol has greater effects on contractile function of rat skeletal muscles than clenbuterol. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, v. 283, n. 6, p. R1386–R1394, 1 dez. 2002.

SALAZAR-DEGRACIA, A. *et al.* Effects of the beta2 agonist formoterol on atrophy signaling, autophagy, and muscle phenotype in respiratory and limb muscles of rats with cancer-induced cachexia. *Biochimie*, v. 149, p. 79–91, 2018.

SASAKI, H. *et al.* Disposition and metabolism of formoterol fumarate, a new bronchodilator, in rats and dogs. *Xenobiotica*, v. 12, n. 12, p. 803–812, 30 jan. 1982.

SATO, S. *et al.* Intracellular β 2-adrenergic receptor signaling specificity in mouse skeletal muscle in response to single-dose β 2-agonist clenbuterol treatment and acute exercise. *The Journal of Physiological Sciences*, v. 63, n. 3, p. 211–218, 13 maio 2013.

SATO, S. *et al.* Muscle Plasticity and β 2-Adrenergic Receptors: Adaptive Responses of β 2-Adrenergic Receptor Expression to Muscle Hypertrophy and Atrophy. *Journal of Biomedicine and Biotechnology*, v. 2011, p. 1–10, 2011.

SCHIAFFINO, S.; REGGIANI, C. Fiber Types in Mammalian Skeletal Muscles. *Physiological Reviews*, v. 91, n. 4, p. 1447–1531, out. 2011.

SENDTNER, M. Motoneuron Disease. *Handbook of Experimental Pharmacology*. [S.l: s.n.], 2014. v. 220. p. 411–441.

SHIEH, P. B.; OH, S. J. Congenital Myasthenic Syndromes. *Neurologic Clinics*, v. 36, n. 2, p. 367–378, maio 2018.

SILVA, R. C. *et al.* Reduced expression of VACHT increases renal fibrosis. *Pathophysiology*, v. 23, n. 3, p. 229–236, set. 2016.

SPENDIFF, S. *et al.* Modulation of the Acetylcholine Receptor Clustering Pathway Improves Neuromuscular Junction Structure and Muscle Strength in a Mouse Model of Congenital Myasthenic Syndrome. *Frontiers in Molecular Neuroscience*, v. 13, n. December, p. 1–13, 17 dez. 2020.

STIFANI, N. Motor neurons and the generation of spinal motor neuron diversity. *Frontiers in Cellular Neuroscience*, v. 8, n. OCT, p. 1–22, 2014.

TSENTSEVITSKY, A. N.; KOVYAZINA, I. V.; BUKHARAEVA, E. A. Diverse Effects of Noradrenaline and Adrenaline on the Quantal Secretion of Acetylcholine at the Mouse Neuromuscular Junction. *Neuroscience*, v. 423, p. 162–171, dez. 2019.

VALADÃO, P. A. C. *et al.* Muscle atrophy is associated with cervical spinal motoneuron loss in BACHD mouse model for Huntington's disease. *European Journal of Neuroscience*, v. 45, n. 6, p. 785–796, mar. 2017.

VALADÃO, P. A. C. *et al.* Neuromuscular synapse degeneration without muscle function loss in the diaphragm of a murine model for Huntington's Disease. *Neurochemistry International*, v. 116, p. 30–42, jun. 2018.

VALDEZ, G. *et al.* Shared Resistance to Aging and ALS in Neuromuscular Junctions of Specific Muscles. *PLoS ONE*, v. 7, n. 4, p. e34640, 2 abr. 2012.

VAN LIEFFERINGE, J. *et al.* Are vesicular neurotransmitter transporters potential treatment targets for temporal lobe epilepsy? *Frontiers in Cellular Neuroscience*, v. 7, n. AUG, p. 1–24, 2013.

VANHAESEBROUCK, A. E. *et al.* β 2-Adrenergic receptor agonists ameliorate the adverse effect of long-term pyridostigmine on neuromuscular junction structure. *Brain*, v. 142, n. 12, p. 3713–3727, 1 dez. 2019.

VAROQUI, H.; ERICKSON, J. D. Active Transport of Acetylcholine by the Human Vesicular Acetylcholine Transporter. *Journal of Biological Chemistry*, v. 271, n. 44, p. 27229–27232, nov. 1996.

WEBSTER, R. Animal Models of the Neuromuscular Junction, Vitally Informative for Understanding Function and the Molecular Mechanisms of Congenital Myasthenic Syndromes. *International Journal of Molecular Sciences*, v. 19, n. 5, p. 1326, 29 abr. 2018.

WEBSTER, R. G. *et al.* Effect of salbutamol on neuromuscular junction function and structure in a mouse model of DOK7 congenital myasthenia. *Human Molecular Genetics*, v. 29, n. 14, p. 2325–2336, 11 ago. 2020.

WOOD, S. J.; SLATER, C. Safety factor at the neuromuscular junction. *Progress in Neurobiology*, v. 64, n. 4, p. 393–429, jul. 2001.

WOOD, S. J.; SLATER, C. R. The contribution of postsynaptic folds to the safety factor for neuromuscular transmission in rat fast- and slow-twitch muscles. *The Journal of Physiology*, v. 500, n. 1, p. 165–176, 1 abr. 1997.

YANKELEVITCH-YAHAV, R. *et al.* The Forced Swim Test as a Model of Depressive-like Behavior. *Journal of Visualized Experiments*, n. 97, p. 1–7, 2 mar. 2015.

ZAYIA, L; TADI, P. 23 de Julho de 2023. Disponível em: <<https://www.ncbi.nlm.nih.gov/books/NBK554616/>>.

ZHOU, H.-C. *et al.* Activation of β 2-adrenoceptor enhances synaptic potentiation and behavioral memory via cAMP-PKA signaling in the medial prefrontal cortex of rats. *Learning & Memory*, v. 20, n. 5, p. 274–284, 17 maio 2013.

8 ANEXOS

8.1 Artigo 1: Influence of β_2 -adrenergic selective agonist formoterol on the motor unit of a mouse model of a congenital myasthenic syndrome with complete VACHT deletion.

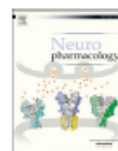
Neuropharmacology 260 (2024) 110116



Contents lists available at ScienceDirect

Neuropharmacology

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



Influence of β_2 -adrenergic selective agonist formoterol on the motor unit of a mouse model of a congenital myasthenic syndrome with complete VACHT deletion

Leonardo Rossi ^a, Bárbara I. Mota ^a, Priscila A.C. Valadão ^a, Matheus P.S. Magalhães-Gomes ^{a,b},
Bruna S. Oliveira ^a, Silvia Guatimosim ^c, Luiz C.C. Navegantes ^b, Aline S. Miranda ^a,
Marco A.M. Prado ^{d,e,f,g}, Vânia F. Prado ^{d,e,f,g}, Cristina Guatimosim ^{a,*}

^a Departamento de Morfologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

^b Departamento de Ciências Básicas, Faculdade Ciências Médicas de Minas Gerais, FCMMG, Belo Horizonte, Brazil

^c Departamento de Fisiologia e Biofísica, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

^d Roberts Research Institute, Schulich School of Medicine & Dentistry, University of Western Ontario, London, Canada

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

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

^g Brain and Mind Institute, University of Western Ontario, London, Canada

^{*} Departamento de Fisiologia, Escola de Medicina, Universidade de São Paulo, Ribeirão Preto, São Paulo, Brazil

ARTICLE INFO

Handling Editor: Prof F De Felice

Keywords:
Neuromuscular junction
 β_2 -adrenergic agonist
Formoterol
Congenital myasthenic syndrome

ABSTRACT

Congenital Myasthenic Syndromes (CMS) are a set of genetic diseases that affect the neuromuscular transmission causing muscular weakness. The standard pharmacological treatment aims at ameliorating the myasthenic symptom by acetylcholinesterase inhibitors. Most patients respond well in the short and medium term, however, over time the beneficial effects rapidly fade, and the efficacy of the treatment diminishes. Increasing evidence shows that β_2 -adrenergic agonists can be a suitable choice for the treatment of neuromuscular disorders, including CMS, as they promote beneficial effects in the neuromuscular system. The exact mechanism on which they rely is not completely understood, although patients and animal models respond well to the treatment, especially over extended periods. Here, we report the use of the long-lasting specific β_2 -adrenergic agonist formoterol in a myasthenic mouse model (mnVACHT-1D), featuring deletion of VACHT (Vesicular Acetylcholine Transporter) specifically in the α -motoneurons. Our findings demonstrate that formoterol treatment (300 μ g/kg/day, sc) for 30 days increased the neuromuscular junction area, induced skeletal muscle hypertrophy and altered fibre type composition in myasthenic mice. Interestingly, β_2 -adrenergic agonists have shown efficacy even in the absence of ACh (acetylcholine). Our data provide important evidence supporting the potential of β_2 -adrenergic agonists in treating neuromuscular disorders of pre-synaptic origin and characterized by disruptions in nerve-muscle communication, through a direct and beneficial action within the motor unit.

1. Introduction

CMS are a group of genetic disorders that affect the NMJ (neuromuscular junction) due to mutations in different proteins. They often cause disruption of neuromuscular communication, leading to muscle weakness and the classical fatigability seen in patients with the disease. Several CMS gene mutations have been identified so far, affecting proteins in three different locations at the NMJ: pre-synaptic site, synaptic

cleft, and post-synaptic site. Mutations in genes encoding post-synaptic proteins are the most common, encompassing almost 50% of all cases and include mutations in different subunits of the nAChR (nicotinic acetylcholine receptor) that lead to deficits in either function or dynamic of the receptor. Mutations leading to synaptic cleft alterations can be related to AChE (acetylcholinesterase) function and extracellular matrix protein. Additionally, pre-synaptic alterations are related to mutations in proteins associated with ACh (synthesis or release machinery

* Corresponding author. Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas, H2-210. Av. Pres. Antônio Carlos, 6627, Pampulha, Belo Horizonte, 31270-901, Brazil.

E-mail address: cguati@icb.ufmg.br (C. Guatimosim).

<https://doi.org/10.1016/j.neuropharm.2024.110116>

Received 5 May 2024; Received in revised form 9 August 2024; Accepted 13 August 2024

Available online 14 August 2024

0028-3908/© 2024 Published by Elsevier Ltd.

(Finsterer, 2019; Ramdas and Beeson, 2021; Ohno et al., 2023; Pugliese et al., 2023).

Several transgenic animal models have been developed to study CMS, providing opportunities to identify disease mechanisms and test pharmacological treatments (Prado et al., 2006; Webster et al., 2013, 2020; McMacken et al., 2019; Vanhaesebrouck et al., 2019). Our research group has introduced a pre-synaptic CMS mouse model (mnVACHT-KD) with selective deletion of VACHT specifically in α -motoneurons (α -MNs), resulting in loss of muscle mass and strength (Joviano-Santos et al., 2021). One advantage of this mouse model, compared to others, is that they allow the identification of MNs with VACHT deletion through the red fluorescent protein TdTomato, allowing for separate identification from those without VACHT deletion (Joviano-Santos et al., 2021).

Storage of ACh into synaptic vesicles by VACHT is a limiting step in the cholinergic neurotransmission (Prado et al., 2013). Minor alterations in VACHT expression can significantly compromise neurotransmission (Edwards, 2007; Prado et al., 2013). Indeed, VACHT is essential for life, as evidenced by the observation that VACHT-knockout mice die soon after birth (de Castro et al., 2009). Furthermore, mutations in the VACHT gene have been identified in humans (O'Grady et al., 2016; Aran et al., 2017; Marina et al., 2021), highlighting the importance of studying absence/dysfunction of VACHT and its association with CMS.

AChE inhibitors (anticholinesterase medication) are the most commonly used drugs for the treatment of CMS, regardless of the origin of the disease (pre, synaptic or post-synaptic). These drugs provide a short-term control of myasthenic symptoms (Ramdas and Beeson, 2021). We have previously performed a short-term treatment (one week) of mnVACHT-KD mice with the central-acting anticholinesterase donepezil and observed a significant improvement in muscle strength (Joviano-Santos et al., 2021). However, while cholinesterase inhibitors initially produce a significant effect, some patients may experience a decreased efficacy of the drug over time (Morsch et al., 2013; Richtsfeld et al., 2013; Vanhaesebrouck et al., 2019). Recently, new pharmacological strategies have emerged, such as the β_2 -adrenergic agonists. Khan et al. (2016) have highlighted the close relationship between the sympathetic nervous system and the NMJ and showed the presence of β_2 -adrenergic receptors (β_2 -AR) at pre-synaptic and post-synaptic elements, leading to subsequent studies on the use of β_2 -adrenergic agonists. These studies have shown that those drugs not only produce beneficial effects in CMS models of post-synaptic origin but also ameliorate adverse effects of cholinesterase inhibitors (McMacken et al., 2019; Vanhaesebrouck et al., 2019; Webster et al., 2020).

Here we investigated the effects of the β_2 -adrenergic agonist formoterol on the NMJ and other components of the motor unit in a pre-synaptic CMS mouse model (mnVACHT-KD mice). We provide original evidence that formoterol administration induced an increase in the synaptic area of the NMJ, muscle hypertrophy and changed fibre type. Therefore, formoterol had a positive effect on NMJ structure by increasing its size and probably mitigating the detrimental effects of reduced ACh release due to VACHT deletion at neuromuscular junction. Our findings shed some light in β_2 -adrenergic agonist potential mechanisms of action in neuromuscular disorders. Importantly, these results have implications for both autoimmune and genetic myasthenia, where anticholinesterase medication is a standard treatment.

2. Materials and methods

2.1. Animals

All experimental procedures were performed following the guidance of the Universidade Federal de Minas Gerais (UFMG) Ethical Committee for Animal Experimentation and received approval number under No 81/2021. The mice used in our experiments were littermates VACHT-flox/flox^{+/+}/Cre^{-/-}/Tomato^{-/-} (WT) and VACHT-flox/flox^{+/+}/Cre^{+/+} Tomato^{+/+} (mnVACHT-KD) (Joviano-Santos et al., 2021) aged 3

months. These mice were randomly divided into four groups for treatment: WT^{vehic}, WT^{for}, mnVACHT-KD^{vehic} and mnVACHT-KD^{for}. We used two different mice cohort: the first one was to evaluate behavioural tasks and NMJ, and the second one was to evaluate the MNs and skeletal muscle. We choose to use female mice due to the short lifespan of the male mice (Joviano-Santos et al., 2021). Mice were housed in ventilated cages placed in an animal care facility at 24 °C $\pm$ 2 °C, 12 h light/dark cycle with water and food provided *ad libitum*.

2.2. Pharmacological treatment

Mice received a daily injection subcutaneously (s.c) of formoterol (300 μ g/kg) or vehicle (saline 0.9% + DMSO 0.45%) for 30 consecutive days, being the last injection administered in the 30th day, before euthanasia. Formoterol dose was based on previous study from Gonçalves et al. (2019). Behavioural tasks were performed before (baseline) and after the last injection (D30). Animals were daily inspected for any irritation in the site of injection and none was detected during the treatment period.

2.3. Motor behavioural tests

2.3.1. Open field

To assess spontaneous locomotor activity, the animals were submitted to the open field test as described elsewhere (Kljakic et al., 2022). Each animal was gently placed in the cage and allowed to explore the arena for 30 min. The total distance and the mean velocity travelled in the cage was recorded. Video tracking was performed by an infrared-sensitive video camera installed in the top unit of each cage. The X-Y coordinates of each mouse were acquired using EthoVision software (EthoVision HTP 2.1.2.0, based on EthoVision XT 4.1, Noldus Information Technology, Wageningen, The Netherlands) (Kljakic et al., 2022).

2.3.2. Wire hang

The wire hang test was used to assess motor strength as described elsewhere (Janickova et al., 2017). Briefly, the mice were placed on a metal grid 45 cm above a large tub filled to the top with wood-chip bedding. Mice were prompted to grip the wires with fore-paws by gently vibrating the cage lid. The cage lid was then inverted and the latency to fall from the cage lid was recorded with a 180-s cut-off time (repeated three times) (Janickova et al., 2017).

2.3.3. Grip force

The grip force test is a well-known and validated test to measure the overall muscular strength of mice and rats. Grip-force in the forelimbs and all limbs were measured using Grip Strength Meter (Insight Equipamentos, Ribeirão Preto, SP) as previously described (Kolimyk et al., 2013). Mice were allowed to grasp the smooth pull bar with forelimbs and also with the all-limbs by holding their tails. Mice were then pulled backward in the horizontal plane and the peak force (N) applied to the bar was recorded. Three trials were performed per mouse within the same session and the average of the three trials was recorded (Kolimyk et al., 2013). All grip force test trials were gently and carefully conducted in order to safeguard from any tail injuries. We thus hold the animal tail in the base (thicker section) to ensure no "whip effect" will happen, even if the animal for some reason tries to jump. We pull the animal backwards using very little force and gradually, so the animal holds the bar and when the force increases to a point it cannot hold, it just let go and the maximum force is displayed in the equipment screen.

2.3.4. Rotarod

Rotarod was used to analyse mice balance, coordination and resistance to the spinning rod. We used a rotarod protocol previously described (Prado et al., 2006) with minor changes. Mice were previously presented to the apparatus 24h before the test and let walk in normal

speed (5 rpm) three times for 5 min and 5 min of interval. In the following day, animals were placed in the rotating rod for 5 min, while the rotating speed increased upon a maximum of 20 rpm. The number of falls were acquired for each animal tested.

2.4. Muscle morphometrical and fibre type analyses

The extensor digitorum longus (EDL) and soleus (SOL) muscles from all mice were surgically excised after anaesthesia (i.p. injection of ketamine (50 mg/kg) and xylazine (5 mg/kg) followed by transcardiac perfusion with PBS 1x. The muscles were then cut in the mid-belly region and immersed in O.C.T (Tissue Plus, Fisher Health Care), and fresh frozen in isopentane (M32631, Sigma-Aldrich) cooled with liquid nitrogen and stored at -80°C . Samples were then sectioned using a cryostat (Leica CM3050S), and 10 μm cross sections were collected on gelatine-coated slides. The slides containing tissue sections underwent a blocking step with 3% BSA + 0.1% Triton X-100 for 30 min followed by 5% normal goat serum + 0.1% Triton X-100 for 30 min at room temperature. Sections were then incubated with Wheat Germ Agglutinin (WGA) Alexa Fluor 647 Conjugate (W32466, Invitrogen), a well-known probe that binds to select carbohydrates present in cell membranes. Here WGA-Alexa Fluor 646 was used to stain the skeletal muscle plasma membrane and washed three times with PBS (Rossi et al., 2023). Sections were mounted using ProLong Gold Antifade Mountant Medium (P36934, Thermo Fisher Scientific).

The fibre type composition analysis was performed according to (Magalhães-Gomes et al., 2016). Samples (10 μm) were sectioned using a cryostat (Leica CM3050S) and collected on gelatine-coated slides. Muscle sections were blocked 30 min at room temperature (RT) with 3% BSA (Sigma-Aldrich), 5% goat serum (Sigma-Aldrich), and 0.1% Triton X-100 (Sigma-Aldrich) diluted in PBS 1x. Muscle sections were then incubated overnight (ON) at 4°C with the following primary antibodies (diluted in 3% BSA, 5% goat serum), all raised in mice: type 1 (Leica Microsystems Cat# NCL-MHCs Lot# RRID:AB_563898; 1:250); type 2A (DSHB Cat# SC-71 Lot# RRID:AB_2147165; 1:100), type 2X (DSHB Cat# BP-35 Lot# RRID:AB_2274680, which recognizes all types of muscle fibres except for the 2X; 1:100), and type 2B (DSHB Cat# BF-P3 Lot# RRID:AB_2266724; 1:100). Slides were washed three times with PBS 1x and incubated for 1 h at room temperature with secondary antibodies all diluted 1:1000 in the same blocking solution: Alexa 488 goat anti-mouse IgG1 (Thermo Fisher Scientific Cat# A-21121 Lot# RRID:AB_2535764) for types 1, 2A and 2X antibodies and Alexa 488 goat anti-mouse IgM (Thermo Fisher Scientific Cat# A-21042 Lot# RRID:AB_2535711) for type 2B antibody. The slides were washed 3 times again with PBS 1x and mounted using ProLong Gold Antifade Mountant Medium (P36934, Thermo Fisher Scientific). Images were acquired using Zeiss LSM 880 confocal microscope (Zeiss OMBH, Germany), using 40x oil immersion objective (NA 1.2), He-Ne (647 nm) and Argon (488 nm) lasers were used for WGA and fibre typing, respectively. The different types of muscle fibres were counted using ImageJ (NIH) and each fibre type was expressed as a percentage of the total number of fibres counted in a whole muscle section.

2.5. Neuromuscular junction analysis

Mice were anesthetized (i.p. injection of ketamine (50 mg/kg) and xylazine (5 mg/kg) and transcardiac perfused by 20 mL of PBS 1x followed by 20 mL of 4% PFA. Muscles were surgically removed and stored soaked in PBS at 4°C . Whole-mount EDL and SOL was used to evaluate the NMJ from all groups. The NMJ staining protocol has been previously described by Valadão et al. (2017). Briefly, pre-synaptic element was stained using a monoclonal primary antibody against synaptotagmin (1:250 dilution, ZNP1S, DSHB) for 12 h, followed by the secondary antibody IgG2A goat anti-mouse conjugated with AlexaFluor 488 (1:800 dilution, A21131, Life Technologies) together with tetramethylrhodamine conjugated α -bungarotoxin 647 (1:800 dilution, B35450, Life

Technologies) for labelling the postsynaptic nicotinic acetylcholine receptors (nAChRs). Images were acquired using the Z-stack imaging tool from Zeiss LSM 880 confocal microscope equipped with 63x oil immersion objective (NA 1.4), and the lasers Argon (488 nm) and He-Ne (543 and 633 nm) were used for synaptotagmin, TdTomato and α -bungarotoxin, respectively. 20 NMJs per animal were considered for analysis using the ImageJ polygon tool.

Colocalization indexes were calculated dividing pre-synaptic element area by the post-synaptic element area and multiplying by 100, to obtain the percentage of the colocalization area. The fragmentation indexes were obtained using the method of particles analysis previously described in Valadão et al. (2018). Images were transformed into a binary pattern and then we used the "particles analysis" tool from ImageJ to obtain the total island of fragments. We analysed only the post-synaptic element and a minimum of 5 islands were used as the criteria for considering the NMJ fragmented.

2.6. Motoneurons morphometrical analysis

Spinal cords (lumbar segment) from transcardiac perfused mice were collected and stored in PFA 4% for 12 h at 4°C . In the following day, the tissues were immersed in sucrose solution (30%) for three days, excised from the vertebrae, and embedded in O.C.T frozen in isopentane cooled with liquid nitrogen. The O.C.T blocks were then sectioned using a cryostat (30 μm thickness) and collected on gelatine-coated slides. Spinal cord sections were blocked with 3% BSA + 0.5% Triton X-100 for 30 min followed by 5% donkey serum + 0.5% Triton X-100 for 30 min at room temperature and incubated for 12 h with a primary antibody against osteopontin (goat anti-OPN; 1:100 dilution, BDO0615031, RD Systems) at 4°C . Sections were washed three times with PBS + 0.5% Triton X-100, and incubated with DAPI to label all cells nuclei, and mounted with ProLong Gold. Images from both strains were acquired using the Z-stack imaging tool from Zeiss LSM 880 confocal microscope, (63x oil immersion objective (NA 1.4), He-Ne (543 nm) and Argon (488 nm) lasers were used for TdTomato and OPN, respectively) and the total number of cells (DAPI+), motor neuron (OPN+), and the motor neurons area analyzed using ImageJ (NIH).

2.7. Statistical analysis

All data were analysed using the software R and GraphPad Prism 8.0.2. All data were analysed for normality by Shapiro-Wilk normality test. One-Way ANOVA (Tukey post-test), Two-Way ANOVA (Tukey post-test) or Kruskal-Wallis (Dunn's post-test) were chosen when appropriate based on the normality of the samples. Values of $p \leq 0.05$ were considered significant.

3. Results

3.1. In vivo treatment with the β_2 -AR agonist formoterol increases NMJ area in mnVAcHt-KD mice muscles

We administered the β_2 -adrenergic agonists formoterol to myasthenic mice with specific deletion of VAcHt in α -MNs and examined two different muscles with distinct fibre compositions and twitching properties: the fast glycolytic extensor digitorum longus (EDL, Fig. 1A) and the slow oxidative soleus (SOL, Fig. 1D). Analyses were performed on NMJs labelled with synaptotagmin (green-presynaptic site) and α -bungarotoxin (red-postsynaptic site), for WT and mnVAcHt-KD mice, both treated and non-treated with formoterol. NMJs from the formoterol-treated mnVAcHt-KD mice group were bigger compared to the untreated group in the EDL muscle. Quantification of several NMJs from each experimental condition confirmed this observation, revealing a significant increase in EDL NMJ size in mnVAcHt-KD mice treated with formoterol compared to untreated counterparts (Fig. 1B and C) (pre-synaptic: *** $p < 0.0001$, Kruskal-Wallis H test; $H = 46.68$, df (4-1); post-

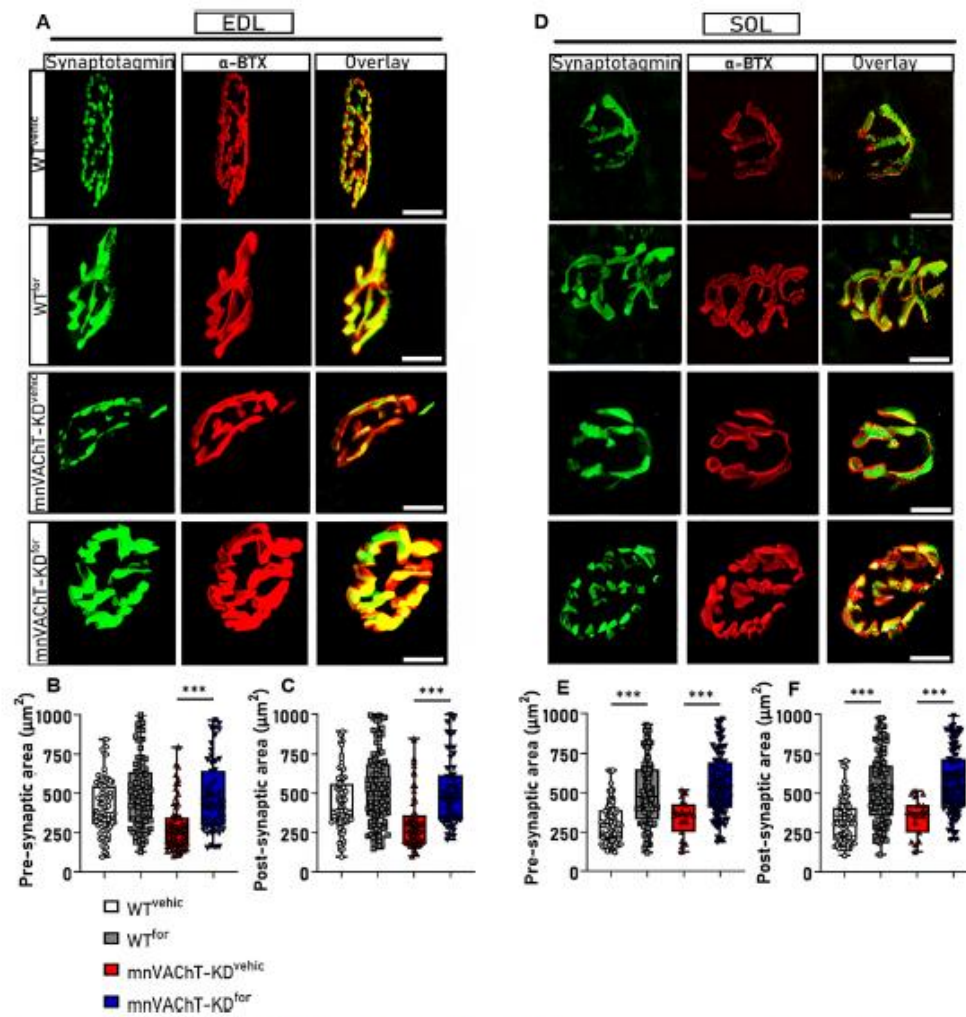


Fig. 1. Formoterol increased the size of NMJs in WT and mnVACHT-KD mice. **A**–Representative panel of pre- and post-synaptic elements showing that formoterol increased the NMJ area of EDL muscle in mnVACHT-KD^{for} mice; **B** and **C**–The quantification of EDL pre- (***) and post-synaptic (p < 0.0001) elements areas, respectively, confirmed that Formoterol increased NMJ synaptic terminals area in mnVACHT-KD^{for} mice, when compared to mnVACHT-KD^{vehic} counterpart; **D**–Representative panel of pre- and post-synaptic elements showing that formoterol increased the NMJ area of SOL muscle in WT^{for} and mnVACHT-KD^{for} mice; **E** and **F**–The quantification of SOL pre- (***) and post-synaptic (***) elements areas, respectively, shows that formoterol increased NMJ synaptic terminals area either in WT^{for} and in mnVACHT-KD^{for} mice, when compared to the vehicle counterparts. Synaptotagmin (green) represents pre-synaptic element and α-BTX (red) represents post-synaptic element. Results represents the median, n = 5 animals per group. Scale bar: 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

synaptic: ***p < 0.0001, Kruskal-Wallis H test; H = 47.31, df (4-1). Similarly, in the SOL muscle (Fig. 1D), formoterol increased the size of NMJs in mnVACHT-KD mice (Fig. 1E and F) (pre-synaptic: ***p = 0.0003, Kruskal-Wallis H test; H = 36.59, df (4-1); post-synaptic: ***p = 0.0002, Kruskal-Wallis H test; H = 35.70, df (4-1)).

One interesting feature of the mnVACHT-KD mouse model for CMS is the possibility to identify MNs with VACHT deletion (TdTomato⁺) and those without VACHT deletion (TdTomato⁻) within the same muscle. This capability allows us to compare compromised and healthy NMJs from the same animal (Joviano-Santos et al., 2021). Therefore, we next compared the effect of formoterol treatment in NMJs with and without

VACHT deletion in the EDL and SOL muscles from mnVACHT-KD mice. We observed that formoterol increased the size of both pre- and post-synaptic elements on VACHT-deleted NMJs (TdTomato⁺) in the EDL (Fig. 2A–C) (pre-synaptic: ***p = 0.0004, Kruskal-Wallis H test; H = 23.68, df (4-1); post-synaptic: ***p < 0.0001, Kruskal-Wallis H test; H = 33.22, df (4-1), as well as in the SOL muscle (Fig. 2D–F) (pre-synaptic: **p = 0.0043, Kruskal-Wallis H test; H = 46.33, df (4-1); post-synaptic: **p = 0.0054, Kruskal-Wallis H test; H = 44.48, df (4-1). Interestingly, in the SOL muscle, formoterol also increased the size of both pre- and post-synaptic elements on NMJs with VACHT (TdTomato⁻) (Fig. 2D–F) (pre-synaptic: *p = 0.0329, Kruskal-Wallis H test; H = 46.33, df (4-1);

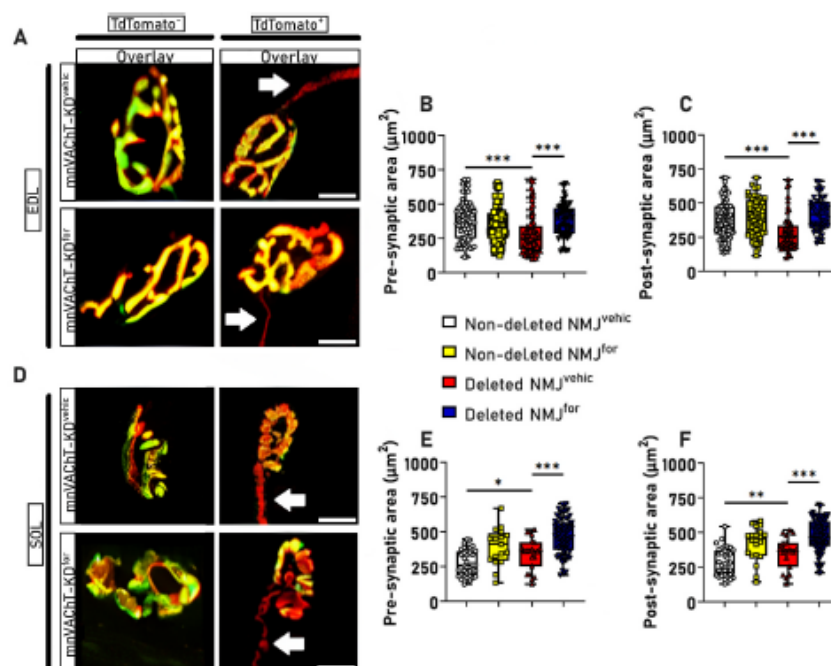


Fig. 2. Formoterol increased the size of VACHT deleted NMJs in fast-twitch EDL and slow-twitch SOL muscles. **A**– Representative panel of pre- and post-synaptic elements of EDL muscle. Left two panels show VACHT non-deleted NMJs (TdTomato⁺) and right two panels show VACHT deleted-NMJs (TdTomato⁺). White arrow highlights the presence of TdTomato⁺ axon. A clear increase in the size of NMJs can be seen in VACHT deleted-NMJs from mnVACHT-KD^{for} mice, in comparison with its vehicle counterpart. **B** The quantification of VACHT non-deleted and deleted-NMJs EDL pre-synaptic (*** $p < 0.0001$) area. **C**– The quantification of VACHT non-deleted and deleted-NMJs EDL post-synaptic (*** $p < 0.0001$) area. **D**– Representative panel of pre- and post-synaptic elements of SOL muscle. Left two panels indicate VACHT non-deleted NMJs (TdTomato⁺) and right two panels indicate VACHT-deleted NMJs (TdTomato⁺). White arrow highlights the presence of TdTomato⁺ axon. In contrast to EDL previously, formoterol increased NMJs area either in the non-deleted and deleted-NMJs, in comparison with its vehicle counterpart. **E** The quantification of VACHT non-deleted and deleted-NMJs SOL pre-synaptic (** $p = 0.0043$) area. **F**– The quantification of VACHT non-deleted and deleted-NMJs SOL post-synaptic (** $p = 0.0054$) area. Results represents the median, $n = 5$ animals per group. Scale bar: 50 μ m.

post-synaptic: ** $p = 0.0032$, Kruskal-Wallis H test; $H = 44.48$, $df (4-1)$. We also analysed the synaptic elements colocalization and post-synaptic fragmentation, and we did not observe any alterations regarding these two parameters in any group (data not shown). These are two important parameters once colocalization gives information on whether pre-synaptic and post-synaptic elements are communicating correctly while fragmentation allows the analysis of discontinuity of post-synaptic nAChRs.

3.2. Formoterol induced hypertrophy and change of muscle fibre type in EDL but not SOL muscle in mnVACHT-KD mice

It has been described that β_2 -AR plays a role in skeletal muscle physiology and plasticity (Lynch and Ryall, 2008; Sato et al., 2011). Given our observation that formoterol increased NMJ sizes in both oxidative and glycolytic muscles, we next investigated whether this effect would be accompanied or not by skeletal muscle fibres hypertrophy.

Indeed, formoterol induced hypertrophy in the EDL muscles fibres of WT and mnVACHT-KD mice, as shown in the representative panel and the quantification of the cross-sectional area (Fig. 3A and C) (WT^{vehic} x WT^{for}, *** $p < 0.0001$; mnVACHT-KD^{vehic} x mnVACHT-KD^{for}, *** $p < 0.0001$; Kruskal-Wallis H test; $H = 109.1$, $df (4-1)$). Interestingly, such muscle fibre hypertrophy was not observed in the SOL of mnVACHT-KD mice, only in WT (Fig. 3B and D) (WT^{vehic} x WT^{for}, *** $p < 0.0001$, Kruskal-Wallis H test; $H = 339.4$, $df (4-1)$).

As formoterol treatment increased the size of EDL muscle fibres in mnVACHT-KD mice, we asked whether it could also change muscle fibre type composition in muscles. We observed a decrease in the percentage of type IIA fibres in the EDL muscle of mnVACHT-KD^{for} compared to mnVACHT-KD^{vehic} (Fig. 3E and F) (one-way ANOVA, Tukey's multiple comparisons test: *** $p < 0.0001$, $F(7,12) = 36.51$) while no changes in fibre composition were observed in the SOL muscle (Fig. 3G and H).

3.3. Formoterol did not induce alterations in α -MNs

Electrical impulses generated from lower α -MN located in the spinal cord ventral horn drive skeletal muscle fibres contractions through the NMJs. However, it is recognized that exists a mutual influence between MNs and skeletal muscles (Li et al., 2008; Chakkalakal et al., 2010; Liu and Chakkalakal, 2021). Thus, we investigated whether formoterol could induce changes in α -MNs from the lumbar segment of the spinal cord. We focused on the lumbar segment of the spinal cord since α -MNs from this region control the contraction of both EDL and SOL muscles. Fig. 4A–D shows representative panels of sections analysed. Quantitative analysis showed that formoterol did not induce alterations in the size of the α -MNs in either WT or mnVACHT-KD mice (Fig. 4E). Importantly, the number of α -MNs remained consistent across all groups (data not shown), indicating no evidence of neuronal degeneration or neuronal death in mnVACHT-KD mice.

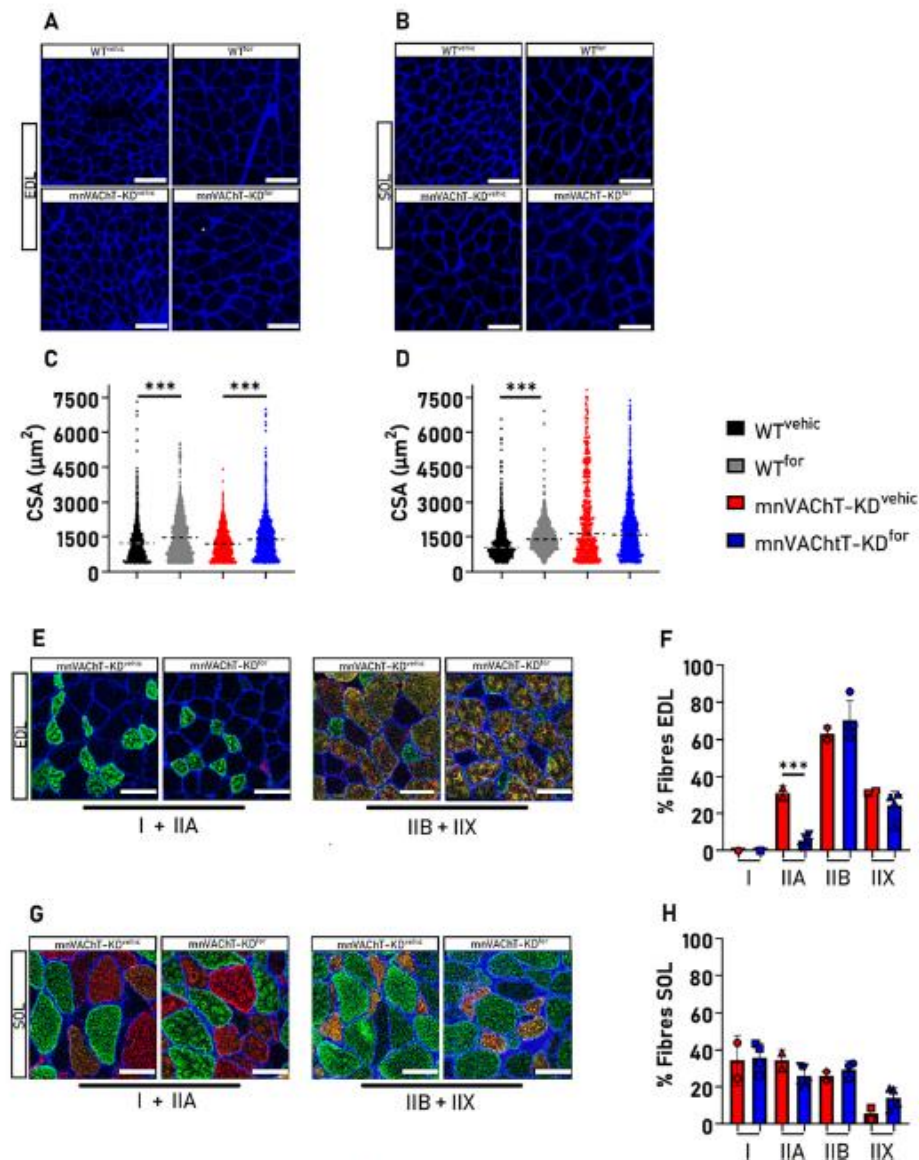


Fig. 3. Formoterol induced skeletal muscle hypertrophy and fibre type changes in myoathenic mice. **A**–Representative panel of EDL muscle CSA showing that formoterol treatment was capable of producing skeletal muscle hypertrophy in WT^{for} and mnVACHT-KD^{for} mice; **B**–Representative panel of SOL muscle CSA showing that formoterol only produced skeletal muscle hypertrophy in the WT^{for} mice muscle; **C**–The quantification shows that formoterol increased the EDL CSA of WT^{for} (*** $p < 0.0001$) and mnVACHT-KD^{for} (*** $p < 0.0001$) mice; **D**–The quantification shows that formoterol increased the SOL CSA of WT^{for} (*** $p < 0.0001$) but not mnVACHT-KD^{for} mice; **E**–Representative panel of EDL muscle fibre type composition showing a reduction in percentage of IIA fibres in the mnVACHT-KD^{for} group. Left two panels show fibre types I (red) and IIA (green) and right two panels show fibre types IIB (double staining in orange) and IIX (negative staining); **F**–Fibre type composition analysis confirms that formoterol induced a decrease in the percentage of IIA fibres in mnVACHT-KD^{for} EDL (*** $p < 0.0001$); **G**–Representative panel of SOL muscle fibre type composition showing that formoterol did not alter the SOL fibre type percentage. Left two panels show fibre types I (red) and IIA (green) and right two panels show fibre types IIB (double staining in orange) and IIX (negative staining); **H**–The SOL fibre type composition analysis confirms that formoterol did not alter fibres percentage in the slow-twitch muscle in mnVACHT-KD mice. Results represent the median for CSA and mean $\pm$ SD for fibre type composition. $n = 4$ animals per groups, one muscle slice per animal for each analysis. *** $p < 0.0005$; ** $p < 0.005$; * $p < 0.05$. Scale bar: 10 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

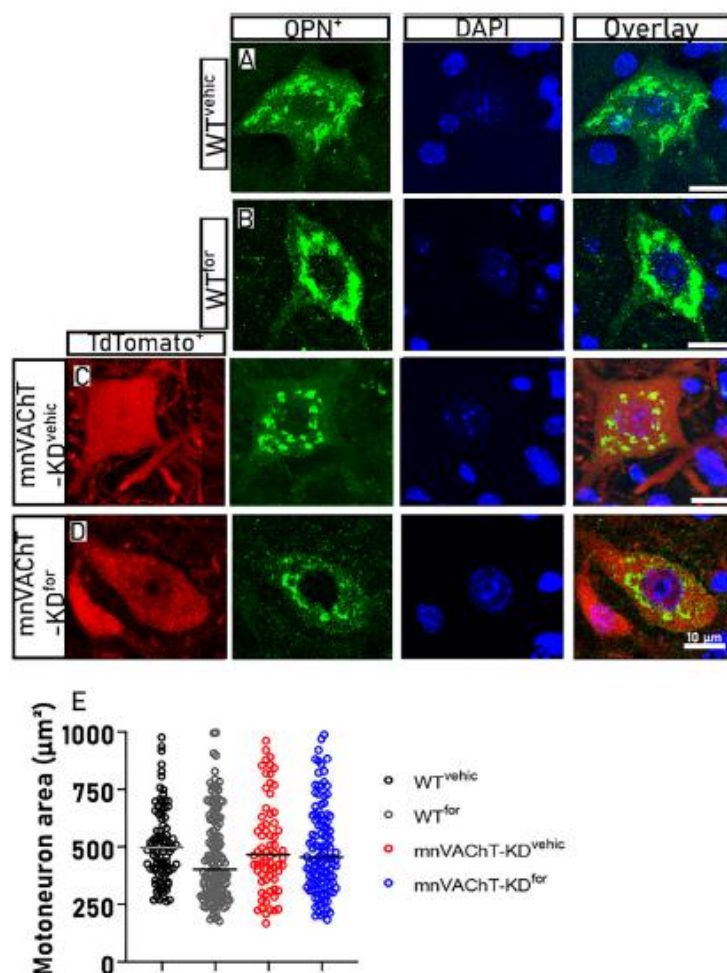


Fig. 4. Formoterol did not induce alterations in the lumbar α -MNs of WT and mnVACHT-KD mice. A, B, C and D- Representative images of WT^{vehic}, WT^{for}, mnVACHT-KD^{vehic} and mnVACHT-KD^{for} lumbar α -MNs, respectively, show that formoterol was not capable of inducing any alteration in the size of the α -MNs; E- The quantitative analysis of the area of lumbar α -MNs shows that all groups have the same α -MN size. Tdtomato⁺ identifies the deleted- α -MN. Deleted (Tdtomato⁺) and non-deleted (Tdtomato⁻) MNs, OPN⁺ and DAPI⁺ MNs were considered for analysis. We used immunostaining for OPN, a specific marker for α -MNs, and DAPI. Tdtomato fluorescence in red indicates α -MN with the deletion of VACHT, green fluorescence shows all the α -MN and blue represents DAPI staining. Kruskal-Wallis test with Dunn's multiple comparisons test was used to analyze α -MN area. Results represents the median, n = 4 animals per group. Scale bar: 10 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Formoterol did not ameliorate the myasthenic symptoms of mnVACHT-KD mice

We have previously shown that mnVACHT-KD mice exhibit motor impairment as early as two months of age (Joviano-Santos et al., 2021). Given the direct effect of formoterol on NMJs and muscle fibres in limb muscles, as described above, we asked if formoterol treatment could reverse muscle weakness in the mnVACHT-KD myasthenic mouse model. To assess this, mnVACHT-KD and WT mice underwent various motor tests to establish baseline performance, were treated with formoterol daily for one month and subsequently retested on motor behaviour (see methods). Fig. 5 illustrates that despite mnVACHT-KD mice presenting reduced motor performance before treatment, formoterol did not

improve forelimbs grip force (A), all-limbs grip force (B), wire-hang performance (C) or the number of falls on the rotarod (D). The only parameter that presented a statistically significant difference was the total distance travelled in the open field, and the improvement was similar in all groups (Fig. 5 E) (Two-way ANOVA, Tukey's multiple comparison test: *** $p < 0.0001$, $F(1,28) = 51.16$; WT^{vehic} baseline x WT^{vehic} D30, * $p = 0.0078$; WT^{for} baseline x WT^{for} D30, * $p = 0.0331$; mnVACHT-KD^{vehic} baseline x mnVACHT-KD^{vehic} D30, * $p = 0.0153$; mnVACHT-KD^{for} baseline x mnVACHT-KD^{for} D30, * $p = 0.0778$).

4. Discussion

Even though most of CMGs are related to post-synaptic alterations

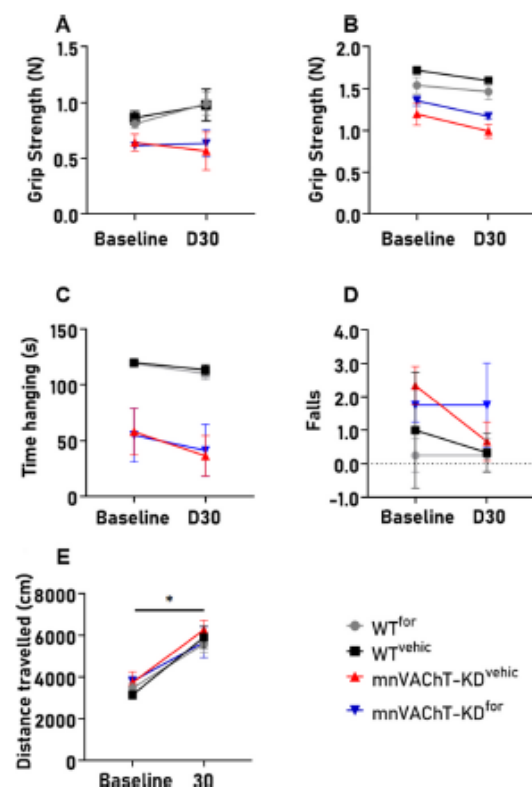


Fig. 5. Formoterol was not able to ameliorate myasthenic symptoms in mnVACHT-KD mice. All mice had their motor behavioural analysis performed before (baseline) and after (D30) in the following tests: A- Forelimb grip strength test; B- All-limbs grip strength test; C- Wire-hang (time hanging); D- Rotarod (number of falls); E- Open field test (total distance travelled) (WT^{vehic} baseline x WT^{vehic} D30: * $p = 0.0078$; WT^{for} baseline x WT^{for} D30: * $p = 0.0331$; mnVACHT-KD^{vehic} baseline x mnVACHT-KD^{vehic} D30: * $p = 0.0183$; mnVACHT-KD^{for} baseline x mnVACHT-KD^{for} D30: $p = 0.0778$). Results represent the mean $\pm$ SD, $n = 5$ animals per group.

such as nAChR clustering and signalling (Ramdas and Beeson, 2021), pre-synaptic changes also lead to CMS and recently, mutations in VACHT gene have been reported in human patients (O'Grady et al., 2016; Aran et al., 2017; Marina et al., 2021). In this work, we injected for 30 days formoterol, a long-lasting and specific β_2 -adrenergic agonist in a mouse CMS model with VACHT deletion in α -MNs to understand the action of this drug at the motor unit level.

The function of VACHT is to store ACh in synaptic vesicles, therefore, in the absence of VACHT, less ACh is released upon the arrival of the action potential. Indeed, a mouse model with knockdown of VACHT (VACHT-KD) exhibits motor dysfunctions and reduced ACh release (Prado et al., 2006). While these mice represent a robust CMS model, they also display various other alterations due to the importance and plethora of ACh functions, such as memory (Prado et al., 2006), cardiovascular (Lara et al., 2010; Durand et al., 2015), renal (Silva et al., 2016) and immune system deficits (Leite et al., 2016). Thus, our research group developed the mnVACHT-KD mouse model, with specific deletion of VACHT only in α -MNs that allows the study of VACHT deficits specifically in the NMJ (Joviano-Santos et al., 2021).

The decrease in VACHT protein is well known to cause muscle

weakness and motor impairment. The VACHT-KD model displays motor alterations due to VACHT knockdown (Prado et al., 2006; Magalhães-Gomes et al., 2018) and we have previously shown that mnVACHT-KD mice developed motor dysfunctions as early as 2 months of age (Joviano-Santos et al., 2021). Furthermore, we have shown that administration of a central acting cholinesterase inhibitor can partially alleviate these symptoms (Joviano-Santos et al., 2021). Although cholinesterase inhibitors are highly effective in the short and medium terms, continuous stimulation of nAChR is known to induce receptor internalization and desensitization resulting in a significant reduction of their therapeutic properties (Morsch et al., 2013; Richtsfeld et al., 2013).

Recent studies have highlighted the potential of β_2 -adrenergic agonists to induce alterations in the NMJ structure. For instance, the β_2 -adrenergic agonist salbutamol improved post-synaptic AChR clustering and synaptic contact size in a zebrafish model of Dok-7 deficiency (McMacken et al., 2018), increased AChR clusters in C2C12 cells (Clayton et al., 2018), and increased synaptic area in a mouse model of ColQ myasthenic syndrome (McMacken et al., 2019). Interestingly, salbutamol was also able to increase the number of NMJ in a mouse model of Dok-7 CMS (Webster et al., 2020). It is expected that β_2 -adrenergic agonist may directly affect NMJ organization, morphology, and/or function, given the presence of β_2 -ARs in this region (Khan et al., 2016; Rudolf et al., 2024). Furthermore, chemical sympathectomy resulted in NMJ abnormalities in both pre- and post-synaptic elements, which were reversed by the administration of β_2 -adrenergic agonist (Khan et al., 2016). However, to our knowledge, there have been no studies on the effects of formoterol or other β_2 -adrenergic agonists in a CMS mouse model of pre-synaptic origin.

In this study, we treated a pre-synaptic CMS (mnVACHT-KD) mice with formoterol and we observed an increase in NMJs in both fast and slow muscles (Fig. 1). We also compared VACHT deleted and VACHT non-deleted NMJs from mnVACHT-KD mice individually in the same muscle and the results showed a similar increase in NMJ size with formoterol (Fig. 2). These findings are intriguing because it represents the first evidence demonstrating that even in the VACHT-deleted NMJ, where there is no release of ACh, the sympathetic nervous system plays a crucial role via β_2 ARs, modifying NMJs structure regardless of cholinergic content. Indeed, recent studies have indicated that epinephrine and norepinephrine have distinct effects at the mouse NMJ (Tsentevisky et al., 2019; Khuzakhmetova and Bukharaeva, 2021; Bukharaeva et al., 2021), and sympathomimetics can regulate ACh release at the NMJ through various receptors, including β_2 AR (Tsentevisky et al., 2019). Electrophysiology and imaging experiments have shown that fenoterol, a β_2 -adrenergic agonist, can increase ACh release at the NMJ (Gafurova et al., 2024). Our results suggest that formoterol has a direct effect on the NMJ. Importantly, formoterol did not produce deleterious effects on NMJ structure, as we did not observe any alterations in synaptic elements' colocalization and post-synaptic fragmentation (data not shown). This is an important observation as synaptic terminal colocalization and fragmentation are signals of NMJ integrity loss due to chronic stimulation and receptor internalization/loss, a well-known feature of cholinesterase inhibitor drugs (Hudson et al., 1985; Richtsfeld et al., 2013; Morsch et al., 2013; Vanhaesebrouck et al., 2019).

There is evidence that β_2 -ARs are also involved in skeletal muscle physiology (Lynch and Ryall, 2008) and hypertrophy (Sato et al., 2011; Gonçalves et al., 2019) via activation of cAMP signalling (Gonçalves et al., 2019). In our study, we found that formoterol induced hypertrophy in muscle fibres from the fast-type EDL but not in the slow-type SOL from mnVACHT-KD mice. Several studies have shown that β_2 -agonists such as clenbuterol can promote muscle alterations (Hinkle et al., 2002; Gonçalves et al., 2012; Sato et al., 2013). Formoterol has been shown to promote various effects on skeletal muscle, including anabolic actions in cachectic rats (Busquets et al., 2012), hypertrophy, increase in β_2 AR content, activation of the Akt-mTOR pathway and downregulation of muscle-specific Ub-ligases (E3s), atrogen-1/MAFbx (muscle atrophy

F-box), and MuRF1 (muscle RING finger-1) (Joazeiro et al., 2013; Gonçalves et al., 2019). Additionally, it has been reported to decrease muscle wasting in a rheumatoid arthritis rat model (Gómez-Sanmiguel et al., 2016), and decrease atrophy and oxidative stress in uremic mice (Higashihara et al., 2021). It is known that all muscles do not respond identically to stimulation by β -adrenergic agonists. For instance, fast-twitch muscles are more responsive to the hypertrophic effects of β_2 -adrenoceptor agonists than slow-twitch muscles (Lynch and Ryall, 2008). Moreover, a previous finding from our group demonstrated that the gastrocnemius, a fast muscle and closely localized with SOL on the posterior paw was atrophied in VACHT-KD mice, resulting in load increase of the SOL muscle, which drove a compensatory hypertrophy of this muscle (Magalhães-Gomes et al., 2018). If the SOL muscle is already hypertrophied (which we can see in the representative images), formoterol could not be able to promote further hypertrophy. Added, these are possible explanations regarding the absence of hypertrophy of SOL after the treatment.

Moreover, formoterol treatment induced alterations in the mnVACHT-KD EDL fibre type composition, decreasing type IIA percentage (Fig. 3E and F). The reduction of a small fibre such as IIA is a possible explanation to the overall increase in the muscle CSA in mnVACHT-KD EDL, since it is expected that together with the reduction of a medium-low oxidative fibre like IIA, an increase of a fast-glycolytic IIB could happen. Although it is shown that β_2 -agonists can promote fibre type switch, especially towards a glycolytic profile (Lynch and Ryall, 2008) we did not observe this type of alteration in fibre composition. This might be due to two possible factors, not mutually excluding: i) the fibre switch may be still undergoing, and with more time we may be able to quantify it; ii) the dose of formoterol used. Other works also used low doses of formoterol (100 μ g/kg) for the same period and did not observe any alteration regarding fibre type composition in WT and *mdx* mice (Gehrig et al., 2010).

We also analysed the lumbar α -MNs pool in the spinal cord, as the lumbar segment is responsible for controlling the EDL and SOL muscle activity. Although we did not find any alterations in the lumbar α -MNs area, it is already known that β_2 -AR are present in spinal cord MNs (Atlas and Melamed, 1978; Patterson and Hanley, 1987; Mizukami, 2004), and that β_2 -adrenergic agonists promote several alterations in neurons (Bartus et al., 2016). Although studies involving β_2 -adrenergic agonists and the motor unit are still limited, activation of those receptors in neurons is implicated in promoting the synthesis of several proteins involved in neuronal homeostasis (Gelinaz and Nguyen, 2005; Zhou et al., 2013). Administration of β_2 -adrenergic agonists stimulates the production of NGF and BDNF, which mediate survival pathways in neurons (Kaspar et al., 2003) and formoterol is known to stimulate anti-proteolytic pathways (Gonçalves et al., 2019). Notably, the number of α -MNs was consistent across all groups analysed (data not shown), suggesting that there is no neuronal death occurring.

Lastly, formoterol was unable to reverse or improve muscle weakness in mnVACHT-KD mice and to explain such lack of behavioural improvement some points can be discussed to help us to understand why the structural changes induced by formoterol did not positively reflect on motor behaviour of our myasthenic mouse model. Our pre-synaptic type CMS mouse model mnVACHT-KD mice has complete ablation of VACHT expression in 75% of motoneurons, thus the animals develop motor symptoms as early as 2 months of age (Joviano-Santos et al., 2021), and in the third month of age (the age we started to inject formoterol) they are severely myasthenic. Most of the works published so far started the treatment of CMS mice model with β_2 -adrenergic agonist as early as 21 days of age (McMacken et al., 2019). Regardless of the CMS mice model used, starting the treatment this early might have been a good strategy for holding the disease course. Together, the severity of our mice model motor impairment and the moment we chose to start the treatment (3 months of age) are important factors that might contribute to the lack of motor behaviour improvement after the treatment. Although Vanhaesebrouck et al., 2019 also used 3-month-old mice

model for post-synaptic CMS (different from our model which is pre-synaptic) and treated them for 4 weeks, the dose of salbutamol used was very high and the drug was administered via the drinking water. In other words, the drug was always available for the animal. These are important characteristics that must be highlighted once as we mentioned before, can produce receptor desensitization in longer treatments. Furthermore, even using high doses of salbutamol, the increment in the hanging time was marginal compared to the other treatment programs. Another important point to mention is that recently, full agonists such as formoterol, were shown to increase synaptic vesicle recruitment and exocytosis (Gafurova et al., 2024) and we have unpublished data showing that formoterol also increases synaptic vesicle exocytosis in ex vivo neuromuscular preparations. Thus, if more neurotransmitter is released, the chances of binding to its receptors also increases (Santos et al. in preparation). However, in pre-synaptic mouse model for CMS (mnVACHT-KD), the deletion of VACHT highly decreases the amount of ACh released so, even if formoterol increases the number of synaptic vesicles exocytosed, the amount of neurotransmitter released might not be sufficient to generate an incremental motor behaviour performance in myasthenic mice. We thus believe that the best use of β_2 -adrenergic agonist to treat pre-synaptic CMS is in combination with cholinesterase inhibitors. This pharmacological approach may be very beneficial once β_2 -adrenergic agonist may increase ACh release and cholinesterase inhibitors may increase the time ACh stays at the synaptic cleft. We are currently conducting experiments with this regimen of treatment.

5. Conclusion

In conclusion, to the best of our knowledge, this study represents the first investigation into the effects of a β_2 -adrenergic agonist on the entire motor unit in a pre-synaptic origin CMS mouse model, such as mnVACHT-KD. Our findings provide additional scientific evidence supporting the use of β_2 -adrenergic agonist in the management of CMS and highlight the potential therapeutic properties of these drugs. β_2 -adrenergic agonists have emerged as promising candidates for the treatment of neuromuscular disorders, especially in prolonged chronic treatments. Further study is needed to explain the molecular mechanisms through which they exert their effects on the various components of the motor unit.

Ethics approval and consent to participate

All experimental procedures were performed following the guidance of the Universidade Federal de Minas Gerais (UFMG) Ethical Committee for Animal Experimentation and received approval number under No 81/2021.

Consent for publication

Not applicable.

Availability of data

All data generated in this study is available upon reasonable request.

Funding

This work was supported by grants from CNPq (#402567/2021-8 and 442473/2023-0) FAPEMIG (APQ-00092-18), International Society for Neurochemistry (ISN) and Chan Zuckerberg Initiative (CZI) to Cristiana Guatimosim. Luis C Navegantes received support from FAPESP 2018/10009-2; PQ CNPq: 302396/2022-5; CAPES/PROEX. Barbara Mota is supported by a fellowship (#38/2022) from Programa de desenvolvimento da pós-graduação parcerias estratégicas nos estados III) CAPES/FAPEMIG.

CRediT authorship contribution statement

Leonardo Rossi: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization. Bárbara I. Motas: Methodology, Formal analysis. Priscila A.C. Valadão: Methodology, Formal analysis. Matheus P.S. Magalhães-Gomes: Writing – review & editing, Methodology, Formal analysis. Bruna S. Oliveira: Methodology, Formal analysis. Silvia Guatimosim: Writing – review & editing, Supervision, Resources. Luiz C.C. Navegantes: Writing – review & editing, Resources, Methodology, Conceptualization. Aline S. Miranda: Writing – review & editing, Supervision, Resources, Formal analysis. Marco A.M. Prado: Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. Vânia F. Prado: Writing – review & editing, Resources, Methodology, Conceptualization. Cristina Guatimosim: Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

The authors would like to acknowledge the Center of Acquisition and Processing of Images (CAPI) – ICB – Universidade Federal de Minas Gerais for providing the equipment and technical support for experiments.

List of abbreviations

(ACh)	Acetylcholine
(AChE)	Acetylcholinesterase
(CMS)	Congenital Myasthenic Syndrome
(EDL)	Extensor Digitorum Longus
(MAFbx)	Muscle Atrophy P-box
(mnVACHT-KD)	Mice with conditional knockout of VACHT in α -motoneurons
(MuRF1)	Muscle RING finger-1
(nAChR)	Nicotinic Acetylcholine Receptor
(NMJ)	Neuromuscular Junction
(s.c)	Subcutaneously
(SOL)	Soleus
(VACHT)	Vesicular Acetylcholine Transporter
(α -MNs)	α -motoneurons
(β_2 -AR)	β_2 -adrenergic receptor

References

- Aran, A., Segel, R., Kaneshige, K., Gulsuner, S., Renbaum, P., Oliphant, S., Meirson, T., Weinberg-Shukron, A., Hershkovitz, Y., Zeligson, S., Lee, M.K., Samson, A.O., Parsons, S.M., Kling, M.-C., Levy-Lahad, E., Walsh, T., 2017. Vesicular acetylcholine transporter defect underlies devastating congenital myasthenic syndrome. *Neurology* 88, 1021–1028. <https://doi.org/10.1212/WNL.0000000000003720>.
- Atlas, D., Melamed, E., 1978. Direct mapping of β -adrenergic receptors in the rat central nervous system by a novel fluorescent β -blocker. *Brain Res.* 150, 377–385. [https://doi.org/10.1016/0006-8993\(78\)90288-3](https://doi.org/10.1016/0006-8993(78)90288-3).
- Bartus, R.T., Bétourné, A., Basile, A., Peterson, B.L., Glass, J., Boulis, N.M., 2016. β_2 -Adrenoceptor agonists as novel, safe and potentially effective therapies for Amyotrophic lateral sclerosis (ALS). *Neurobiol. Dis.* 85, 11–24. <https://doi.org/10.1016/j.nbd.2015.10.006>.

- Bukharava, E., Khuzakmetova, V., Dmitrieva, S., Tsentsevitsky, A., 2021. Adrenoceptors modulate cholinergic synaptic transmission at the neuromuscular junction. *Int. J. Mol. Sci.* 22, 4611. <https://doi.org/10.3390/ijms22094611>.
- Busquets, S., Toledo, M., Marmont, E., Orpi, M., Capdevila, E., Betancourt, A., López-Soriano, F.J., Argilés, J.M., 2012. Formoterol treatment downregulates the myostatin system in skeletal muscle of cachectic tumour-bearing rats. *Oncol. Lett.* 3, 185–189. <https://doi.org/10.3892/ol.2011.442>.
- Chakkalakal, J.V., Nishimune, H., Ruas, J.L., Spiegelman, B.M., Sanes, J.R., 2010. Retrograde influence of muscle fibers on their innervation revealed by a novel marker for slow motoneurons. *Development* 137, 3489–3499. <https://doi.org/10.1242/dev.053348>.
- Clausen, L., Cossins, J., Beeson, D., 2018. Beta-2 adrenergic receptor agonists enhance AChR clustering in C2C12 myotubes: implications for therapy of myasthenic disorders. *J. Neuromuscul. Dis.* 5, 231–240. <https://doi.org/10.5233/JND-170293>.
- de Castro, B.M., De Jaeger, X., Martins-Silva, C., Lima, R.D.F., Amaral, E., Menezes, C., Lima, P., Neves, C.M.L., Pires, R.G., Gould, T.W., Welch, L., Kushmerick, C., Guatimosim, C., Izquierdo, L., Cammarota, M., Rylett, R.J., Gomez, M.V., Caron, M.G., Oppenheim, R.W., Prado, M.A.M., Prado, V.F., 2009. The vesicular acetylcholine transporter is required for neuromuscular development and function. *Mol. Cell Biol.* 29, 5238–5250. <https://doi.org/10.1128/MCB.00245-09>.
- Durand, M.T., Becari, C., Tezini, G.C.S.V., Fazan, R., Oliveira, M., Guatimosim, S., Prado, V.F., Prado, M.A.M., Salgado, H.C., 2015. Autonomic cardiovascular control in mice with reduced expression of the vesicular acetylcholine transporter. *Am. J. Physiol. Heart Circ. Physiol.* 309, H655–H662. <https://doi.org/10.1152/ajpheart.00114.2015>.
- Edwards, R.H., 2007. The neurotransmitter cycle and quantal size. *Neuron* 55, 835–858. <https://doi.org/10.1016/j.neuron.2007.09.001>.
- Finsterer, J., 2019. Congenital myasthenic syndromes. *Orphanet J. Rare Dis.* 14, 57. <https://doi.org/10.1186/s13023-019-1025-5>.
- Gafurova, C.R., Tsentsevitsky, A.N., Fedorov, N.S., Khaziev, A.N., Malomouzh, A.I., Petrov, A.M., 2024. β_2 -adrenergic regulation of the neuromuscular transmission and its lipid-dependent switch. *Mol. Neurobiol.* <https://doi.org/10.1007/s12035-024-03991-2>.
- Gehrig, S.M., Koopman, R., Naim, T., Tjoarkarfa, C., Lynch, G.S., 2010. Making fast-twitch dystrophic muscles bigger protects them from contraction injury and attenuates the dystrophic pathology. *Am. J. Pathol.* 176, 29–33. <https://doi.org/10.2353/ajpath.2010.090760>.
- Gellinas, J.N., Nguyen, P.V., 2005. β -Adrenergic receptor activation facilitates induction of a protein synthesis-dependent late phase of long-term potentiation. *J. Neurosci.* 25, 3294–3303. <https://doi.org/10.1523/JNEUROSCI.4175-04.2005>.
- Gómez-SanMiguel, A.B., Gomez-Moreira, C., Nieto-Esna, M.P., Fernández-Galar, C., Villanúa, M.A., Martín, A.L., López-Calderón, A., 2016. Formoterol decreases muscle wasting as well as inflammation in the rat model of rheumatoid arthritis. *Am. J. Physiol. Endocrinol. Metab.* 310, E925–E937. <https://doi.org/10.1152/ajpendo.00503.2015>.
- Gonçalves, D.A.F., Silveira, W.A., Lira, E.C., Graça, F.A., Paula-Gomes, S., Zanon, N.M., Kettelhut, I.C., Navegantes, L.C.G., 2012. Clenbuterol suppresses proteasomal and lysosomal proteolysis and atrophy-related genes in denervated rat soleus muscles independently of Akt. *Am J Physiol Metab* 302, E123–E133. <https://doi.org/10.1152/ajpendo.00188.2011>.
- Gonçalves, D.A., Silveira, W.A., Manfredi, L.H., Graça, F.A., Armani, A., Bertagaglia, E., O'Neill, B.T., Lautherbach, N., Machado, J., Nogara, L., Pereira, M.G., Arcidiacono, D., Realdon, S., Kahn, C.R., Sandri, M., Kettelhut, I.C., Navegantes, L.C.G., 2019. Insulin/IGF1 signalling mediates the effects of β_2 -adrenergic agonist on muscle proteostasis and growth. *J Cachexia Sarcopenia Muscle* 10, 455–475. <https://doi.org/10.1002/jcsm.12395>.
- Higashihara, T., Nishi, H., Takemura, K., Watanabe, H., Maruyama, T., Inagi, R., Tanaka, T., Nangaku, M., 2021. β_2 -adrenergic receptor agonist counteracts skeletal muscle atrophy and oxidative stress in uremic mice. *Sci. Rep.* 11, 9130. <https://doi.org/10.1038/s41598-021-88438-7>.
- Hinkle, R.T., Hodge, K.M.B., Cody, D.B., Sheldon, R.J., Koblika, E.K., Isfort, R.J., 2002. Skeletal muscle hypertrophy and anti-atrophy effects of clenbuterol are mediated by the β_2 -adrenergic receptor. *Muscle Nerve* 25, 729–734. <https://doi.org/10.1002/mus.10092>.
- Hudson, C.S., Foster, R.E., Kahng, M.W., 1985. Neuromuscular toxicity of pyridostigmine bromide in the diaphragm, extensor digitorum longus, and soleus muscles of the rat. *Toxicol. Sci.* 5, 260–269. <https://doi.org/10.1093/toxsci/5.6part2.260>.
- Janickova, H., Rosborough, K., Al-Onaizi, M., Kljakic, O., Guzman, M.S., Gros, R., Prado, M.A.M., Prado, V.F., 2017. Deletion of the vesicular acetylcholine transporter from pedunculopontine/laterodorsal tegmental neurons modifies gait. *J. Neurochem.* 140, 787–798. <https://doi.org/10.1111/jnc.13910>.
- Joassard, O.R., Amirouche, A., Gallot, Y.S., Desgeorges, M.M., Castells, J., Durieux, A.-C., Berthon, P., Freysenet, D.G., 2013. Regulation of Akt-mTOR, ubiquitin-proteasome and autophagy-lysosome pathways in response to formoterol administration in rat skeletal muscle. *Int. J. Biochem. Cell Biol.* 45, 2444–2455. <https://doi.org/10.1016/j.biocel.2013.07.019>.
- Joviano-Santos, J.V., Kljakic, O., Magalhães-Gomes, M.P.S., Valadão, P.A.C., Oliveira, L.R., Prado, M.A.M., Prado, V.F., Guatimosim, C., 2021. Motoneuron-specific loss of VACHT mimics neuromuscular defects seen in congenital myasthenic syndrome. *FEBS J.* 288, 5331–5349. <https://doi.org/10.1111/febs.15825>.
- Kaspar, B.K., Ildad, J., Sherkat, N., Rothstein, J.D., Gage, F.H., 2003. Retrograde viral delivery of IGF-1 prolongs survival in a mouse ALS model. *Science* 301 (80), 839–842. <https://doi.org/10.1126/science.1086137>.
- Khan, M.M., Lustrino, D., Silveira, W.A., Wild, F., Straka, T., Issop, Y., O'Connor, E., Cox, D., Reischl, M., Marquardt, T., Labelt, D., Labelt, S., Fenoit, E., Molgó, J., Lochmüller, H., Witzemann, V., Kettelhut, I.C., Navegantes, L.C.G., Pozzan, T.,

- Rudolf, R., 2016. Sympathetic innervation controls homeostasis of neuromuscular junctions in health and disease. *Proc. Natl. Acad. Sci. USA* 113, 746–750. <https://doi.org/10.1073/pnas.1524272113>.
- Khurakmetova, V., Bukharava, E., 2021. Adrenaline facilitates synaptic transmission by synchronizing release of acetylcholine quanta from motor nerve endings. *Cell. Mol. Neurobiol.* 41, 395–401. <https://doi.org/10.1007/s10571-020-00840-3>.
- Kljakic, O., Hogan-cann, A.E., Yang, H., Dover, B., Al-onazi, M., Prado, M.A.M., Prado, V.F., 2022. Chemogenetic activation of VGLUT3-expressing neurons decreases movement. *Eur. J. Pharmacol.* 935, 175298. <https://doi.org/10.1016/j.ejphar.2022.175298>.
- Kolinsnyk, B., Guzman, M.S., Raulic, S., Fan, J., Magalhaes, A.C., Feng, G., Gros, R., Prado, V.F., Prado, M.A.M., 2013. ChAT-ChR2-EYFP mice have enhanced motor endurance but show deficits in attention and several additional cognitive domains. *J. Neurosci.* 33, 10427–10438. <https://doi.org/10.1523/JNEUROSCI.0395-13.2013>.
- Lara, A., Damasceno, D.D., Pires, R., Gros, R., Gomes, E.R., Gavioli, M., Lima, R.F., Guimarães, D., Lima, P., Bueno, C.R., Vasconcelos, A., Roman-Campos, D., Menezes, C.A.S., Silvestre, R.A., Salemi, V.M., Mady, C., Caron, M.G., Ferreira, A.J., Brum, P.C., Resende, R.R., Cruz, J.S., Gomez, M.V., Prado, V.F., de Almeida, A.P., Prado, M.A.M., Guatimosim, S., 2010. Dysautonomia due to reduced cholinergic neurotransmission causes cardiac remodeling and heart failure. *Mol. Cell Biol.* 30, 1746–1756. <https://doi.org/10.1128/MCB.00996-09>.
- Leite, H.R., Oliveira-Lima, O.C., de, Pereira, L. de M., Oliveira, V.E. de M., Prado, V.F., Prado, M.A.M., Pereira, G.S., Massensini, A.R., 2016. Vesicular acetylcholine transporter knock down-mice are more susceptible to inflammation, c-Fos expression and sickness behavior induced by lipopolysaccharide. *Brain Behav. Immun.* 57, 282–292. <https://doi.org/10.1016/j.bbi.2016.05.005>.
- Li, X., Dong, X., Luo, S., Zhang, B., Lee, D., Ting, A.K.L., Neiswander, H., Kim, C., Carpenter-hyland, E., Gao, T., Xiong, W., Mei, L., 2008. Retrograde regulation of motoneuron differentiation by muscle β -catenin. *Nat. Neurosci.* 11. <https://doi.org/10.1038/nn2053>.
- Liu, W., Chaikakalak, J. V., n.d. The Composition, Development, and Regeneration of Neuromuscular Junctions, 1st ed. Myogenesis in Development and Disease. Elsevier Inc. <https://doi.org/10.1016/b978-0-12-370800-5>.
- Lynch, G.S., Ryall, J.G., 2008. Role of β -adrenoceptor signaling in skeletal muscle: implications for muscle wasting and disease. *Physiol. Rev.* 88, 729–767. <https://doi.org/10.1152/physrev.00028.2007>.
- Magalhães-Gomes, M.P.S., Motta-Santos, D., Schettino, L.P.L., Andrade, J.N., Bastos, C.P., Guimarães, D.A.S., Vaughan, S.K., Martinelli, P.M., Guatimosim, S., Pereira, G.S., Coimbra, C.C., Prado, V.F., Prado, M.A.M., Valdez, G., Guatimosim, C., 2018. Fast and slow-twitching muscles are differentially affected by reduced cholinergic transmission in mice deficient for VACHT: a mouse model for congenital myasthenia. *Neurochem. Int.* 120, 1–12. <https://doi.org/10.1016/j.neuint.2018.07.002>.
- Marina, A., Della, Arit, A., Schara-Schmidt, U., Depienne, C., Gangfu, A., Köbel, H., Sickmann, A., Freier, E., Köhlschmidt, N., Hentschel, A., Weis, J., Czech, A., Grüneboom, A., Roos, A., 2021. Phenotypic and myopathological consequences of compound heterozygous missense and nonsense variants in *SLC18A3*. *Cells* 10. <https://doi.org/10.3390/10123481>.
- McMacken, G., Cox, D., Roos, A., Müller, J., Whittaker, R., Lochmüller, H., 2018. The β -adrenergic agonist salbutamol modulates neuromuscular junction formation in zebrafish models of human myasthenic syndromes. *Hum. Mol. Genet.* 27, 1556–1564. <https://doi.org/10.1093/hmg/ddy062>.
- McMacken, G.M., Spendiff, S., Whittaker, R.G., O'Connor, E., Howarth, R.M., Boczonadi, V., Horvath, R., Slater, C.R., Lochmüller, H., 2019. Salbutamol modifies the neuromuscular junction in a mouse model of ColQ myasthenic syndrome. *Hum. Mol. Genet.* 28, 2339–2351. <https://doi.org/10.1093/hmg/ddz059>.
- Mizukami, T., 2004. Immunocytochemical localization of β -Adrenergic receptors in the rat spinal cord and their spatial relationships to tyrosine hydroxylase-immunoreactive terminals. *Kurume Med. J.* 51, 175–183. <https://doi.org/10.2739/kurumemedj.51.175>.
- Morsch, M., Reddel, S.W., Ghazanfari, N., Toyka, K.V., Phillips, W.D., 2013. Pyridostigmine but not 3,4-diaminopyridine exacerbates ACh receptor loss and myasthenia induced in mice by muscle-specific kinase autoantibody. *J. Physiol.* 591, 2747–2762. <https://doi.org/10.1113/jphysiol.2013.251827>.
- O'Grady, G.L., Verschuren, C., Yuen, M., Webster, R., Menezes, M., Fock, J.M., Pride, N., Best, H.A., Benavides Damm, T., Turner, C., Lek, M., Engel, A.G., North, K. N., Clarke, N.F., MacArthur, D.G., Kamsteeg, E., Cooper, S.T., 2016. Variants in *SLC18A3*, vesicular acetylcholine transporter, cause congenital myasthenic syndrome. *Neurology* 87, 1442–1448. <https://doi.org/10.1212/WNL.0000000000003179>.
- Ohno, K., Ohkawara, B., Shen, X.M., Selcen, D., Engel, A.G., 2023. Clinical and pathologic features of congenital myasthenic syndromes caused by 35 genes—a comprehensive review. *Int. J. Mol. Sci.* <https://doi.org/10.3390/ijms24043730>.
- Patterson, S.L., Hanley, M.R., 1987. Autoradiographic evidence for β -adrenergic receptors on capsaicin-sensitive primary afferent terminals in rat spinal cord. *Neurosci. Lett.* 78, 17–21. [https://doi.org/10.1016/0304-3940\(87\)90554-4](https://doi.org/10.1016/0304-3940(87)90554-4).
- Prado, V.F., Martins-Silva, C., de Castro, B.M., Lima, R.F., Barros, D.M., Amaral, E., Ramsey, A.J., Sotnikova, T.D., Ramirez, M.R., Kim, H.G., Rossato, J.I., Koenen, J., Quan, H., Cota, V.R., Moraes, M.F.D., Gomez, M.V., Guatimosim, C., Wetsel, W.C., Kushmerick, C., Pereira, G.S., Gainetdinov, R.R., Izquierdo, I., Caron, M.G., Prado, M.A.M., 2006. Mice deficient for the vesicular acetylcholine transporter are myasthenic and have deficits in object and social recognition. *Neuron* 51, 601–612. <https://doi.org/10.1016/j.neuron.2006.08.005>.
- Prado, V.F., Roy, A., Kolinsnyk, B., Gros, R., Prado, M.A.M., 2013. Regulation of cholinergic activity by the vesicular acetylcholine transporter. *Biochem. J.* 450, 265–274. <https://doi.org/10.1042/BJ20121662>.
- Pugliese, A., Holland, S.H., Rodolico, C., Lochmüller, H., Spendiff, S., 2023. Presynaptic congenital myasthenic syndromes: understanding clinical phenotypes through in vivo models. *J. Neuromuscul. Dis.* 10, 731–759. <https://doi.org/10.3233/JND-221646>.
- Ramdas, S., Beeson, D., 2021. Congenital myasthenic syndromes: where do we go from here? *Neuromuscul. Disord.* 31, 943–954. <https://doi.org/10.1016/j.nmd.2021.07.000>.
- Richtsfeld, M., Yanuhara, S., Pink, H., Ebner, M., Martyn, J.A.J., 2013. Prolonged administration of pyridostigmine impairs neuromuscular function with and without down-regulation of acetylcholine receptors. *Anesthesiology* 119, 412–421. <https://doi.org/10.1097/ALN.0b013e318291c02e>.
- Rossi, L., Santos, K.S., Mota, B.I., Pimenta, J., Oliveira, B., Machado, C.A., Fernandes, H. B., Barbosa, L.A., Rodrigues, H.A., Marques, G., Gomes-Martins, G.A., Chalmowicz, G.F., Queiroz-Junior, C.M., Chaves, I., Tapia, J.C., Teixeira, M.M., Costa, V.V., Miranda, A.S., Guatimosim, C., 2023. Neuromuscular defects after infection with a beta coronavirus in mice. *Neurochem. Int.* 169, 105567. <https://doi.org/10.1016/j.neuint.2023.105567>.
- Rudolf, R., Kettelhut, L.C., Navegantes, L.C.C., 2024. Sympathetic innervation in skeletal muscle and its role at the neuromuscular junction. *J. Muscle Res. Cell Motil.* <https://doi.org/10.1007/s10974-024-09665-9>.
- Sato, S., Shirato, K., Mitsuhashi, R., Inoue, D., Kizaki, T., Ohno, H., Tachiyashiki, K., Imazumi, K., 2013. Intracellular β 2-adrenergic receptor signalling specificity in mouse skeletal muscle in response to single-dose β 2-agonist clenbuterol treatment and acute exercise. *J. Physiol. Sci.* 63, 211–218. <https://doi.org/10.1007/s12576-013-0253-z>.
- Sato, S., Shirato, K., Tachiyashiki, K., Imazumi, K., 2011. Muscle plasticity and β 2-adrenergic receptors: adaptive responses of β 2-adrenergic receptor expression to muscle hypertrophy and atrophy. *J. Biomed. Biotechnol.* 1–10. <https://doi.org/10.1155/2011/729598>.
- Silva, R.C., Terra, F.F., Guise, Y.F., Prado, M.A.M., Prado, V.F., Hiyane, M.L., Costa Malheiros, D.M.A., Prado, C.M., Camara, N.O.S., Braga, T.T., 2016. Reduced expression of VACHT increases renal fibrosis. *Pathophysiology* 23, 229–236. <https://doi.org/10.1016/j.pathophys.2016.07.002>.
- Tsentsvetitsky, A.N., Kovyzina, I.V., Bukharava, E.A., 2019. Diverse effects of noradrenaline and adrenaline on the quantal secretion of acetylcholine at the mouse neuromuscular junction. *Neuroscience* 423, 162–171. <https://doi.org/10.1016/j.neuroscience.2019.10.049>.
- Valadão, P.A.C., de Aragão, B.C., Andrade, J.N., Magalhães-Gomes, M.P.S., Foureaux, G., Joviano-Santos, J.V., Nogueira, J.C., Ribeiro, F.M., Tapia, J.C., Guatimosim, C., 2017. Muscle atrophy is associated with cervical spinal motoneuron loss in BACHD mouse model for Huntington's disease. *Eur. J. Neurosci.* 45, 785–796. <https://doi.org/10.1111/ejn.13510>.
- Valadão, P.A.C., Gomes, M.P.S.M., Aragão, B.C., Rodrigues, H.A., Andrade, J.N., Garcias, R., Joviano-Santos, J.V., Luiz, M.A., Camargo, W.L., Naves, L.A., Kushmerick, C., Cavalcante, W.L.G., Gallacci, M., de Jesus, I.C.G., Guatimosim, S., Guatimosim, C., 2018. Neuromuscular synapse degeneration without muscle function loss in the diaphragm of a murine model for Huntington's Disease. *Neurochem. Int.* 116, 30–42. <https://doi.org/10.1016/j.neuint.2018.03.007>.
- Vanhaesebrouck, A.E., Webster, R., Maxwell, S., Rodriguez Cruz, P.M., Cossins, J., Wickens, J., Liu, W.-W., Cetin, H., Cheung, J., Ramjattan, H., Palace, J., Beeson, D., 2019. β 2-Adrenergic receptor agonists ameliorate the adverse effect of long-term pyridostigmine on neuromuscular junction structure. *Brain* 142, 3713–3727. <https://doi.org/10.1093/brain/awz322>.
- Webster, R.G., Cossins, J., Lashley, D., Maxwell, S., Liu, W.W., Wickens, J.R., Martinez-Martinez, P., de Baets, M., Beeson, D., 2013. A mouse model of the slow channel myasthenic syndrome: neuromuscular physiology and effects of ephedrine treatment. *Exp. Neurol.* 248, 286–298. <https://doi.org/10.1016/j.expneurol.2013.06.012>.
- Webster, R.G., Vanhaesebrouck, A.E., Maxwell, S.E., Cossins, J.A., Liu, W., Ueta, R., Yamanashi, Y., Beeson, D.M.W., 2020. Effect of salbutamol on neuromuscular junction function and structure in a mouse model of DOK7 congenital myasthenia. *Hum. Mol. Genet.* 29, 2325–2336. <https://doi.org/10.1093/hmg/ddaa116>.
- Zhou, H.C., Sun, Y.Y., Cai, W., He, X.T., Yi, F., Li, B.M., Zhang, X.H., 2013. Activation of β 2-adrenoceptor enhances synaptic potentiation and behavioral memory via cAMP-PKA signaling in the medial prefrontal cortex of rats. *Learn. Mem.* 20, 274–284. <https://doi.org/10.1101/lm.030411.113>.

8.2 Artigo 2: Motoneuron-specific loss of VACHT mimics neuromuscular defects seen in congenital myasthenic syndrome.

Motoneuron-specific loss of VACHT mimics neuromuscular defects seen in congenital myasthenic syndrome

Julliane V. Joviano-Santos^{1,2} , Omela Kljakic^{1,3}, Matheus P. S. Magalhães-Gomes^{2,4} ,
Priscila Aparecida C. Valadão², Leonardo R. de Oliveira², Marco A. M. Prado^{1,3,5,6} ,
Vania F. Prado^{1,3,5,6} and Cristina Guatimosim² 

¹ Roberts Research Institute, Schulich School of Medicine & Dentistry, University of Western Ontario, London, Canada

² Departamento de Morfologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil

³ Department of Anatomy and Cell Biology, Schulich School of Medicine & Dentistry, University of Western Ontario, London, Canada

⁴ Departamento de Medicina, Faculdade Ciências Médicas de Minas Gerais, FCMMG, Belo Horizonte, Brazil

⁵ Department of Physiology and Pharmacology, Schulich School of Medicine & Dentistry, University of Western Ontario, London, Canada

⁶ Brain and Mind Institute, University of Western Ontario, London, Canada

Keywords

acetylcholine; alpha-motoneurons;
congenital myasthenic syndromes;
neuromuscular dysfunction; vesicular
acetylcholine transporter

Correspondence

V. F. Prado, Roberts Research Institute,
1151 Richmond St. N, N6A 5B7, The
University of Western Ontario, London, ON,
Canada
Tel: +1 519 9315777; Ext. 24888 or 24889
E-mail: vprado@roberts.ca
C. Guatimosim, Departamento de
Morfologia, Instituto de Ciências Biológicas,
Universidade Federal de Minas Gerais, Av.
Antônio Carlos, 6627, Belo Horizonte, MG,
Brazil
Tel: +55 3134092524
E-mail: cguati@icb.ufmg.br

(Received 29 May 2020, revised 3 March
2021, accepted 16 March 2021)

doi:10.1111/febs.15825

Motoneurons (MNs) control muscle activity by releasing the neurotransmitter acetylcholine (ACh) at the level of neuromuscular junctions. ACh is packaged into synaptic vesicles by the vesicular ACh transporter (VACHT), and disruptions in its release can impair muscle contraction, as seen in congenital myasthenic syndromes (CMS). Recently, *VACHT* gene mutations were identified in humans displaying varying degrees of myasthenia. Moreover, mice with a global deficiency in VACHT expression display several characteristics of CMS. Despite these findings, little is known about how a long-term decrease in VACHT expression *in vivo* affects MNs structure and function. Using Cre-loxP technology, we generated a mouse model where VACHT is deleted in select groups of MNs (mnVACHT-KD). Molecular analysis revealed that the VACHT deletion was specific to MNs and affected approximately 50% of its population in the brainstem and spinal cord, with alpha-MNs primarily targeted (70% in spinal cord). Within each animal, the cell body area of VACHT-deleted MNs was significantly smaller compared to MNs with VACHT preserved. Likewise, muscles innervated by VACHT-deleted MNs showed atrophy while muscles innervated by VACHT-containing neurons appeared normal. In addition, mnVACHT KD mice had decreased muscle strength, were hypoactive, leaner and exhibited kyphosis. This neuromuscular dysfunction was evident at 2 months of age and became progressively worse by 6 months. Treatment of mutants with a cholinesterase inhibitor was able to improve some of the motor deficits. As these observations mimic what is seen in CMS, this new line could be valuable for assessing the efficacy of potential CMS drugs.

Abbreviations

ACh, acetylcholine; AChRs, acetylcholine receptors; ChAT, choline acetyltransferase; ChR2, channelrhodopsin-2; CMS, congenital myasthenic syndromes; MNs, motoneurons; NMJ, neuromuscular junctions; OPN, osteopontin; PFA, paraformaldehyde; SD, standard deviation; SEM, standard error of mean; SOL, soleus; STM, sternomastoid; Syn, synaptophysin; TA, tibialis anterior; VACHT, vesicular ACh transporter; WT, wild-type.

Introduction

The proper functioning of the muscular system is highly dependent on the spinal cord, a crucial region of the central nervous system. Localized within the anterior horn of the spinal cord are motoneurons (MNs), output cells responsible for muscle contraction [1]. MNs are specifically found in the motor pool and can exist in gamma and alpha forms [2]. Gamma-MNs are less prevalent but because they innervate intrafusal muscle fibers, they can modulate the sensitivity of muscle spindles to stretch [3]. In contrast, alpha-MNs help innervate the force-generating extrafusal muscle fibers at neuromuscular junctions (NMJ) [4]. Any disruption in the connection between these alpha-MNs and skeletal muscles may cause deficient contraction and weakness as seen, for example, in myasthenia.

Myasthenic disorders are a group of NMJ diseases that have diverse pathogenesis, ranging from genetic to autoimmune mechanisms [5,6]. For instance, congenital myasthenic syndromes (CMS) are inherited disorders caused by mutations in genes essential for the formation and/or development of the NMJ [7,8]. CMS are rare and under-diagnosed disorders that are associated with severe alterations in neuromuscular impulse transmission. This alteration often triggers the characteristic skeletal muscle weakness and fatigability of CMS. Correspondingly, CMS can cause significant disability to patients and can even be fatal [6]. CMS are divided into three different categories based on the location of the affected protein within the NMJ. These include presynaptic, synaptic, and postsynaptic mutation proteins [8–10]. In regard to CMS with presynaptic cause, the protein defects can disrupt acetylcholine (ACh) release from the nerve terminals [8,11,12].

Motoneurons release the neurotransmitter ACh at the vertebrate NMJ to trigger skeletal muscle contraction [13]. ACh is synthesized by the enzyme choline acetyltransferase (ChAT) and then stored into releasable vesicles by the vesicular ACh transporter (VACHT) (reviewed by Prado *et al.* [14]). ACh synthesis and release are crucial for the formation and development of the NMJ as well as for muscle fibers. ACh also contributes to the centralization of NMJ at the end plate and determines the correct site of synapses in muscles [15,16]. Decreased VACHT expression, which leads to reduced ACh storage and release, impairs muscle contractility [17,18]. Mutations in the *VACHT* gene were recently identified in humans displaying varying degrees of myasthenia [19–21].

As a result, mouse models with altered ACh release are important tools to investigate the morphological, physiological, and behavioral consequences of reduced

cholinergic transmission. One example is the VACHT-KD model, where mice have a global decrease in VACHT expression [18]. VACHT-KD mice are myasthenic and display neuromuscular alterations [17,22,23], as well as cognitive impairments [17], visceral organ dysfunctions [24–26], and increased inflammatory responses [27].

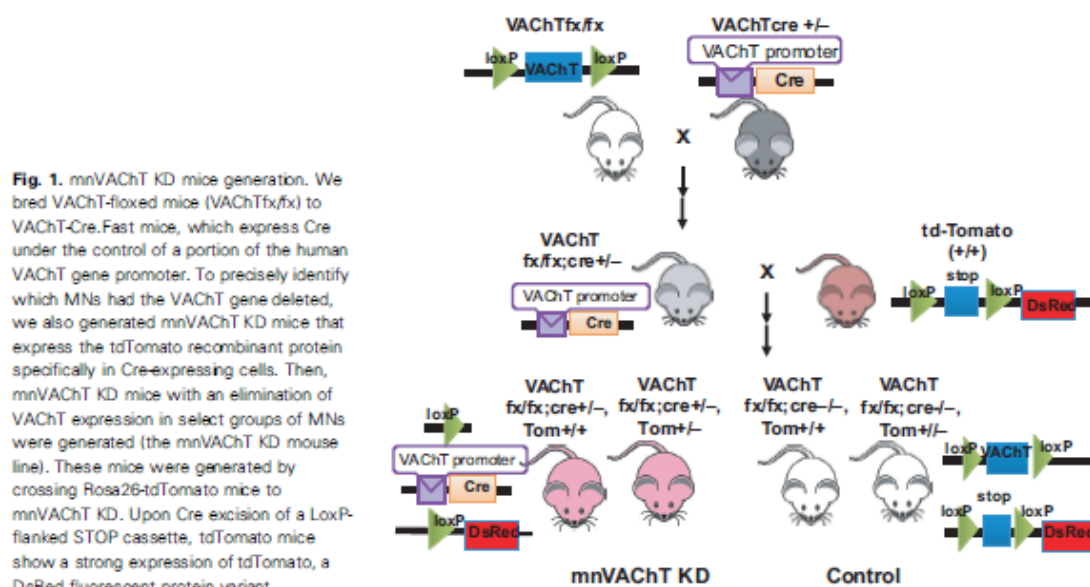
In this study, we generated a mouse model with decreased VACHT expression specifically in MNs, to investigate how a long-term decrease of VACHT expression influences motor function *in vivo*. We find that these mice present with a loss of muscle mass and strength that mimics CMS. Consequently, we suggest that this specific transgenic mouse model could be a valuable tool for assessing the efficacy of therapeutic drugs in treating myasthenia-like pathology.

Results

Generation of mice with VACHT deletion restricted to a group of motoneurons

To generate mice with cholinergic deficiency restricted to a select group of MNs, we used Cre-loxP technology. Specifically, we bred VACHT-floxed mice [28] to VACHT-Cre.Fast mice, which express Cre under the control of a portion of the human VACHT gene promoter [29]. As the promoter region is not complete, Cre expression in VACHT-Cre.Fast mice is restricted to a subset of cholinergic neurons in the somatomotor nucleus, but is absent in visceromotor and other central and peripheral cholinergic neurons [29]. To identify which MNs express the Cre enzyme in these VACHT-mutant mice (VACHT^{flx/flx;Cre+/-} mice, Fig. 1), we crossed them to tdTomato reporter mice. The tdTomato reporter allele has a loxP-flanked STOP cassette that prevents transcription of the downstream reporter protein in cells that do not express Cre. In Cre-expressing cells, the STOP cassette is deleted and strong expression of tdTomato, a DsRed fluorescent protein, is observed. VACHT-mutant mice expressing tdTomato were named mnVACHT KD.

Analysis of VACHT^{flx/wt;Cre+} × VACHT^{flx/wt} intercrosses revealed that the recovery of VACHT^{flx/flx;Cre+} pups did not follow a Mendelian frequency. Out of 100 pups born from 13 litters, 60 (60%) were heterozygous (VACHT^{flx/wt;Cre+} or VACHT^{flx/wt}) 28 (28%) were wild-type (WT; VACHT^{wt/wt;Cre+} or VACHT^{wt/wt}), and 12 (12%) were homozygous (VACHT^{flx/flx;Cre+} or VACHT^{flx/flx}), instead of the expected 50%, 25% and 25% ratios, respectively. The observed reduced frequency of homozygous mice (12% instead of 25%) suggested that VACHT deficiency affected survival of



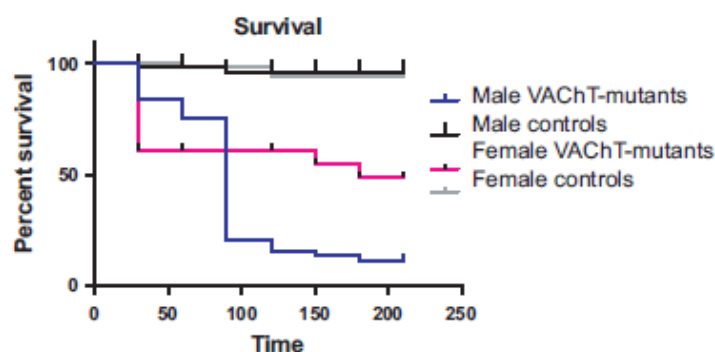
embryos. Notably, male mice had a significantly higher mortality rate compared to females. Specifically, while only 11% of VAcHT-mutant males (VAcHT^{flox/flox;cre+}) were alive after 210 days (total period evaluated), 55% of VAcHT-mutant females were alive (Fig. 2; $\chi^2(3) = 98.91$, $P < 0.0001$).

To investigate whether Cre is selectively expressed in cholinergic MNs of the mnVAcHT KD mice, we performed immunofluorescence analysis in the whole brain, brainstem, and spinal cord regions. Cre expression was assessed by the presence of DsRed fluorescence (tdTomato) while cholinergic cells were identified by ChAT staining. Sagittal and coronal slices revealed minimal colocalization between ChAT and tdTomato in brain areas such as the striatum, cortex, and

hippocampus (Fig. 3A,B). A small number of non-cholinergic cells exhibited Cre expression (Fig. 3A); however, this small ectopic Cre expression does not interfere with our analysis. Colocalization between tdTomato and ChAT in brainstem motor nuclei was approximately 49% (Fig. 3A). In the spinal cord, tdTomato was present in approximately 55% of cholinergic neurons in the cervical segments and 52% in the lumbar segments (Fig. 3A,B). These results indicate that Cre is primarily expressed in MNs of mnVAcHT KD mice.

Immunoblot analysis confirmed that in these mutant mice, VAcHT protein is preserved in brain areas (striatum and cortex) while it is decreased in the brainstem and spinal cord (Fig. 4A–E). Specifically, there was no

Fig. 2. Survival curve considering male and female VAcHT mutants. Percent of survival among the time, during ~ 250 days. Approximately 80% of male VAcHT mutants and 40% of female VAcHT mutants died within 100 days. No differences were seen between male and female controls with ~ 100% of survival during the total number of days analyzed.



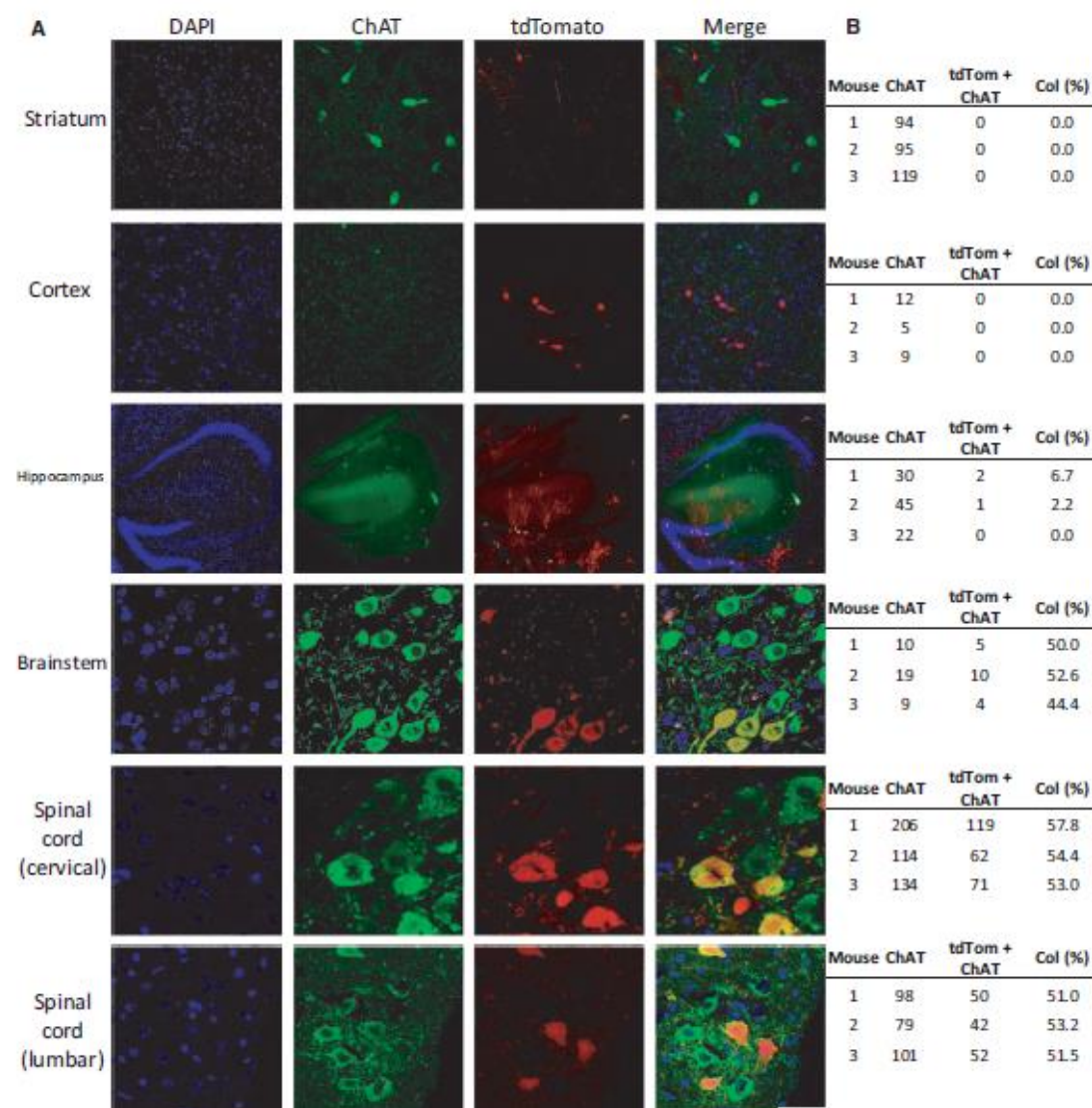


Fig. 3. The mnVAcHt KD mice have Cre expression and VAcHt deletion specifically in MNs. (A) Immunostained sections for DAPI (blue), ChAT (green) and tdTomato (red) in mnVAcHt KD- mice. (B) Quantitative analysis of colocalization between ChAT staining and tdTomato in the striatum, cortex, hippocampus, and brainstem from three different sagittal sections, and in the anterior horn of spinal cord cervical and lumbar segments from three different transversal sections. $N = 3$ individual animals. Scale bar: 100 μm .

change in VAcHt expression in the striatum (control: 99.3 ± 14.4 versus mnVAcHt KD: 85.0 ± 16.8 ; $P = 0.2424$) and cortex (control: 100.0 ± 13.7 versus mnVAcHt KD: 97.4 ± 16.4 ; $P = 0.4686$). In the brainstem, we observed a $\sim 40\%$ decrease in VAcHt expression (control: 105.5 ± 8.6 versus mnVAcHt

KD: 61.8 ± 13.1 ; $P = 0.0206$); a $\sim 30\%$ decrease in the spinal cord cervical region (control: 101.7 ± 6.2 versus mnVAcHt KD: 70.5 ± 13.5 ; $P = 0.0465$); and a $\sim 50\%$ decrease in the spinal cord lumbar region (control: 109.7 ± 17.7 versus mnVAcHt KD: 50.3 ± 16.2 ; $P = 0.0476$). Together, these data confirm

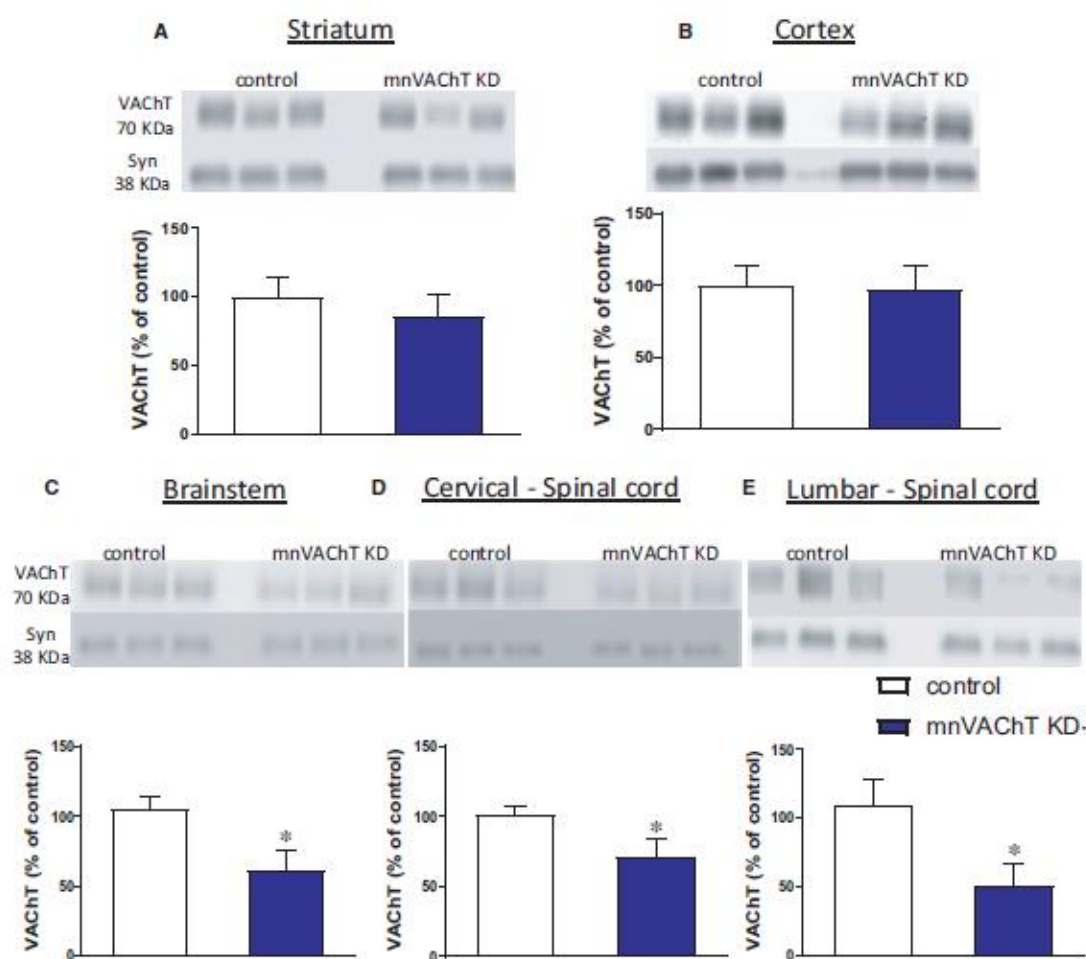


Fig. 4. The mnVACHT KD mice have VACHT reduction specifically in motor areas. Representative immunoblot and quantitative analysis of VACHT expression in the (A) striatum, (B) cortex, (C) brainstem, (D) cervical spinal cord segments, and (E) lumbar spinal cord segments of control (VACHT^{flx/flx}-Tomato) and mnVACHT KD mice. Data are shown as mean ± SEM; *N* = 6 animals per group; **P* < 0.05; Student's *t*-test.

that VACHT was selectively deleted on most MNs and was preserved in brain cholinergic neurons.

VACHT deletion in alpha-motoneurons

We then investigated if Cre was equally expressed on different MN subtypes. It has been shown that ostepontin (OPN) is an alpha MN marker in the mouse spinal cord whereas the transcription factor ERR is a gamma MN marker [2,30,31]. Thus, we used immunofluorescence to determine the MN subtype that expressed the Cre-recombinase and therefore had undergone VACHT deletion. The analyses revealed

that in both, cervical and lumbar spinal cord segments, ~ 74% and 69% respectively, tdTomato MNs colocalized with OPN labeling (for alpha-MNs). Conversely, we could not detect colocalization between tdTomato and ERR labeling (Fig. 5A–C). Collectively, these analyses revealed that mnVACHT KD mice showed VACHT deletion mainly at alpha-MNs.

MNs with VACHT deletion show reduced soma area

We then investigated whether the absence of VACHT expression influences the morphology of alpha-MNs.

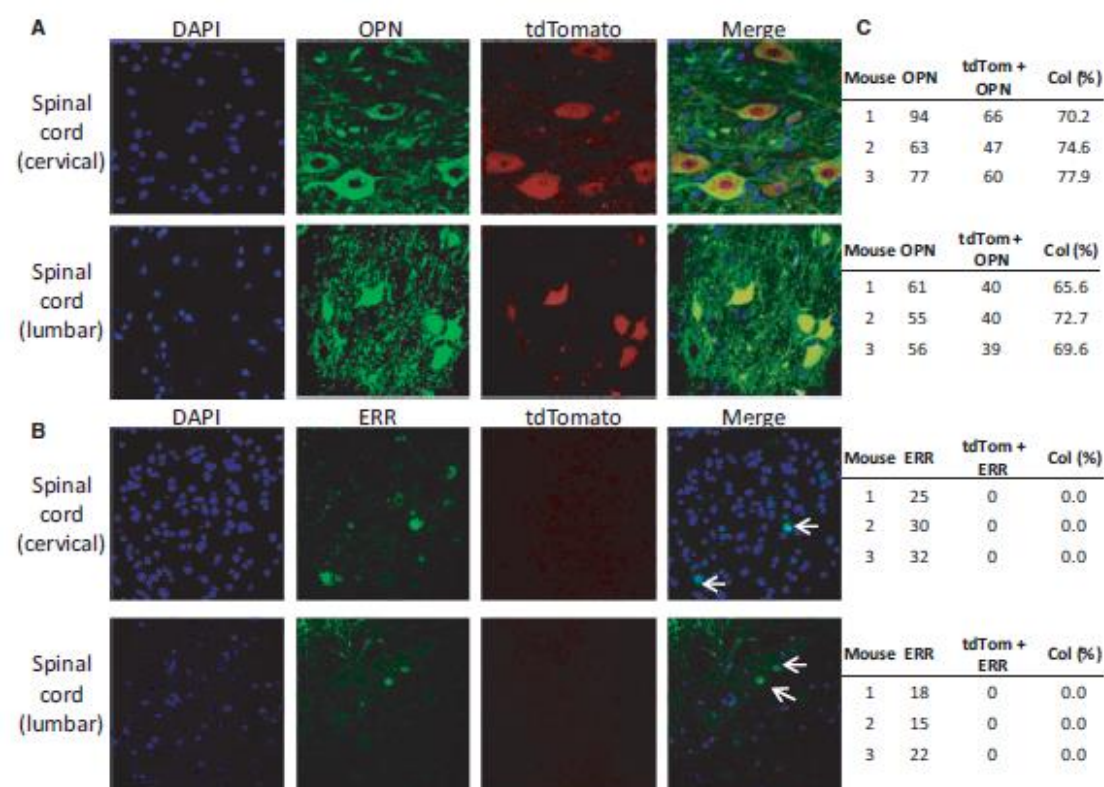
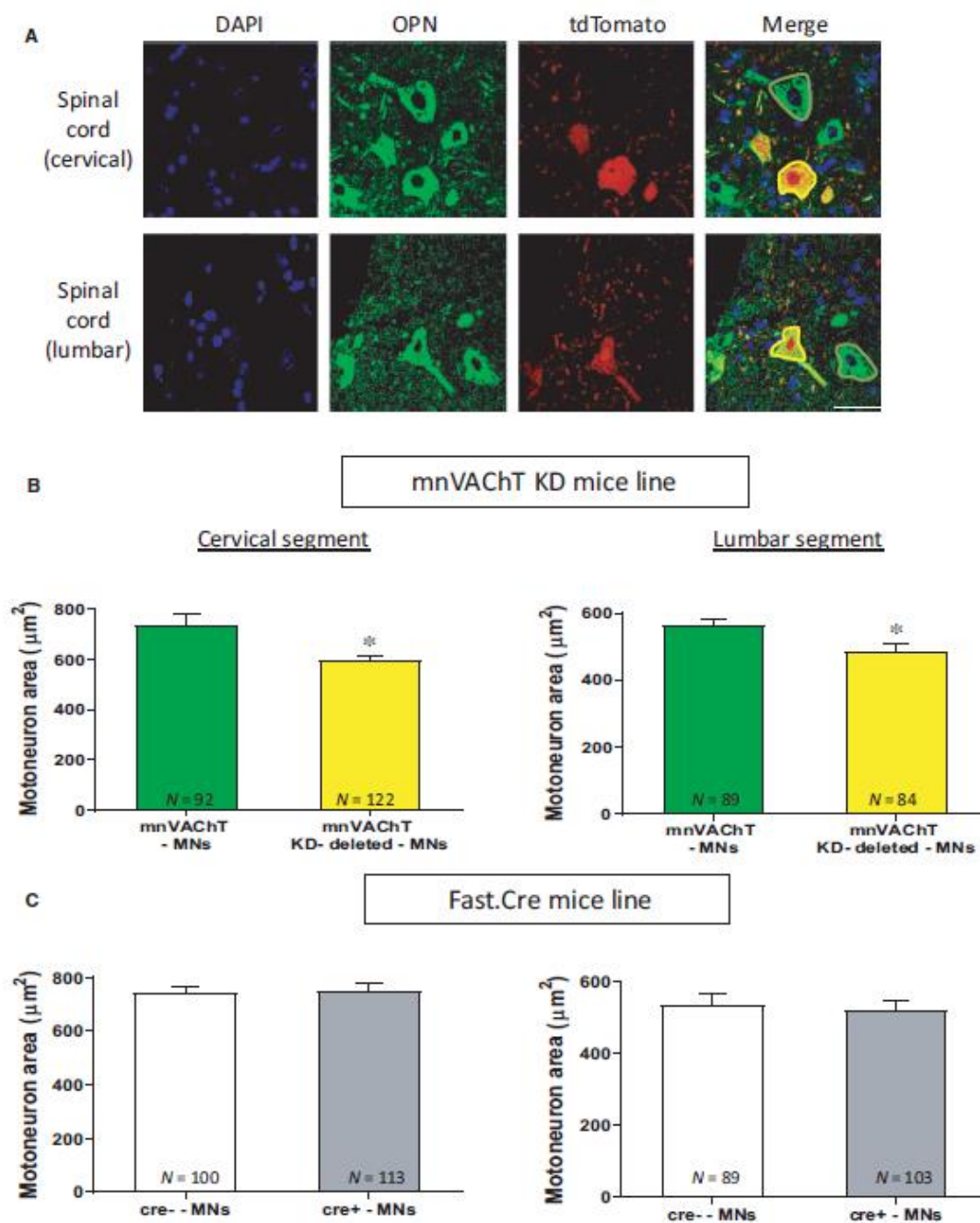


Fig. 5. The mnVAcHt KD animals have Cre preference and VAcHt deletion in alpha-MNs. Spinal cord immunostained sections for DAPI (blue), (A) OPN-alpha marker or (B) ERR-gamma marker (green) and tdTomato (red) in mnVAcHt KD mice. (C) Quantitative analysis of colocalization between OPN or ERR staining and tdTomato in the anterior horn of cervical and lumbar spinal cord segments from 3 different transversal sections. The arrows show gamma MNs. Data are shown as mean $\pm$ SEM; $N = 3$ individual animals; Student's t -test. Scale bar: 100 μ m.

Within the same animal (the mnVAcHt KD- mouse), the soma area from OPN and DsRed-double-stained MNs (mnVAcHt KD- deleted - MNs) was measured and compared to that of OPN-labeled MNs that did not show DsRed fluorescence (mnVAcHt - MNs). Both at the cervical and at lumbar segments of the spinal cord, the soma area of mnVAcHt KD- deleted - MNs was significantly reduced when compared with mnVAcHt - MNs (cervical segment; mnVAcHt -

MNs: 740.5 ± 37.8 versus mnVAcHt KD- deleted - MNs: 596.3 ± 18.3 ; $P = 0.0215$; and lumbar segment; mnVAcHt - MNs: 562.5 ± 21.7 versus mnVAcHt KD- deleted - MNs: 487.9 ± 22.4 ; $P = 0.0228$; area in μ m², Fig. 6A,B). Importantly, the reduction in size of alpha-MNs was not due to Cre presence, as Fast.Cre line mice, which express Cre on MNs but did not have the VAcHt gene deleted, did not show alpha-motoneuron size differences when compared to the

Fig. 6. Alpha-motoneuron atrophy in the mnVAcHt KD animals. (A) Spinal cord immunostained sections for DAPI (blue), OPN (green), and tdTomato (red) in mnVAcHt KD mice. The colocalization between the labeling is shown in yellow. Quantitative analysis of motoneuron area in cervical and lumbar spinal cord segments in (B) mnVAcHt KD mouse line and (C) Fast.Cre mouse line. The areas delimited by the green color indicate MNs with VAcHt (mnVAcHt - MNs) and those with the yellow color indicate MNs that had VAcHt deletion (mnVAcHt KD- deleted - MNs). Data are shown as mean $\pm$ SEM; The N value for the total number of MNs analyzed is displayed in the respective bar graph, from 4 different animals per group (~ 20 images analyzed from three different sections); * $P < 0.05$; Student's t -test. Scale bar: 100 μ m.



respective control mice (Fig. 6C). In all, our results indicate that a reduction in VAcHT leads to alpha-motoneuron atrophy.

Muscle fibers innervated by MNs without VAcHT expression show signs of atrophy

As alpha-MNs control muscle activity, we investigated the effect of VAcHT deletion on skeletal muscles of mnVAcHT KD mice. We chose three different muscles for the analyses which display differences in their biochemistry and are innervated by MNs from distinct segments of the spinal cord: sternomastoid (STM), tibialis anterior (TA), and soleus (SOL; STM is hybrid fast, TA is fast glycolytic, and SOL is slow oxidative muscle [32]). Synaptophysin (Syn) immunofluorescence was used to visualize the presynaptic terminal in all muscle fibers and then make the cross-sectional area quantification. TdTomato fluorescence was used to identify muscles fibers innervated by MNs with VAcHT deletion. We observed that in the same animal (the mnVAcHT KD mouse), the cross-sectional area of myofibers was decreased when they were innervated by MNs with a VAcHT deletion (mnVAcHT KD- deleted - myofibers) compared to MNs with VAcHT preservation (mnVAcHT-myofibers), Fig. 7A. A statistical difference was observed in all muscles analyzed: STM: $P < 0.0001$; TA: $P < 0.0001$ and SOL: $P = 0.0490$, Fig. 7B). The frequency distribution of the cross-sectional area is displayed in Fig. 7C. We suggest that the reduction in muscle fibers could be associated with a lack of ACh release.

Neuromuscular junction analyses in mnVAcHT KD mice

NMJs from VAcHT-KD mice were pre- and postsynaptically stained with antisynaptotagmin and α -btX, respectively, to assess the size of these NMJ. Fig. 8A, 8B, and 8C shows representative images of presynaptic nerve terminals (green), postsynaptic ACh receptors (AChRs) stained with Alexa 555 α -btX (blue), and the merge of both green and blue signals for STM muscle. Fig. 8D, 8E, and 8F shows the same labeling for TA muscle. Fig. 8G–I shows the images considering SOL muscle. In red, we can observe tdTomato signal at the nerve fibers. Finally, Fig. 8J–L shows the graphical quantification of the analysis of the pre- and postsynaptic areas of the NMJ of the STM, TA, and SOL muscles. Our results showed that the pre- and postsynaptic areas of the STM and SOL muscles did not differ between the deleted NMJ (mnVAcHT KD- deleted

- NMJ) and not deleted (mnVAcHT - NMJ), in the same animal. However, for the TA muscle, it was observed that there is an increase in the size of the pre- and postsynaptic areas of the deleted NMJ when compared to the nondeleted NMJ, in the same animal: presynaptic [mnVAcHT - NMJ: 488.2 ± 236.4 versus mnVAcHT KD- deleted - NMJ: 746.3 ± 239.6 ; $P = 0.0037$; area in μm^2 , median $\pm$ standard deviation (SD)] and postsynaptic (mnVAcHT - NMJ: 508.1 ± 235.7 versus mnVAcHT KD- deleted - NMJ: 773.5 ± 243.6 ; $P = 0.0051$; area in μm^2 , median $\pm$ SD, see Fig. 8K).

mnVAcHT KD mice display motor dysfunctions

We investigated muscle function in two different cohorts of mnVAcHT KD mice using the wire-hang, grip-force, and open-field tests. Two-month-old mnVAcHT KD mice showed deficits in the wire-hang test which were more pronounced in 6-month mutants (Fig. 9A, 2-month-old cohort; control: 59.4 ± 0.3 versus mnVAcHT KD: 47.1 ± 4.2 ; $P = 0.0077$; and 6-month-old cohort; control: 58.9 ± 0.9 versus mnVAcHT KD: 22.0 ± 6.3 ; $P < 0.0001$). Similar progressive deficits were observed when mice were analyzed in the grip-force test (Fig. 9B, 2-month-old cohort; control: 89.4 ± 5.7 versus mnVAcHT KD: 69.1 ± 4.6 ; $P = 0.0075$; and 6-month-old cohort; control: 88.7 ± 3.6 versus mnVAcHT KD: 37.2 ± 3.9 ; $P < 0.0001$). Locomotor activity of two-month-old mutants did not differ from controls; however, 6-month-old mutants showed a significant decrease in movement (Fig. 9C, 2-month-old cohort; control: 3295 ± 380 versus mnVAcHT KD: 3969 ± 449 ; $P = 0.1150$; and 6-month-old cohort; control: 3559 ± 290.4 versus mnVAcHT KD: 1484 ± 365.9 ; $P = 0.0002$). Interestingly, at 2 months of age rearing activity was already significantly decreased in the mnVAcHT KD mice analyzed (Fig. 9D, 2-month-old cohort; control: 225 ± 19 versus mnVAcHT KD: 30 ± 10 ; $P < 0.0001$; and 6-month-old cohort; control: 151 ± 21 versus mnVAcHT KD: 33 ± 10 ; $P = 0.0007$). To note, the body weight of two-month-old mutants did not differ from controls while 6-month-old mutants were significantly leaner than controls (Fig. 9E, 2-month-old cohort; control: 20.0 ± 0.5 versus mnVAcHT KD: 19.4 ± 0.5 ; $P = 0.3106$; and 6-month-old cohort; control: 23.3 ± 0.8 versus mnVAcHT KD: 19.2 ± 0.3 ; $P = 0.0004$). Figure 9F,G shows the horizontal locomotor activity over time. Additionally, we found that at 6 months of age we were able to observe kyphosis in the mnVAcHT KD-deleted mice (Fig. 9H).

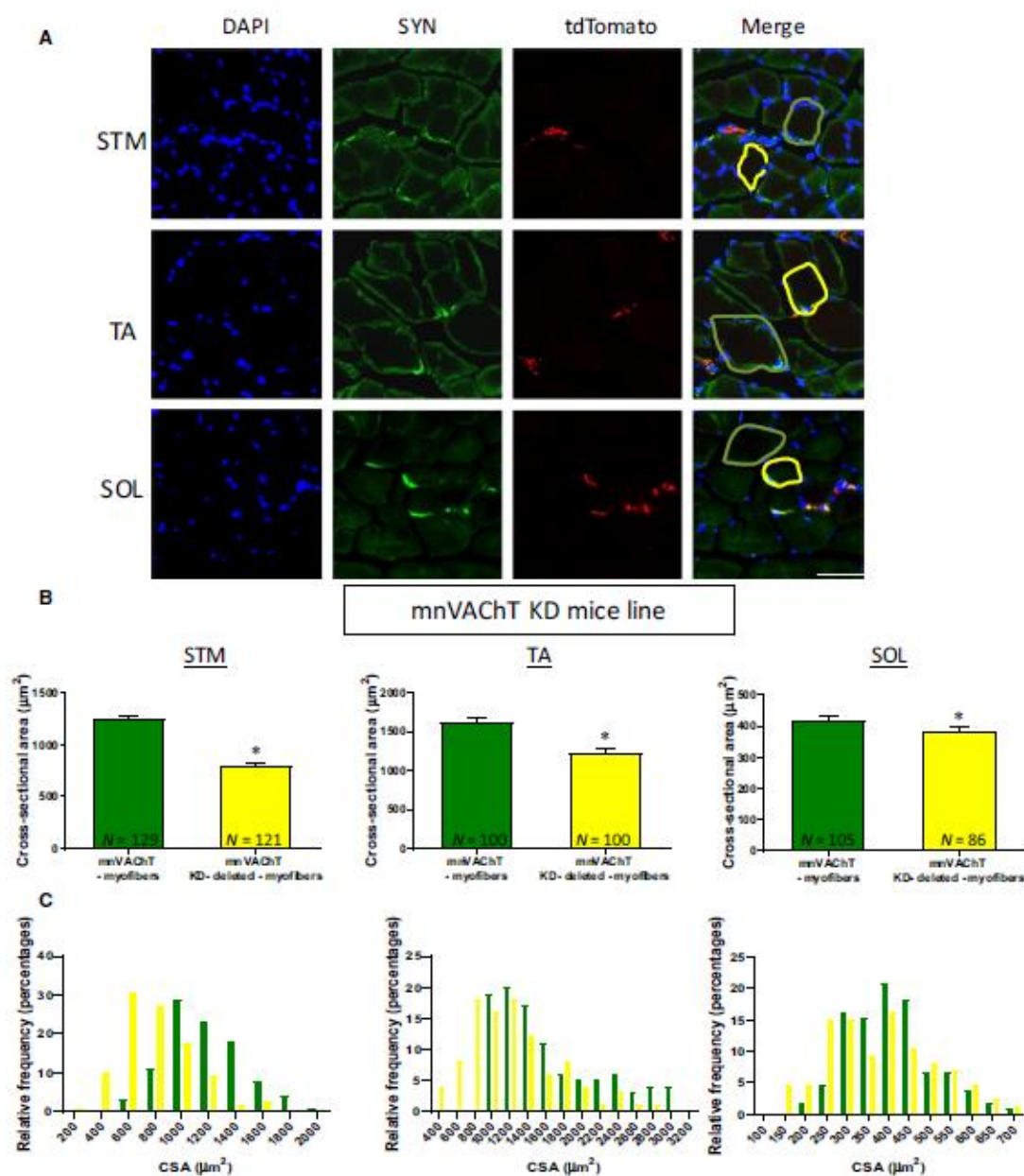


Fig. 7. Muscle atrophy in the mnVAcHt KD animals. (A) Muscle immunostained sections for DAPI (blue) Syn (green) and tdTomato (red) in mnVAcHt KD mice. The colocalization between the labeling is shown in yellow. (B) Quantitative analysis of muscle cross-sectional area in STM, TA, and SOL muscles. The areas delimited by the green color indicate myofibers with VAcHt (mnVAcHt - myofibers) and those with the yellow color indicate myofibers that had VAcHt deletion (mnVAcHt KD-deleted - myofibers). (C) Frequency distribution of the cross-sectional area. Data are shown as mean $\pm$ SEM; The *N* value for the total myofibers area analyzed is displayed in the respective bar graph, from four different animals per group ($\sim$ 20 images analyzed from three different sections); **P* < 0.05; Student's *t*-test. Scale bar: 100 μ m. CSA, cross-sectional area.

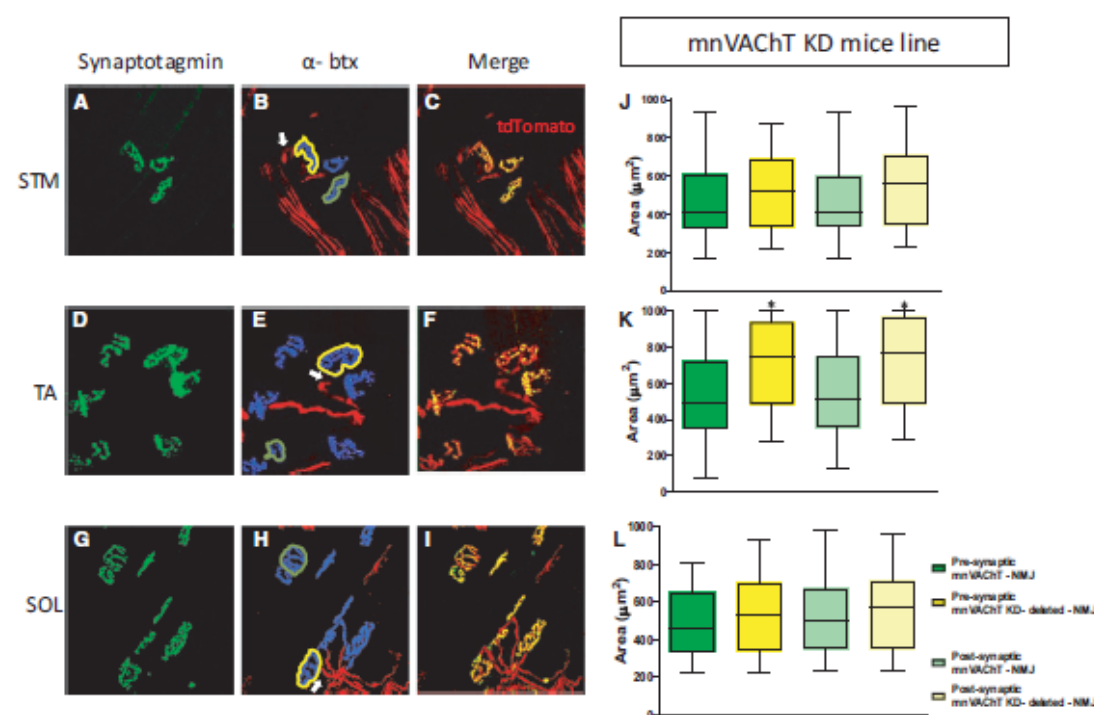


Fig. 8. The neuromuscular junction (NMJ) areas in mnVAcHT KD animals. (A–I) Representative NMJs images of STM, TA, and SOL muscles. Presynaptic terminals labeled with an Alexa-488 Synaptotagmin antibody (green). Postsynaptic AChRs labeled with Alexa-555 α-btx (blue). In these images, the white arrows indicate where the VAcHT was deleted (nerves in red marked with tdTomato signal). The areas delimited by the green color indicate NMJ with preserved VAcHT (mnVAcHT - NMJ) and those with the yellow color, indicate NMJ that had VAcHT deletion (mnVAcHT KD-deleted - NMJ). Scale bar: 50 μm. (J–L) Graphical quantification of the pre- and postsynaptic areas for STM, TA, and SOL muscles (presynaptic: * $P = 0.0037$; postsynaptic: * $P = 0.0051$; Mann–Whitney test); the results represent the median $\pm$ SD from 25 NMJs per animal. $N = 4$ individual animals.

Cholinesterase inhibitor treatment improves muscle strength in mutant mice

To determine whether we could rescue/improve the neuromuscular deficits observed in mnVAcHT KD-deleted mice, we treated 6-month-old mutants with donepezil, a noncompetitive reversible cholinesterase inhibitor. Figure 10A shows a timeline for the treatment. Treatment with donepezil improved muscle strength in the grip-force test and also the number of rearings in the open-field test. We found that the transgenic mice exhibited significant improvements in the grip-force test ($P = 0.0011$) and in rearing count ($P = 0.0228$). However, we found that locomotor activity was unaltered following drug treatment, as seen in Fig. 10B, for grip-force; Fig. 10C, for distance traveled and 10D, for rearings. Figure 10E–G shows the final values analyzed at day 5 after donepezil treatment. It was observed significant difference when

comparing animals treated with saline versus donepezil considering maximal strength ($P = 0.0046$) and number of rearing ($P = 0.0429$). Figure 10H–J displays a line graph showing individual data for each animal in baseline and after 5 days treatment with donepezil.

Discussion

The present study uses a novel mouse model which has a selective deletion of VAcHT in motor neurons (mnVAcHT KD line mice) to provide a detailed analysis of the neuromuscular changes that occur following long-term cholinergic deficiency in MNs. We show that in mnVAcHT KD mice, VAcHT protein was reduced in the brainstem and in alpha-MNs from the spinal cord but was preserved in other cholinergic brain regions. The reduction in VAcHT expression affected

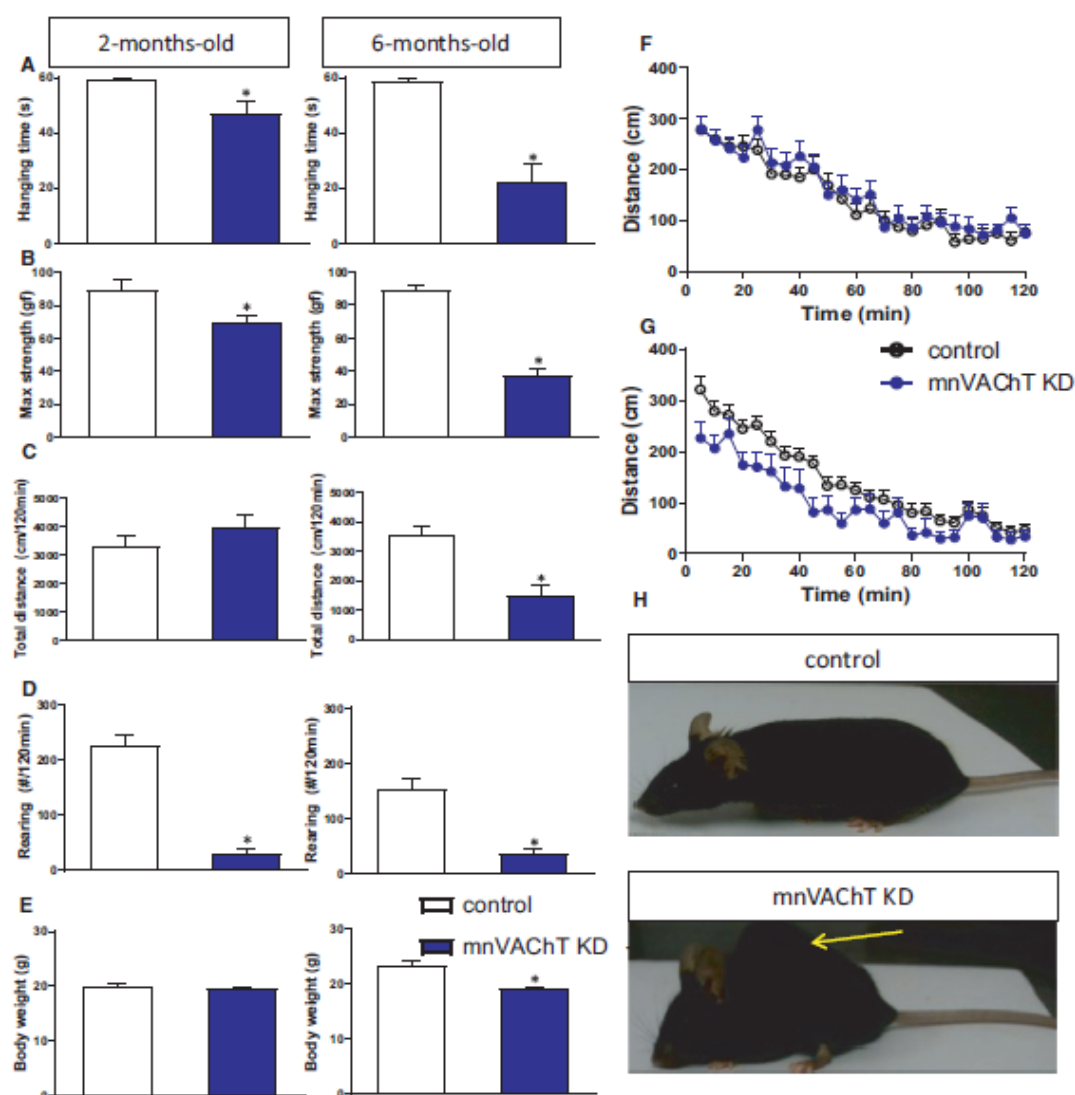


Fig. 9. The mnVAcHT KD animals have *in vivo* neuromuscular dysfunction. Control (VAcHTflox/flox-Tomato) and mnVAcHT KD mice were assessed on a battery of behavioral tests including (A) wire-hang, (B) grip-force, (C) locomotor activity, (D) rearing at 2 or 6 months. (E) Body weight at 2 and 6 months. Horizontal locomotor activity in the open-field test at (F) 2 months and (G) 6 months. (H) Physical appearance of control and mutant animals. The mnVAcHT KD mouse was leaner than the control and showed a hunched posture (arrow). Data are shown as mean $\pm$ SEM; $N = 16$ and $N = 15$ animals per group at 2 months of age; $N = 24$ and $N = 14$ animals per group at 6 months of age; * $P < 0.05$; Student's *t*-test.

both alpha-MNs and muscle structures. However, we do not detect statistical alterations considering the NMJs area (in pre- or postsynaptic terminals), except for an increase in TA NMJ. Mutant mice showed

neuromuscular deficits that were evident at 2 months of age and progressively worsened. Importantly, treatment with the cholinesterase inhibitor donepezil significantly ameliorated the behavioral deficits.

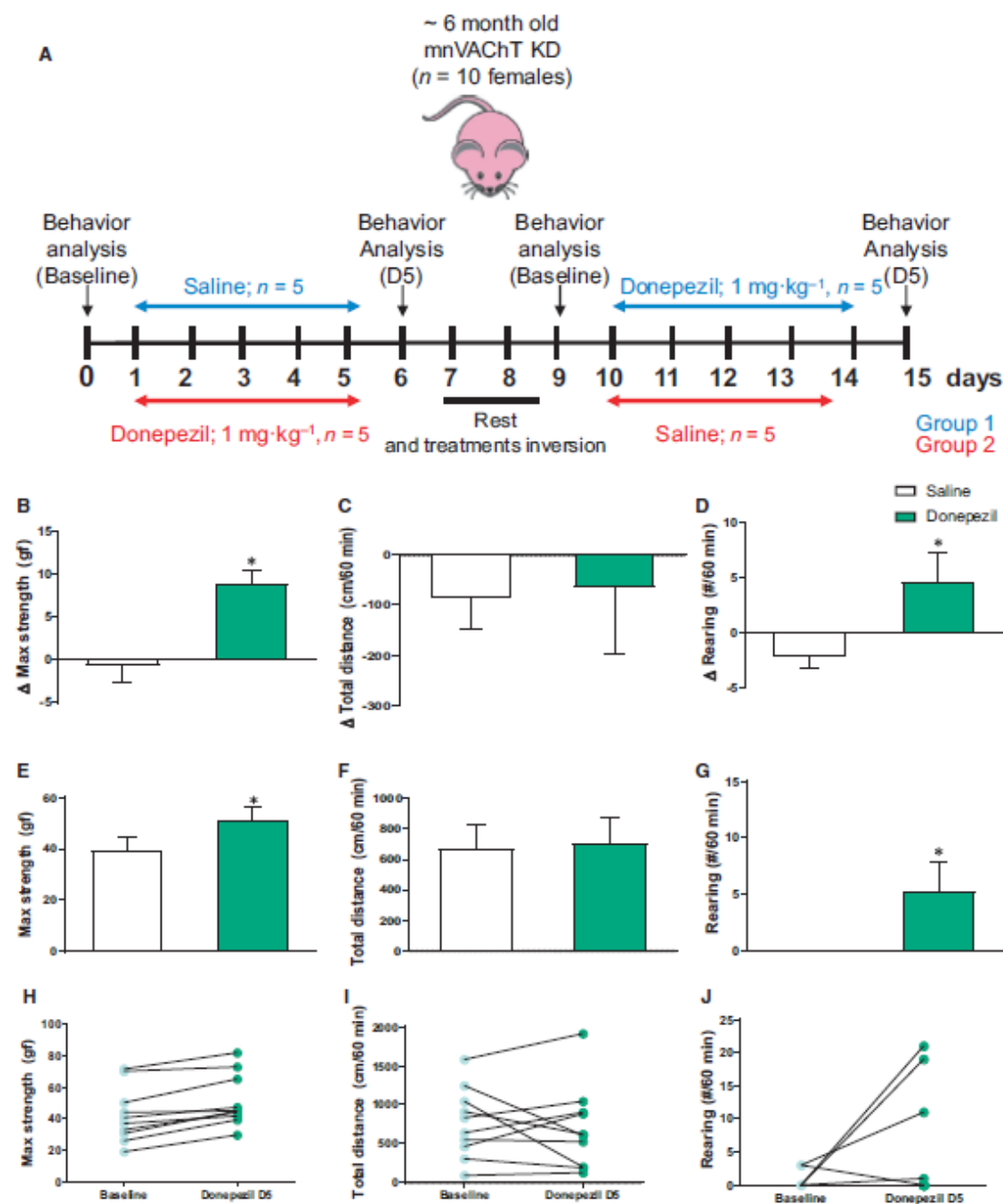


Fig. 10. Cholinesterase inhibitor treatment in the mnVAcHt KD animals. (A) Timeline showing the groups and injections of saline or donepezil (1 mg·kg⁻¹). Mice were assessed on grip-force, locomotor activity, and rearing after intraperitoneal injections of saline or donepezil. (B–D) Variation (Δ) between day 5 of injection and day 1. (E–G) Final values at day 5 comparing saline and donepezil groups. (H–J) Line graph showing individual data for each animal in baseline and after 5 days treatment with donepezil (D5). Data are shown as mean ± SEM; *N* = 10 animals; **P* < 0.05; Student's *t*-test.

Although the global VAcHT-KD homozygous mice [17], which also show neuromuscular deficits, can be used as a tool to study the impact of ACh release on neuromuscular function, the systemic effects observed in VAcHT-KD homozygous mice, including renal and cardiac deficiencies [24,25], are major confounders for the analysis of behavioral deficits. As VAcHT expression is abolished selectively in alpha-MNs, neuromuscular deficits can be clearly ascertained in the mnVAcHT KD mouse model. Similar to previous reports on other mouse lines with deficient cholinergic transmission [17,18,33], mnVAcHT KD showed muscular damage. Noteworthy, mnVAcHT KD mice individually have MNs that lack VAcHT expression as well as MNs with intact VAcHT levels. We found that muscle fibers innervated by MNs lacking VAcHT are smaller in comparison with muscle fibers innervated by VAcHT-expressing MNs. These results support previous findings which show that a reduction in cholinergic neurotransmission on motor plates promotes muscle fiber atrophy [34]. In particular, it is possible that the deficiency in cholinergic signaling decreases muscle activity and that leads to muscle fiber atrophy. Relatedly, mice with a global decrease in VAcHT expression exhibit downregulation of some genes important for skeletal muscle cell differentiation and function [35]. These molecular changes could be responsible for the observed atrophy in these global VAcHT-KD mice. In our present investigation, considering a specific-deficient cholinergic model (mnVAcHT KD mice), we observed a reduction in MNs soma size. It has been previously shown that cholinergic tone is essential to maintaining cellular structures and organization. It was demonstrated that mice with a global increase in cholinergic tone exhibit a significant enlargement in the size of striatal cholinergic interneurons [36]. This study used mice expressing the channelrhodopsin-2 (ChR2) protein under the control of the ChAT promoter (ChAT-ChR2) [36]. This caused mice to have extra copies of the VAcHT gene and therefore an increase in cholinergic tone (a hypercholinergic phenotype). VAcHT overexpression profoundly altered the shape, organization, and distribution of VAcHT in subcellular compartments (promoting ultrastructural remodeling) [36]. Based on this concept that cellular configuration is influenced by VAcHT, it is plausible to suggest that ACh influences intracellular organization. In our present study, we found that our cholinergic deficient mouse model (mnVAcHT KD) had a reduction in the soma size of MNs. It is possible that a lack of VAcHT induced an intracellular redistribution of

organelles which led to changes in MN soma size. Further studies at the ultrastructure level are required to elucidate the exact relationship between VAcHT and intracellular structures of MNs.

In our experiments, despite the atrophy of STM, TA, and SOL myofibers and reduction of MNs soma in mnVAcHT KD- deleted cells, there is a trend toward an increase in the area of NMJs for STM and SOL, and a significant increase in NMJ area in TA (see Fig. 8). This is in line with previous reports using an alternative model of reduced cholinergic activity in murine embryos after deletion of ChAT enzyme [16,33]. Moreover, increased muscle innervation was further observed with pharmacological intervention by others [34,37–40]. An increase in NMJ area might be associated with increased innervation branching and fasciculation, and there is evidence that preterminal AChRs regulate nerve branching *in vitro* [41]. It is also plausible that this enlargement of TA muscle NMJs might be a compensation for a possible reduced ACh release due to VAcHT deletion in MNs in mnVAcHT mouse line.

When examining muscle strength in our mutant mouse line, we found that mnVAcHT KD mice have muscle weakness as early as 2 months of age—observed as deficits in the wire-hang, grip-force as well as decreased rearing behavior in locomotor boxes. Although rearing activity is frequently used as an exploratory behavior parameter [42], the decreased rearing in mnVAcHT KD mice likely reflects the fact that mutant mice have difficulties in standing up on both hind paws, in a vertical upright posture. This difficulty can be attributed to the observed atrophy of the hind limb TA and SOL muscles. Notably, the SOL muscle is an important postural muscle which has a higher degree of resistance to fatigue due to its preponderance of oxidative type 1 and 2A fibers [43]. It allows the maintenance of an upright posture related to its function as an anti-gravity muscle [44].

Importantly, we showed that treatment with the cholinesterase inhibitor donepezil was able to ameliorate some motor phenotypes. Although donepezil treatment did not significantly improve general locomotor activity, it did improve muscle strength measured by the grip-force task and by rearing activity. Taking into consideration the main mechanism of action that donepezil has, we propose that this drug was capable of maintaining ACh levels in the synaptic cleft through the inhibition of cholinesterases. Thus, a higher level of ACh would be available to interact with the receptors of the muscle membrane, thereby triggering muscle contraction.

Future studies will be necessary to test whether a longer treatment could be more effective and whether earlier interventions would prevent neuromuscular deficits. Likewise, it will be important to compare the effect of other cholinesterase inhibitors (for instance rivastigmine, galantamine, or pyridostigmine) to that of donepezil. Notably, donepezil is a noncompetitive acetylcholinesterase inhibitor, with long-lasting activity and a higher selectivity for acetylcholinesterase as compared to butyrylcholinesterase [45]. Moreover, pyridostigmine has no central action as it does not cross the blood–brain barrier [46].

In short, mnVACHT KD mice, because of their unique, selective, and specific VACHT deletion in alpha-MNs, offer some advantages for the investigation of neuromuscular deficits caused by cholinergic dysfunction including (a) allows the investigation of the effects ACh loss in neurons and/or muscles using the same animal as a control because not all neurons will be VACHT-depleted; (b) as Cre expression does not depend on a viral injection, the results obtained from mnVACHT KD mice do not have viral-induced inflammation; (c) there are no confounder behavioral effects from general cholinergic deficiency; and (d) mice develop normally and survive for a long period of time, allowing for longitudinal studies.

Misawa [29] showed that in somatomotor neurons Cre-mediated recombination is often not 100%. Consequently, it is possible that the surviving mice still have some ACh release in motor neurons. Cre expression in the mice may be marginal and therefore not enough to trigger a complete recombination (100%) of the loxP-flanked target DNA. Furthermore, the number of cells that undergo Cre recombination follows a sigmoidal curve as a function of time, which can ultimately reach a saturable level. We also cannot exclude the fact that some genes can be subjected to random allelic inactivation, including inactivation of the Cre construct. Nonetheless, an intrinsic property of the allele cannot be discarded. Considering these possibilities, the Cre recombination frequency not reaching 100% is presumable.

In summary, mnVACHT KD mice exhibit a progressive impairment of neuromuscular function induced by a precise reduction in ACh release in alpha-MNs. The alterations in the synapses caused by a lack ACh release and the subsequent muscle defects resemble some of the features described in myasthenic patients [6–8]. Importantly, neuromuscular deficits could be ameliorated by treatment with donepezil. Thus, these mutant mice represent a valuable model to assess the efficacy of potential therapeutic compounds or repair strategies for CMS with a presynaptic cause.

Materials and methods

Animals

All experiments followed the Canadian Council of Animal Care guidelines for care and use of animals, and the animal protocol approved by the University of Western Ontario (protocols# 2016-103 and 2016-104). The generation of VACHT^{fllox/flox} mice has been previously described [28], and the behavior of these mice does not differ from WT littermates [28]. Mice with an elimination of VACHT expression at selected groups of MNs (mnVACHT KD) were generated by breeding VACHT^{fllox/flox} mice with VACHT-Cre.Fast mice [Tg(SLC18A3-cre)K^{Misa}; Riken Bio Resource Center, Ibaraki, Japan; Stock#: RBRC01515] [29]. The mnVACHT KD line created has a C57BL/6J background. To identify MNs that had the *VACHT* gene deleted, we generated mnVACHT KD mice that express the tdTomato recombinant protein specifically in Cre-expressing cells (mn-VACHT-KD-Tomato). These mice were generated by crossing Rosa26-tdTomato mice (B6. Cg-Gt (ROSA) 26Sortm14 (CAG-tdTomato)Hze/J, Jax# 007914) [47] to mnVACHT KD. Upon Cre excision of a LoxP-flanked STOP cassette, tdTomato mice show strong expression of tdTomato, a DsRed fluorescent protein variant [48]. The mice used in our experiments were littermates: VACHT^{fllox/flox}-Tomato (VACHT^{fllox/flox}; Cre^{-/-}; Tomato^{+/+}) and mn-VACHT-KD-Tomato [VACHT^{fllox/flox}; Cre^{+/+}; Tomato^{+/+}; (mnVACHT KD)].

Study design

Behavioral analysis was performed on mice at 2 and 6 months of age. Most experiments were done on female mice as males exhibited a shorter lifespan. Two- and 6-month-old mutant mice and the controls performed the wire-hang, grip-force, and open-field tests. Six-month-old mice were then treated with a cholinesterase inhibitor and re-assessed on the grip-force and open-field tests. Upon completion of the behavioral tasks, the 6-month-old mice were euthanized and the brain and spinal cord were collected. Molecular analysis was only completed on these 6-month-old mice.

Mice were housed in groups of two to four per cage in a temperature-controlled room (23 °C) in a 12-h light/12-h dark cycle, with food and water provided *ad libitum*. Mice were randomized for behavioral tests and the experimenter was blind to genotypes and pharmacological treatments. Experiments were performed between 9 am and 6 pm and mice were tested during the light cycle.

Immunofluorescence and Western Blots

For immunofluorescence, mice were deeply anesthetized with a mixture of ketamine (100 mg·kg⁻¹) plus xylazine

(20 mg·kg⁻¹) and perfused transcardially with 4% paraformaldehyde (PFA) (w/v) in PBS. The brain and spinal cord were then removed and incubated in 4% PFA overnight at 4 °C. Brains were sectioned using a vibratome (40 µm thick), and spinal cords were immersed in OCT compound and sectioned using a cryostat (30 µm thick). All samples (brains and spinal cords) were washed with PBS and then blocked for 1 h at room temperature in PBS with 3% BSA (w/v) + 0.5% Triton X-100 (v/v) + 5% donkey or goat serum (depending on the primary antibody) (v/v). Sections were then incubated overnight in the same blocking solution at 4 °C with one of the following antibodies: (a) goat anti-ChAT (1 : 100; Catalog# AB144P; Millipore Corporation, Burlington, MA, USA); (b) goat anti-OPN (1 : 1000; Catalog# AF808; R&D Systems, Minneapolis, MN, USA); (c) mouse anti-ERR γ -human (ERR; 1 : 100; Catalog# PP-H6812-00; Perseus Proteomics, Tokyo, Japan); or (d) mouse anti-Cre-Recombinase (Clone 2D8; 1 : 100; Catalog# MAB3120; Sigma-Millipore, Burlington, MA, USA). Samples were washed three times for 5 min in PBS + 0.5% Triton X-100 (v/v) and incubated with the secondary antibody (1 : 500; Alexa Fluor 488 or 633; ThermoFisher, Waltham, MA, USA) in blocking solution for 2 h. Finally, sections were washed three times for 10 min in PBS then mounted on slides with Fluoroshield containing DAPI (Genetex, Irvine, CA, USA). Samples were visualized using a Leica TCS SP58 confocal system (63 $\times$ objective, 488-nm Ar laser, and 633-nm HeNe laser were used for fluorophores excitation). Images were acquired through z series sections collected with 2.0-µm optical slices.

Western blots were performed as described elsewhere [28,48]. Briefly, tissues were collected (striatum, cortex, brainstem, cervical and lumbar segments of the spinal cord), frozen, and kept at -80 °C until use. For extract preparation, tissue samples were homogenized in RIPA lysis buffer supplemented with protease and phosphatase inhibitors [49]. Extracts were then centrifuged (10 000 g, for 20 min) at 4 °C. Next, 25 µg of protein was separated by SDS/PAGE followed by electronic transfer into a polyvinylidene difluoride membrane (Millipore, Darmstadt, Germany). After blocking with 5% dry milk (w/v) for 1 h, membranes were incubated with the following primary antibodies overnight at 4 °C: (a) rabbit anti-VAcHT (1 : 3000; Synaptic Systems, Goettingen, Germany) and (b) mouse anti-Syn (1 : 500; Sigma-Aldrich, Oakville, ON, Canada). Syn immunoreactivity was used to correct the protein load as both VAcHT and Syn are membrane proteins present in synaptic vesicles [49,50]. Membranes were washed three times for 10 min in Tris-buffered saline + 0.1% Tween 20 (v/v) and incubated with a secondary antibody, for 1 h (1 : 10 000; rabbit-HRP Catalog# ab11427; Abcam, Cambridge, MA, USA or 1 : 5000; Mouse-IgG-perox; Catalog# SAB3701095; Sigma-Millipore). After the secondary incubation, the membranes were washed three times for 10 min

in Tris-buffered saline + 0.1% Tween 20 (v/v). The band intensity was quantified using FLUOROCHEM software (Thermo Fisher Scientific).

Muscle analyses

Sternomastoid, TA, and SOL muscles were collected, immersed in OCT compound, and sectioned using a cryostat (10 µm thick). In addition to the antibodies described above, a rabbit anti-Syn (1 : 100; Catalog# ab32594; Abcam) antibody was also used. The same immunofluorescence procedure described was performed. For all morphological analyses and area measurements, we used IMAGEJ software (NIH, Bethesda, MD, USA). We chose three different muscles for the analyses: STM, TA, and SOL that display differences in their biochemistry. As discussed by Kammoun *et al.* [32], STM is a hybrid fast muscle, TA is fast glycolytic, and SOL is a slow oxidative muscle, and due to these compositions, they may display selective vulnerabilities. Besides, they are innervated by MNs from different segments of the spinal cord (cervical for STM, and lumbar for TA and SOL). In addition, STM is innervated by some MNs from the brainstem.

Neuromuscular junction analyses

The protocol used has been previously described by Valadão *et al.* [51]. In summary, STM, TA, and SOL muscles were dissected from the mnVAcHT KD mice. The STM and SOL muscles were blocked using 3% BSA, 5% goat serum, and 0.5% Triton X-100 for 30 min at room temperature. Subsequently, the muscles were incubated with antisynaptotagmin antibody (1 : 250; antisynaptotagmin, anti-mouse, IgG2A, Developmental Studies Hybridoma Bank; Catalog# 3H2 2D7 RRID: AB_528483) in the blocking solution. The incubation step with the primary antibody occurred overnight, at 4 °C, and then, the muscles were washed three times with PBS and incubated for 1 h at room temperature with Alexa 555- α -bungarotoxin (α -btx; 1 : 1000; Catalog# T1175 Molecular Probes, Carlsbad, CA, USA; Invitrogen, Waltham, MA, USA; T1175 RRID: AB_2313931) together with the secondary antibody (1 : 1000, Alexa-488 goat anti-mouse IgG2A; Invitrogen, Catalog# A-21141 also A21141 RRID: AB_141626). Next, the samples were washed three times with PBS and whole-mounted using ProLongTM Gold Antifade Mountant (Catalog# P10144, Molecular Probes, Life Technologies, Carlsbad, CA, USA). Due to its size, the TA muscle was firstly frozen in liquid nitrogen and then was sliced using a Cryostat (Leica Biosystems CM350S, Wetzlar, Germany) at 30-µm sections. Next, the same procedures described were performed. The NMJ images were acquired using a 63-oil immersion objective (NA: 1.4) attached to a laser scanning confocal microscope (ZEISS LSM 880; Zeiss GmbH, Jena, Germany). The argon (488 nm) and helium-neon (He-Ne,

543 nm) lasers were used to excite the samples. The optical sections of the Z-series were collected at 2.0- μ m intervals. The images were obtained at Centro de Aquisição e Processamento de Imagens (CAPI- ICB/UFG). Images analyses were performed using the IMAGEJ software, and the tool polygon selection was used to circle the pre- and postsynaptic terminals, separately. We compared the pre- and postsynaptic areas of the VAcHT- deleted NMJs to the VAcHT- nondeleted NMJs, in the same animal ($n = 4$ individual animals). A total of 100 NMJs were analyzed for each muscle. It is important to highlight that in this mice model we can observe a VAcHT-deleted and a VAcHT-nondeleted NMJ at the same image, due to the Tomato labeling.

Behavioral tests

Wire-hang and grip-force tests were used to measure muscle strength [52]. Mice were habituated to the behavioral room 30 min before all experiments. For the wire-hang test, each mouse was individually placed at the top of a wire cage lid (22 $\times$ 22 cm), and then, the lid was gently turned upside down and maintained 45 cm above the bench surface for 60 s. The time it took the mouse to fall off the apparatus was recorded. For the grip-force test, a Grip Strength Meter (Columbus Instruments, Columbus, OH, USA) was used as previously described [28]. In short, mice were allowed to grasp the pull bar with their forelimbs and were pulled horizontally backward by their tail. The force applied to the bar immediately before grasp release was recorded. Max strength was automatically registered and expressed in grams/force (gf). All tests were performed three times for each animal with a 20-min interval period between each trial; mean values of the trials were used for analysis.

We further investigated motor performance by measuring spontaneous activity in open-field locomotor boxes. An automated activity monitor (AccuScan Instruments Inc.; Columbus, OH, USA; 20 $\times$ 20 cm platform with 30 cm high walls) was used to measure locomotor activity and number of rearings. Mice were placed in the center of the apparatus and allowed to freely explore the novel environment for 120 min. The distance traveled was recorded in 5-min intervals.

Pharmacological intervention using acetylcholinesterase inhibition

One mouse cohort ($n = 10$, females, 6-month-old) was treated with the acetylcholinesterase inhibitor, donepezil prior to behavioral testing. Intraperitoneal injections of saline or donepezil (1 mg·kg⁻¹) were performed once a day for 5 days. The choice of dose was based on findings from previous studies [53–56]. Grip-force and open-field behavioral tests were assessed before drug treatment began and following it.

Statistical analysis

GRAPHPAD PRISM 8 (GraphPad software, La Jolla, CA, USA) was used for statistical analysis. When the data passed a normal distribution test, the Student's *t*-test was used to compare two experimental groups. The significance level was set to 5% ($P < 0.05$) and the data were expressed as mean $\pm$ standard error of mean (SEM). For NMJ data representation, it was used boxplot with median $\pm$ SD.

Acknowledgements

The authors declare no competing financial interests. We thank Sanda Raulic, Jue Fan, and Matthew Cowan for animal care and technical support. JVS received support from CAPES-PDSE. This work was supported by grants from CNPq PVE (401071/2014-6) and ISN Career re-entry grant to CG. MAMP and VFP received support from the Canadian Institutes of Health Research (PJT 162431, PJT 159781), National Science and Engineering Research Council of Canada (06577-2018 RGPIN), and ALS Society Canada. OK received an Ontario Graduate Student (OGS) Doctoral Award. CG is a CNPq bolsista de produtividade. The authors want to thank CAPI-UFG for all the technical and equipment support.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

JVS, OK, MPSM-G, PACV, and LRO performed experiments and analyzed data; MAMP, VFP, and CG planned experiments, contributed reagents, and made substantial contributions to the conception and design of the work; JVS and OK wrote the paper. JVS, OK, MPSM-G, PACV, LRO, MAMP, VFP, and CG revised the work critically.

Peer Review

The peer review history for this article is available at <https://publons.com/publon/10.1111/febs.15825>.

References

- Manuel M & Zytnicki D (2011) Alpha, beta and gamma motoneurons: functional diversity in the motor system's final pathway. *J Integr Neurosci* **10**, 243–276.
- Fries A, Kaltschmidt J, Ladle D, Sigrist M, Jessell T & Arber S (2009) Gamma and alpha motor neurons

- distinguished by expression of transcription factor Err3. *Proc Natl Acad Sci USA* **106**, 13588–13593.
- 3 Hunt CC & Kuffler SW (1951) Further study of efferent small-nerve fibers to mammalian muscle spindles; multiple spindle innervation and activity during contraction. *J Physiol* **113**, 283–297.
 - 4 Burke RE, Strick PL, Kanda K, Kim CC & Walmsley B (1977) Anatomy of medial gastrocnemius and soleus motor nuclei in cat spinal cord. *J Neurophysiol* **40**, 667–680.
 - 5 Finsterer J, Papić L & Auer-Grumbach M (2011) Motor neuron, nerve, and neuromuscular junction disease. *Curr Opin Neurol* **24**, 469–474.
 - 6 Querol L & Illa I (2013) Myasthenia gravis and the neuromuscular junction. *Curr Opin Neurol* **26**, 459–465.
 - 7 Palace J & Beeson D (2008) The congenital myasthenic syndromes. *J Neuroimmunol* **201**, 2–5.
 - 8 Engel A, Shen X, Selen D & Sine S (2012) New horizons for congenital myasthenic syndromes. *Ann N Y Acad Sci* **1275**, 54–62.
 - 9 Finlayson S, Beeson D & Palace J (2013) Congenital myasthenic syndromes: an update. *Pr Neurol* **13**, 80–91.
 - 10 Hantai D, Nicole S & Eymard B (2013) Congenital myasthenic syndromes: an update. *Curr Opin Neurol* **26**, 561–568.
 - 11 Milone M, Fukuda T, Shen X, Tsujino A, Brengman J & Engel A (2006) Novel congenital myasthenic syndromes associated with defects in quantal release. *Neurology* **66**, 1223–1229.
 - 12 Abicht A, Dusl M, Gallenmüller C, Guergueltcheva V, Schara U, Della Marina A, Wibbeler E, Almaras S, Mihaylova V, von der Hagen M et al. (2012) Congenital myasthenic syndromes: achievements and limitations of phenotype-guided gene-after-gene sequencing in diagnostic practice: a study of 680 patients. *Hum Mutat* **33**, 1474–1484.
 - 13 Birks R, Huxley H & Katz B (1960) The fine structure of the neuromuscular junction of the frog. *J Physiol* **150**, 134–144.
 - 14 Prado V, Roy A, Kolisnyk B, Gros R & Prado M (2013) Regulation of cholinergic activity by the vesicular acetylcholine transporter. *Biochem J* **450**, 265–274.
 - 15 Lin W, Burgess RW, Dominguez B, Pfaff SL, Sanes JR & Lee KF (2001) Distinct roles of nerve and muscle in postsynaptic differentiation of the neuromuscular synapse. *Nature* **410**, 1057–1064.
 - 16 Brandon EP, Lin W, D'Amour KA, Pizzo DP, Dominguez B, Sugiura Y, Thode S, Ko CP, Thal LJ, Gage FH et al. (2003) Aberrant patterning of neuromuscular synapses in choline acetyltransferase-deficient mice. *J Neurosci* **23**, 539–549.
 - 17 Prado VF, Martins-Silva C, De CBM, Lima RF, Barros DM, Amaral E, Ramsey AJ, Sotnikova TD, Ramirez MR, Kim H et al. (2006) Mice deficient for the vesicular acetylcholine transporter are myasthenic and have deficits in object and social recognition. *Neuron* **51**, 601–612.
 - 18 de Castro B, De Jaeger X, Martins-Silva C, Lima R, Amaral E, Menezes C, Lima P, Neves C, Pires R, Gould T et al. (2009) The vesicular acetylcholine transporter is required for neuromuscular development and function. *Mol Cell Biol* **29**, 5238–5250.
 - 19 O'Grady G, Verschuuren C, Yuen M, Webster R, Menezes M, Fock J, Pride N, Best H, Benavides Damm T, Turner C et al. (2016) Variants in SLC18A3, vesicular acetylcholine transporter, cause congenital myasthenic syndrome. *Neurology* **87**, 1442–1448.
 - 20 Aran A, Segel R, Kaneshige K, Gulsuner S, Renbaum P, Oliphant S, Meirson T, Weinberg-Shukron A, HersHKovitz Y, Zeligson S et al. (2017) Vesicular acetylcholine transporter defect underlies devastating congenital myasthenia syndrome. *Neurology* **88**, 1021–1028.
 - 21 Schwartz M, Sternberg D, Whalen S, Afenjar A, Isapof A, Chabrol B, Portnoi M, Heide S, Keren B, Chantot-Bastaraud S et al. (2018) How chromosomal deletions can unmask recessive mutations? Deletions in 10q11.2 associated with CHAT or SLC18A3 mutations lead to congenital myasthenic syndrome. *Am J Med Genet A* **176**, 151–155.
 - 22 Lima R, Prado V, Prado M & Kushmerick C (2010) Quantal release of acetylcholine in mice with reduced levels of the vesicular acetylcholine transporter. *J Neurochem* **113**, 943–951.
 - 23 Rodrigues H, Fonseca MC, Camargo W, Lima P, Martinelli P, Naves L, Prado V, Prado M & Guatimosim C (2013) Reduced expression of the vesicular acetylcholine transporter and neurotransmitter content affects synaptic vesicle distribution and shape in mouse neuromuscular junction. *PLoS One* **8**, e78342.
 - 24 Silva R, Terra F, Guise Y, Prado M, Prado V, Hiyane M, Costa Malheiros D, Prado C, Camara N & Braga T (2016) Reduced expression of VACHT increases renal fibrosis. *Pathophysiology* **23**, 229–236.
 - 25 Lara A, Damasceno DD, Pires R, Gros R, Gomes ER, Gavioli M, Lima RF, Guimaraes D, Lima P, Bueno CR et al. (2010) Dysautonomia due to reduced cholinergic neurotransmission causes cardiac remodeling and heart failure. *Mol Cell Biol* **30**, 1746–1756.
 - 26 Durand M, Becari C, Tezini G, Fazan RJ, Oliveira M, Guatimosim S, Prado V, Prado M & Salgado H (2015) Autonomic cardiocirculatory control in mice with reduced expression of the vesicular acetylcholine transporter. *Am J Physiol Heart Circ Physiol* **309**, H655–H662.
 - 27 Leite H, Oliveira-Lima O, Pereira L, Oliveira V, Prado V, Prado M, Pereira G & Massensini A (2016) Vesicular acetylcholine transporter knock down-mice are more susceptible to inflammation, c-Fos expression and sickness behavior induced by lipopolysaccharide. *Brain Behav Immun* **57**, 282–292.

- 28 Martins-Silva C, De Jaeger X, Guzman M, Lima R, Santos M, Kushmerick C, Gomez M, Caron M, Prado M & Prado V (2011) Novel strains of mice deficient for the vesicular acetylcholine transporter: insights on transcriptional regulation and control of locomotor behavior. *PLoS One* **6**, e1761.
- 29 Misawa H, Nakata K, Toda K, Matsuura J, Oda Y, Inoue H, Tatenos M & Takahashi R (2003) VACHT-Cre.Fast and VACHT-Cre.Slow: postnatal expression of Cre recombinase in somatomotor neurons with different onset. *Genesis* **37**, 44–50.
- 30 Yamamoto T, Higo N, Sato A, Nishimura Y, Oishi T, Murata Y, Yoshino-Saito K, Isa T & Kojima T (2011) SPPI expression in spinal motor neurons of the macaque monkey. *Neurosci Res* **69**, 81–86.
- 31 Misawa H, Hara M, Tanabe S, Niihara M, Moriwaki Y & Okuda T (2012) Osteopontin is an alpha motor neuron marker in the mouse spinal cord. *J Neurosci Res* **90**, 732–742.
- 32 Kammoun M, Cassar-Malek I, Meunier B & Picard B (2014) A simplified immunohistochemical classification of skeletal muscle fibres in mouse. *Eur J Histochem* **58**, 2254.
- 33 Misgeld T, Burgess RW, Lewis RM, Cunningham JM, Lichtman JW & Sanes JR (2002) Roles of neurotransmitter in synapse formation: development of neuromuscular junctions lacking choline acetyltransferase. *Neuron* **36**, 635–648.
- 34 Dahm LM & Landmesser LT (1988) The regulation of intramuscular nerve branching during normal development and following activity blockade. *Dev Biol* **130**, 621–644.
- 35 Magalhães-Gomes M, Motta-Santos D, Schetino L, Andrade J, Bastos C, Guimarães D, Vaughan S, Martinelli P, Guatimosim S, Pereira G *et al.* (2018) Fast and slow-twitching muscles are differentially affected by reduced cholinergic transmission in mice deficient for VACHT: a mouse model for congenital myasthenia. *Neurochem Int* **120**, 1–12.
- 36 Janickova H, Prado VF, Prado MAM, El Mestikawy S & Bernard V (2017) Vesicular acetylcholine transporter (VACHT) over-expression induces major modifications of striatal cholinergic interneuron morphology and function. *J Neurochem* **142**, 857–875.
- 37 Dahm LM & Landmesser LT (1991) The regulation of synaptogenesis during normal development and following activity blockade. *J Neurosci* **11**, 238–255.
- 38 Oppenheim RW, Bursztajn S & Prevette D (1989) Cell death of motoneurons in the chick embryo spinal cord. XI. Acetylcholine receptors and synaptogenesis in skeletal muscle following the reduction of motoneuron death by neuromuscular blockade. *Development* **107**, 331–341.
- 39 Oppenheim RW, Prevette D, D'Costa A, Wang S, Houenou LJ & McIntosh JM (2000) Reduction of neuromuscular activity is required for the rescue of motoneurons from naturally occurring cell death by nicotinic-blocking agents. *J Neurosci* **20**, 6117–6124.
- 40 Hory-Lee F & Frank E (1995) The nicotinic blocking agents d-tubocurarine and alpha-bungarotoxin save motoneurons from naturally occurring death in the absence of neuromuscular blockade. *J Neurosci* **15**, 6453–6460.
- 41 Zheng JQ, Felder M, Connor JA & Poo MM (1994) Turning of nerve growth cones induced by neurotransmitters. *Nature* **368**, 140–144.
- 42 Ennaceur A (2014) Tests of unconditioned anxiety - pitfalls and disappointments. *Physiol Behav* **135**, 55–71.
- 43 Bloemberg D & Quadrilatero J (2012) Rapid determination of myosin heavy chain expression in rat, mouse, and human skeletal muscle using multicolor immunofluorescence analysis. *PLoS One* **7**, e35273.
- 44 Atherton PJ, Greenhaff PL, Phillips SM, Bodine SC, Adams CM & Lang CH (2016) Control of skeletal muscle atrophy in response to disuse: clinical/preclinical contentions and fallacies of evidence. *Am J Physiol Endocrinol Metab* **311**, E594–E604.
- 45 Sugimoto H, Ogura H, Arai Y, Iimura Y & Yamanishi Y (2002) Research and development of donepezil hydrochloride, a new type of acetylcholinesterase inhibitor. *Jpn J Pharmacol* **89**, 7–20.
- 46 Maggi L & Mantegazza R (2011) Treatment of myasthenia gravis: focus on pyridostigmine. *Clin Drug Investig* **31**, 691–701.
- 47 Madisen L, Zwingman T, Sunkin S, Oh S, Zariwala H, Gu H, Ng L, Palmiter R, Hawrylycz M, Jones A *et al.* (2010) A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nat Neurosci* **13**, 133–140.
- 48 Janickova H, Rosborough K, Al-Onaizi M, Kljakic O, Guzman M, Gros R, Prado M & Prado V (2017) Deletion of the vesicular acetylcholine transporter from pedunculopontine/laterodorsal tegmental neurons modifies gait. *J Neurochem* **140**, 787–798.
- 49 Guzman M, De Jaeger X, Raulic S, Souza I, Li A, Schmid S, Menon R, Gainetdinov R, Caron M, Bartha R *et al.* (2011) Elimination of the vesicular acetylcholine transporter in the striatum reveals regulation of behaviour by cholinergic-glutamatergic co-transmission. *PLoS Biol* **9**, e1001194.
- 50 Prado V & Prado M (2002) Signals involved in targeting membrane proteins to synaptic. *Cell Mol Neurobiol* **22**, 565–577.
- 51 Valadão PAC, de Aragão BC, Andrade JN, Magalhães-Gomes M, Foureux G, Joviano-Santos JV, Nogueira JC, Ribeiro FM, Tapia JC & Guatimosim C (2017) Muscle atrophy is associated with cervical spinal motoneuron loss in BACHD mouse model for Huntington's disease. *Eur J Neurosci* **45**, 785–796.

- 52 Brooks S & Dunnett S (2009) Tests to assess motor phenotype in mice: a user's guide. *Nat Rev Neurosci* **10**, 519–529.
- 53 Geerts H, Guillaumat P, Grantham C, Bode W, Anciaux K & Sachak S (2005) Brain levels and acetylcholinesterase inhibition with galantamine and donepezil in rats, mice, and rabbits. *Brain Res* **1033**, 186–193.
- 54 Van Dam D, Abramowski D, Staufenbiel M & De Deyn P (2005) Symptomatic effect of donepezil, rivastigmine, galantamine and memantine on cognitive deficits in the APP23 model. *Psychopharmacol* **180**, 177–190.
- 55 Gavioli M, Lara A, Almeida P, Lima A, Damasceno D, Rocha-Resende C, Ladeira M, Resende R, Martinelli P, Melo M *et al.* (2014) Cholinergic signaling exerts protective effects in models of sympathetic hyperactivity-induced cardiac dysfunction. *PLoS One* **9**, e100179.
- 56 Gawel K, Labuz K, Gibula-Bruzda E, Jenda M, Marszalek-Grabska M, Filarowska J, Silberring J & Kotlinska J (2016) Cholinesterase inhibitors, donepezil and rivastigmine, attenuate spatial memory and cognitive flexibility impairment induced by acute ethanol in the Barnes maze task in rats. *Naunyn-Schmiedeberg Arch Pharmacol* **389**, 1059–1071.

8.3 Artigo 3: Neuromuscular defects after infection with a beta coronavirus in mice.

Neurochemistry International 169 (2023) 105567



Contents lists available at ScienceDirect

Neurochemistry International

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



Neuromuscular defects after infection with a beta coronavirus in mice

Leonardo Rossi^a, Kivia B.S. Santos^a, Barbara I.S. Mota^a, Jordane Pimenta^a, Bruna Oliveira^a,
Caroline A. Machado^a, Heliana B. Fernandes^a, Leticia A. Barbosa^a, Hermann A. Rodrigues^d,
Gabriel H.M. Teixeira^a, Gabriel A. Gomes-Martins^a, Gabriel F. Chaimowicz^a,
Celso Martins Queiroz-Junior^a, Ian Chaves^a, Juan C. Tapia^c, Mauro M. Teixeira^b,
Vivian V. Costa^a, Aline S. Miranda^a, Cristina Guatimosim^{a,*}

^a Department of Morphology, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

^b Department of Biochemistry, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

^c School of Medicine, University of Talca, Talca, Chile

^d Departamento de Ciências Básicas da Vida, Universidade Federal de Juiz de Fora, Campus Governador Valadares, MG, Brazil

ABSTRACT

COVID-19 affects primarily the lung. However, several other systemic alterations, including muscle weakness, fatigue and myalgia have been reported and may contribute to the disease outcome. We hypothesize that changes in the neuromuscular system may contribute to the latter symptoms observed in COVID-19 patients. Here, we showed that C57BL/6J mice inoculated intranasally with the murine betacoronavirus hepatitis coronavirus 3 (MHV-3), a model for studying COVID-19 in BSL-2 conditions that emulates severe COVID-19, developed robust motor alterations in muscle strength and locomotor activity. The latter changes were accompanied by degeneration and loss of motoneurons that were associated with the presence of virus-like particles inside the motoneuron. At the neuromuscular junction level, there were signs of atrophy and fragmentation in synaptic elements of MHV-3-infected mice. Furthermore, there was muscle atrophy and fiber type switch with alteration in myokines levels in muscles of MHV-3-infected mice. Collectively, our results show that acute infection with a betacoronavirus leads to robust motor impairment accompanied by neuromuscular system alteration.

1. Introduction

In December 2019, a novel coronavirus named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was identified in Wuhan, China. By the middle of 2020, coronavirus disease 2019 (COVID-19, named by WHO on Feb 11, 2020), became a global pandemic, affecting individuals all around the world. The virus is a member of the coronavirus family that caused the 2002–2004 SARS and 2012 MERS outbreaks, and primarily spreads through respiratory droplets produced by the infected people. Noteworthy, it can be transmitted even if the infected person has no symptoms. The clinical manifestations of COVID-19 can range from no symptoms to severe ailment. Fever, cough, headache, myalgia, and difficulty of breathing are among the typical symptoms. Severe symptoms, on the other hand, lead to acute respiratory distress syndrome (ARDS), with systemic inflammatory response, and lung-organ failure, which frequently culminates in the patient's death. Additionally, it is important to note that the severity of COVID-19 disease, although varies widely from person to person, seems to be higher in older adults, people with underlying health conditions, or

individuals with compromised immune systems (Del Valle et al., 2020; Lu et al., 2020).

Because of all symptoms described above in humans, animal models for COVID-19 are key to study the disease, providing valuable insights into mechanisms of viral-induced disease (Andrade et al., 2021; Sia et al., 2020; Winkler et al., 2020; Gurumurthy et al., 2020; Rockx et al., 2020). The SARS-CoV-2 virus adsorbs cells via the spike (S) glycoprotein, which has a high affinity (>10 fold more than previous SARS-CoV-1) for ACE2 (angiotensin-converting enzyme 2), localized at the surface of host cells (Bourgonje et al., 2020). Since ACE2 is expressed in several tissues i.e., nasal tissue, lungs, kidneys, liver, blood vessels, gut, brain, immune system, and skeletal muscles; COVID-19 infects not only lung cells but many other cell types and organs throughout the body (Bourgonje et al., 2020; Motta-Santos et al., 2016). Thus, a detailed understanding of the pathophysiological consequences of COVID-19 on various cells and organs remains elusive.

The use of the murine coronavirus has been suggested as a strategy to mimic the key aspects of human coronavirus infection in a biosafety level 2 environment (Albuquerque et al., 2006; Yang et al., 2014;

* Corresponding author.

E-mail address: cguati@icb.ufmg.br (C. Guatimosim).

<https://doi.org/10.1016/j.neuint.2023.105567>

Received 13 April 2023; Received in revised form 14 June 2023; Accepted 19 June 2023

Available online 20 June 2023

0197-0196/© 2023 Elsevier Ltd. All rights reserved.

Andrade et al., 2021). Among them, murine hepatitis coronavirus (MHV) stands out as a natural pathogen of mice (Weiss and Leibowitz, 2011). Different MHV viruses, for instance, MHV-1 and MHV-A59 have initial pulmonary system preferences. On the other hand, MHV-3 is capable of causing severe hepatitis in many strains of mice (Weiss and Leibowitz, 2011; Graff Reis et al., 2022). Thus, MHV viruses-based infection models are valuable tools to investigate preclinical pathophysiological alterations in many physiological systems (Körner et al., 2020), such as nervous system and skeletal muscle, adding the fact that this mouse model requires only a biosafety level 2 laboratory.

Muscle weakness, fatigue, myalgia and muscle injury are symptoms and signs reported by many COVID-19 patients (Wu et al., 2020; Sun et al., 2020; Lahiri et al., 2020; Mao et al., 2020). We hypothesized that infection with SARS-CoV-2 may alter the neuromuscular system and contribute to the latter observations in COVID-19 patients. To this end, in this work, we used a murine beta coronavirus directly injected into the nose (intranasal inoculation) (Andrade et al., 2021) to investigate if MHV-3 infection would affect the neuromuscular system in mice. We characterized the model and described the COVID-19-related symptoms showing that the infection with MHV-3 causes impairment in motor function and performance and that such alterations are in part explained by a decrease in both muscle fiber size (CSA, cross-sectional area) and alterations in muscle fiber type, with neuromuscular junction degeneration (reduced NMJ area, with increased fragmentation). We also observed signs of motoneuron degeneration (cell death and organelles disruptions) along with alterations in myokines, signaling proteins involved in the COVID-19 inflammatory immune response. Overall, our findings are relevant since postural skeletal muscles, along with respiratory muscles, are responsible for conducting forces that allow motor behavior and alveolar ventilation respectively (Gransee et al., 2015). Understanding neuromuscular degeneration due to MHV-3 infection gives mechanistic insights into their collapse during COVID-19 disease.

2. Materials and Methods

2.1. Mouse strains and MHV-3 infection

All experimental procedures were performed following the guidance of the Universidade Federal de Minas Gerais (UFMG) Ethical Committee for Animal Experimentation and received approval number under No 190/2020. Female C57BL/6J wild-type mice (Central Animal Housing of the UFMG) aged 6–7 weeks were randomly divided into two groups: mock and MHV-3. As described in Andrade et al. (2021), mice were anesthetized using intraperitoneal injection of ketamine (50 mg/kg) and xylazine (5 mg/kg), and then given an intranasal inoculation of 30 µl sterile saline solution loaded with MHV-3 at a concentration of 3×10^5 PFU. This viral titer was chosen after a dose-response study showing that induced drastic symptoms at 5 dpi, with few deaths before that time (Andrade-Junior et al., 2021). We chose 3 dpi to represent the intermediate phase and 5 dpi to represent the peak of the pulmonary infection and symptoms. At the end of 5 dpi mice started to quickly perish (Andrade et al., 2021). Mock mice received the same intranasal inoculation but without any MHV-3. Mice were housed in individually ventilated cages placed in an animal care facility at $24 \pm 2^\circ\text{C}$, 12 h light/dark cycle with water and food *ad libitum*.

2.2. Behavioral tests

2.2.1. Open field test

To assess locomotor activity, the animals were submitted to the open field test as described elsewhere (Takahashi et al., 2006). Each animal was gently placed in the cage and allowed to explore the arena for 30 min. The total distance and the mean velocity traveled in the cage was recorded. Video tracking was performed by an infrared-sensitive video camera installed in the top unit of each cage. The X–Y coordinates of each mouse were acquired using EthoVision software (EthoVision HTP

2.1.2.0, based on EthoVision XT 4.1, Noldus Information Technology, Wageningen, The Netherlands) (Takahashi et al., 2006).

2.2.2. Wire hang test

The wire hang test was used to assess motor strength and coordination as described elsewhere (Tanaka et al., 2009). Briefly, the mice were placed on a metal grid 45 cm above a large tub filled to the top with wood-chip bedding. Mice were prompted to grip the wires with forepaws by gently vibrating the cage lid. The cage lid was then inverted and the latency to fall from the cage lid was recorded with a 180-s cut-off time (Tanaka et al., 2009).

2.2.3. Grip force test

Grip-force in the forelimbs and all limbs were measured using Grip Strength Meter (Insight Equipamentos, Ribeirão Preto, SP) as previously described (Kolisnyk et al., 2013). Mice were allowed to grasp the smooth pull bar with forelimbs and also with the all limbs by holding their tails. Mice were then pulled backward in the horizontal plane and the peak force (N) applied to the bar was recorded. Three trials were performed per mouse within the same session and the average of the three trials was recorded (Kolisnyk et al., 2013).

2.3. Muscle morphometrical analyses

MHV-3 infected (COVID-19) and mock mice (PBS) were anesthetized with pentobarbital followed by cervical dislocation. The extensor digitorum longus (EDL), soleus, and sternomastoideus (STM) muscles from both mice were surgically excised, immersed in O.C.T compound (Tissue Plus, Fisher Health Care) inside freezing molds, frozen in isopentane (P36631, Sigma-Aldrich), cooled with liquid nitrogen, and stored at -80°C . Samples were then sectioned using a cryostat (Leica CM3050S), and 10 µm cross sections were collected on gelatin-coated slides. The slides containing tissue sections underwent a blocking step with 3% BSA + 0.1% Triton X-100 for 30 min followed by 5% normal goat serum + 0.1% Triton X-100 for 30 min at room temperature. Sections were then incubated with Wheat Germ Agglutinin (WGA) Alexa Fluor 647 Conjugate (W32466, Invitrogen) and washed three times with PBS. Sections were mounted using ProLong Gold Antifade Mountant Medium (P36934, Thermo Fisher Scientific). Images were acquired using Zeiss LSM 880 confocal microscope (Zeiss OMBH, Germany) equipped with 40x 1.2 NA and 63x 1.3 NA apochromatic objectives and Argon and He-Ne lasers. The total number of fibers and their cross-sectional area were obtained using ImageJ (NIH).

2.4. Neuromuscular junction analysis

MHV-3 infected (COVID-19) and mock mice (PBS) were anesthetized, followed by intracardiac perfusion using 20 ml of PBS followed by 20 ml of paraformaldehyde (PFA) at 4%. Muscles were surgically excised and stored in PBS at 4°C . Whole-mount EDL, soleus, and STM were used to evaluate changes in NMJ from both MHV-3 infected (COVID-19) and mock mice (PBS) controls. The NMJ staining protocol has been previously described (Valadão et al., 2017). Briefly, the pre-synaptic element was stained using a monoclonal primary antibody against synaptotagmin (1:250 dilution, ZNP15, DSHB) for 12 h, followed by the secondary antibody IgG2A goat anti-mouse conjugated with AlexaFluor 488 (1:300 dilution, A21131, Life Technologies) together with tetramethylrhodamine conjugated α -bungarotoxin 647 (1:300 dilution, B35450, Life Technologies) for labeling the postsynaptic acetylcholine receptors (nAChRs). Images were acquired using the Z-stack imaging tool from Zeiss LSM 880 confocal microscope, and 20 NMJs per animal had their pre- and post-synaptic areas assessed by the ImageJ polygon tool as well as the colocalization of pre- and post-synaptic elements, followed by particle analysis to estimate the fragmentation index at the neuromuscular junction terminals.

2.5. Motoneurons morphometrical analysis

MHV-3 infected (COVID-19) and control mice were anesthetized, and intracardially perfused as indicated above. Spinal cord cervical and lumbar segments from both were collected and stored in PFA 4% for 12 h at 4 °C. The next day, the respective spinal cord segments were immersed in sucrose solution (30%) for three days, excised from the vertebrae, and embedded in O.C.T frozen in isopentane cooled with liquid nitrogen. The O.C.T blocks were then sectioned using a cryostat at 30 µm thickness and collected on gelatin-coated slides. The MHV-3 infected (COVID-19) and control sections went through the blocking step with 3% BSA + 0.5% Triton X-100 for 30 min followed by 5% donkey serum + 0.5% Triton X-100 for 30 min at room temperature and incubated for 12 h with a primary antibody against osteopontin (goat anti-OPN; 1:100 dilution, BDO0615081, RD Systems) at 4 °C. Sections were washed three times with PBS + 0.5% Triton X-100, and incubated with DAPI to label all cells, and mounted in ProLong Gold. Images from both strains were acquired using the Z-stack imaging tool from LSM 880 confocal microscope, and the total number of cells (DAPI+), motor neuron (OPN+), and the motor neurons area analyzed using ImageJ (NIH).

2.6. Transmission electron microscopy

For transmission electron microscopy (TEM), we used a protocol previously described (Rodrigues et al., 2013). Briefly, mice were anesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg), transcardially perfused with modified Karnovsky fixative (4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer) and maintained in this solution for at least 24 h at 4 °C. Spinal cord segments from mock and MHV-3 infected mice were removed and the samples were then washed with cacodylate buffer (0.1 M), cut into several fragments, post-fixed in reduced osmium (2% osmium tetroxide containing 1.6% potassium ferrocyanide), contrasted en bloc with uranyl acetate (UA, 2% in deionized water), dehydrated through an ascending series of ethanol solutions and embedded in EPON. Sections of 50 nm were collected on 200 or 300 mesh copper grids and contrasted with lead citrate. Samples were examined with a Tecnai-G2-Spirit FEL/Quanta electron microscope (120 kV Philips).

2.7. ELISA analyses of cytokines/myokines, chemokines and neurotrophic factor

Spinal Cord and muscular tissues were carefully removed and homogenized in an extraction solution (100 mg of tissue per milliliter), containing 0.4 M NaCl, 0.05% Tween 20, 0.5% BSA, 0.1 mM phenyl methyl sulphonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA, and 20 KIU aprotinin, using Ultra-Turrax. Lysates were centrifuged at 13,000g for 10 min at 4 °C, supernatants were collected and stocked at -70 °C until use. The concentration of the Brain-Derived Neurotrophic Factor (BDNF), chemokine CX3CL1 and myokines irisin and interleukin-6 (IL-6) was determined by ELISA (R&D Systems, Minneapolis, MN) in accordance to the manufacturer's instructions. Results are expressed as picogram per 100 mg of tissue. The detection limit of the ELISA assays was around 5 pg/mL.

2.8. qPCR assay

Viral RNA was extracted with kit QIAamp® Viral RNA from MHV-3 infected (COVID-19) and mock mice (PBS) following the manufacturer's guidelines with adaptations that the tissues were macerated in the first step with the lysis buffer. Samples were eluted and quantified in a spectrophotometer (NanoDrop™, Thermo Scientific). cDNA was synthesized starting with 500 ng of total RNA using the kit iScript™ gDNA Clear cDNA Synthesis Kit (BIO-RAD) following the manufacturer's guidelines. The cDNA obtained was diluted 1:10 to be used in the qPCR

assay with Fast SYBR™ Green Master Mix (Applied Biosystems™) and primers in a concentration of 5 nM: Forward primer 5'-CAGATGCTT-GATGATGGCGTAAT-3'; Reverse primer 5'-AGAGTGTCTATCCC-GACTTTCTC-3'. The standard was obtained by extracting RNA from a known amount of PFU from MHV-3. Results were expressed in Arbitrary Units.

2.9. Immunohistochemistry

Slides containing 30 µm cross-sections of 4% PFA fixed spinal cord of mock and MHV-3-infected mice were washed with 1% bovine serum albumin. Then, endogenous peroxidase blockade was performed with 0.3% hydrogen peroxide and unspecific reactions were blocked with protein block (Abcam ab64226). The slides were washed with TBS-Triton X-100 0.1%, treated with fc-blocking reagents (Mouse-on-mouse kit; Vector) and incubated overnight with the primary antibody anti-dsRNA (1:50, Merck MABE1134, Clone nJ2). On the following day, the slides were washed with TBS-Triton X-100 0.1%, and the secondary antibody (biotinylated goat anti-mouse IgG, Mouse-on-mouse kit) was added for 10 min, followed by an avidin-peroxidase procedure. The reaction was revealed with DAB chromogenic solution.

3. Results

3.1. C57BL/6 mice display significant motor changes following MHV-3 infection

To first assess whether the MHV-3 infection leads to motor dysfunction in mice, we performed a series of well-characterized motor tests, including the open field, wire hang, and grip force tests. In the open field test, MHV-3 infected mice at 3 dpi did not show motor alterations with respect to the controls (mock mice), either in the total distance traveled or in the mean velocity reached, respectively (MOCK: 9528 ± 965.1 cm, MHV-3: 9535 ± 1136 cm, $p = 0.9060$; MOCK: 5.29 ± 0.53 cm/s, MHV-3: 5.29 ± 0.63 cm/s, $p = 0.9071$) (Fig. 1A and B). At 5 dpi however, the MHV-3 mice exhibited a significant reduction in both total distances traveled, and average velocity reached, respectively, when compared to mock animals (MOCK: 5492 ± 552.5 cm, MHV-3: 3529 ± 1739 cm $p = 0.0026$; MOCK: 3.06 ± 0.30 cm/s, MHV-3: 1.96 ± 0.96 cm/s, $p = 0.0025$), indicating significant locomotor changes (Fig. 1C and D).

Similarly, in the wire-hang test, the MHV-3 mice did not show significant changes at 3 dpi when compared to the mock animals (MOCK: 174.5 ± 11.65 s, MHV-3: 180.0 ± 0.00 s, $p = 0.1630$). At 5 dpi, the MHV-3 infected mice did not show a reduction in the latency to fall when compared to the control (MOCK: 180.0 ± 0.00 s, MHV-3: 152.0 ± 42.89 s, $p = 0.0669$) (Fig. 1E and F).

Another motor parameter assessed was the grip force for forelimbs followed by all limbs. MHV-3 mice at 3 dpi showed a significant reduction in the grip force in the forelimbs when compared to mock animals (MOCK: 609.6 ± 149.9 N, MHV-3: 502.5 ± 56.61 N, $p = 0.0361$). No significant changes were found in the all-limbs grip force at 3 dpi (MOCK: 1143 ± 168.5 N, MHV-3: 1077 ± 172.2 N, $p = 0.3435$) (Fig. 1G and H). As the infection progresses, i.e. at 5 dpi, the MHV-3 mice displayed a drastic decay in the grip force in both forelimbs (MOCK: 541 ± 139.2 N, MHV-3: 350.1 ± 69.43 N, $p = 0.0003$) and all limbs (MOCK: 1244 ± 185.6 N, MHV-3: 860 ± 160.2 N, $p < 0.0001$) compared to controls (Fig. 1I and J).

3.2. MHV-3 infection induces motoneuron loss at the lumbar spinal cord

One putative cause of motor dysfunction is the damage of one or more components of the motor unit such as motoneurons, neuromuscular junctions and skeletal muscle fibers innervated by them. Therefore, the next set of experiments was designed to address if the MHV-3-associated motor impairment, which is more pronounced at 5 dpi, might

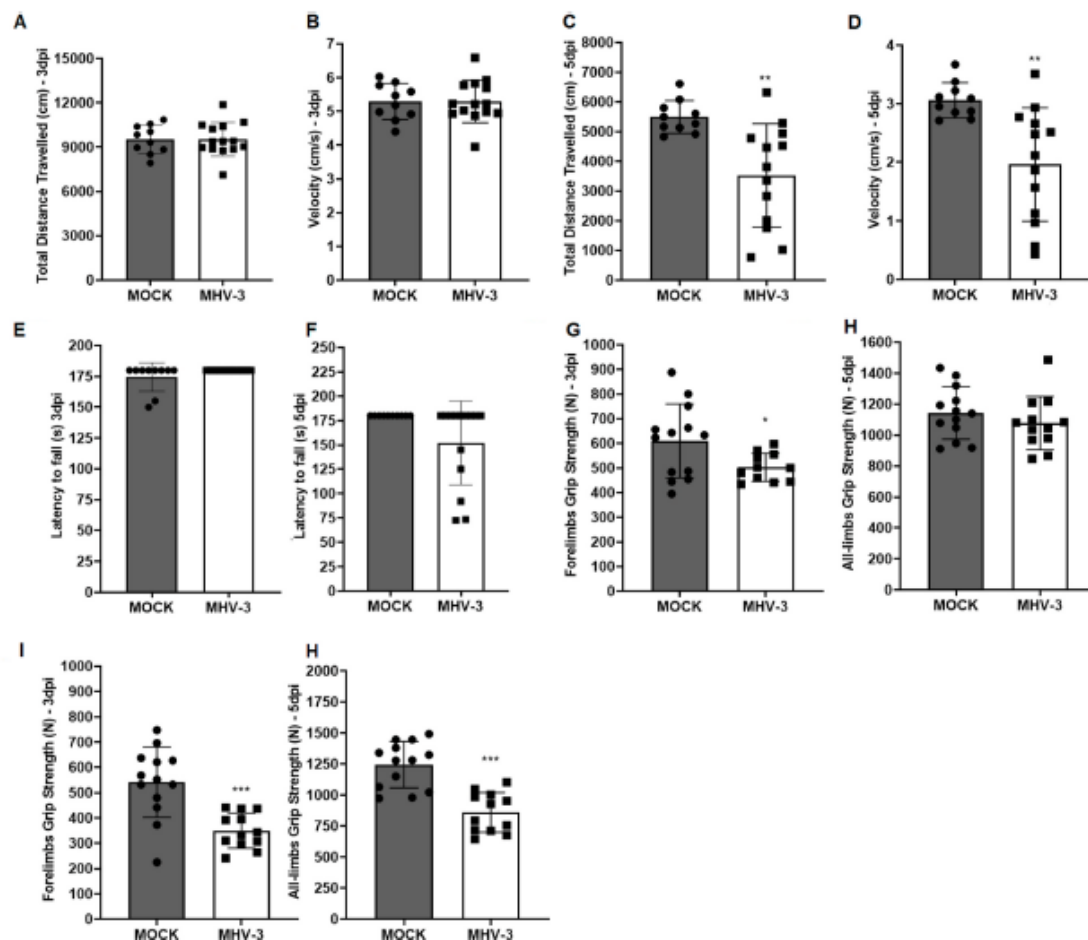


Fig. 1. MHV-3-infected mice showed reduced performance in behavioral tests. (A–B) Total distance traveled and velocity in the Open Field Test, respectively, at 3 dpi. (C–D) Total distance traveled and velocity in the Open Field Test, respectively, at 5 dpi. (E–F) Time sustaining the body weight in the wire hang test, at 3 dpi and 5 dpi, respectively. (G) Forelimbs strength in the grip force test, at 3 dpi. (H) All-limbs strength in the grip force test, at 3 dpi. (I) Forelimbs strength in the grip force test at 5 dpi. (J) All-limbs strength in the grip force test at 5 dpi. For Open Field and wire hang tests $n = 10$ animals for mock and $n = 14$ animals for MHV-3. For grip strength tests $n = 14$ animals for mock and $n = 12$ for MHV-3. Values were compared between groups by unpaired Student's *t*-test and results are presented as mean $\pm$ SD. For MHV-3 compared to mock group * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

result from loss or degeneration of spinal cord motoneurons. Motoneurons loss is known to be related to motor dysfunctions in both aging (Drey et al., 2014) or neurodegenerative diseases (Valadão et al., 2017).

We used DAPI to label all cells and osteopontin (OPN) to specifically stain α -MNs at the spinal segments (see Methods). We observed no differences in the number of OPN⁺ cells (MOCK: 33.67 ± 11.02 cells, MHV-3: 30.75 ± 3.86 cells, $p = 0.6359$) or area (MOCK: $503.6 \pm 190.2 \mu\text{m}^2$, MHV-3: $561.3 \pm 193.2 \mu\text{m}^2$, $p = 0.3290$) in the cervical segments of MHV-3 and mock mice (Fig. 2A–D). We also quantified the total number of DAPI⁺ cells and found no differences between MHV-3 and mock samples (MOCK: 735.3 ± 96.02 cells, MHV-3: 700.3 ± 94.27 cells, $p = 0.6491$) (Fig. 2 E).

Interestingly, a significant reduction of OPN⁺ MNs was found in the lumbar spinal cord of MHV-3 infected mice compared to controls (MOCK: 32.33 ± 1.52 cells, MHV-3: 24.75 ± 2.21 cells, $p = 0.0040$). No significant changes were found in the MNs area (MOCK: 454.1 ± 170.8

μm^2 , MHV-3: $460.4 \pm 180.6 \mu\text{m}^2$, $p = 0.8488$) (Fig. 2F–J). Again, we did not find any difference in DAPI⁺ cells (MOCK: 794.0 ± 162.4 cells, MHV-3: 741.3 ± 50.68 cells, $p = 0.5574$) (Fig. 2 I), supporting the hypothesis of a loss/degeneration of α -MNs in the lumbar spinal cord.

3.3. Molecular and cellular signs of neurodegeneration in the spinal cord of MHV-3 infected mice

Fractalkine (CX3CL1) is a chemokine that is highly expressed in neurons and involved in the neuronal and microglial communication. Importantly, a neuroprotective role has been reported for CX3CL1 in neurodegenerative diseases such as Alzheimer's disease (Pawelec et al., 2020). Neuroprotective effects have been also associated with enhanced expression of the brain derived neurotrophic factor (BDNF) in different areas of the central nervous system, including the spinal cord (Tejeda et al., 2019; Glowacka et al., 2022). To investigate molecular (BDNF and

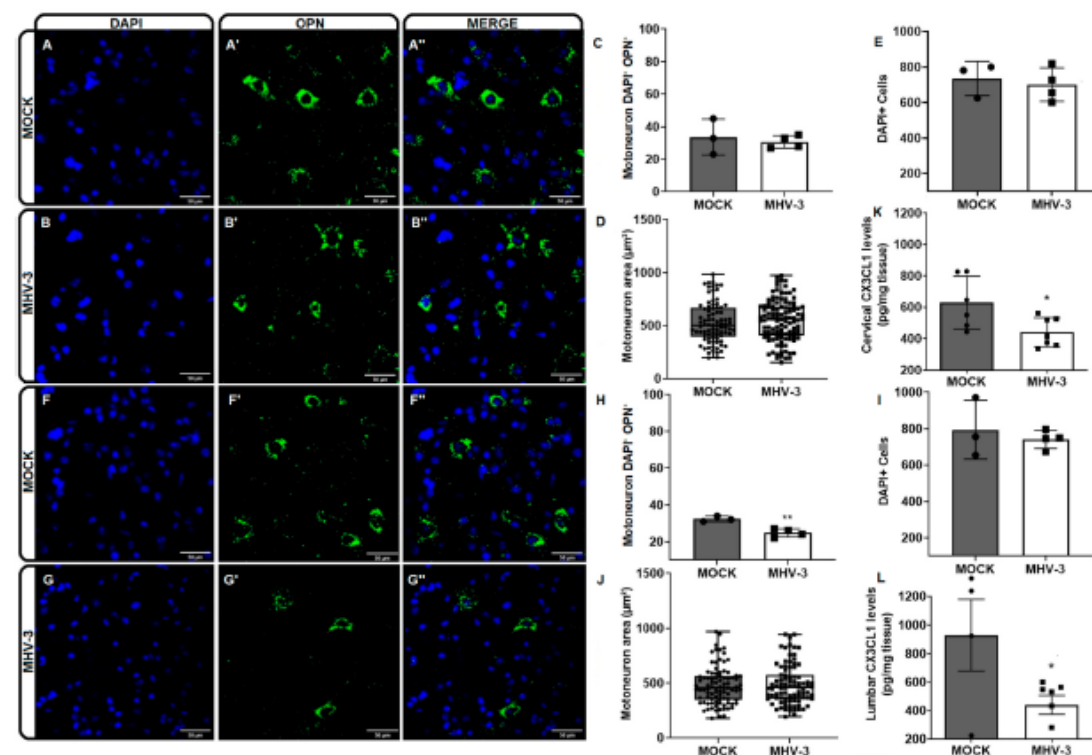


Fig. 2. Reduction in the number of motoneurons and decrease levels of CX3CL1 in MHV-3 mice spinal cord. (A-A'') Representative images of DAPI⁺, OPN⁺ and merged images, respectively, in the cervical segment of mock group. (B-B'') Representative images of DAPI⁺, OPN⁺ and merged images, respectively, in the cervical segment of MHV-3 group. (C-E) Quantification of MNs OPN⁺, MNs area and total DAPI⁺ cells, respectively, in the cervical segment, from mock and MHV-3 (n = 3 for mock and n = 4 for MHV-3; at least 100 MNs were analyzed for each experimental group). (F-F'') Representative images of DAPI⁺, OPN⁺ and merged images, respectively, in the lumbar segment of mock group. (G-G'') Representative images of DAPI⁺, OPN⁺ and merged images, respectively, in the lumbar segment of MHV-3 group. (H-I) Quantification of MNs OPN⁺, MNs area and total DAPI⁺ cells, respectively, in the lumbar segment, from mock and MHV-3 (n = 3 for mock and n = 4 for MHV-3; at least 100 MNs were analyzed for each experimental group). (J) Quantification of CX3CL1 levels of the cervical (n = 6 for mock, and n = 7 for MHV-3) and (K) lumbar (n = 4 animals for mock and n = 7 for MHV-3) segments of the spinal cord. Values were compared between groups by unpaired Student's t-test (mean ± SD) or Mann-Whitney test (median ± SD). For MHV-3 compared to mock group *p < 0.05; **p < 0.005.

CX3CL1) and cellular signs of MNs degeneration, we performed ELISA assays and ultrastructural analysis with TEM in both cervical and lumbar segments of the spinal cord.

Our results showed no signs of disturbances in the BDNF levels either in the cervical or lumbar segments of MHV-3 compared to mock mice (data not shown). Interestingly, a significant reduction in the levels of CX3CL1 in both cervical (MOCK: 630.1 ± 168.3 pg/mg of tissue, MHV-3: 441.9 ± 91.60 pg/mg of tissue, $p = 0.0265$) and lumbar segments (MOCK: 927.8 ± 500.4 pg/mg of tissue, MHV-3: 430.9 ± 176.3 pg/mg of tissue, $p = 0.0309$) was observed between MHV-3 compared to mock mice (Fig. 2 K and L, respectively).

At the ultrastructural level, we were able to identify MHV-3 infected MNs with a viral-like particle. These particles had a characteristic size of 100–150 nm in diameter (Fig. 3 E and F), with a well-recognizable structure that included surface projections corresponding to the spike protein crown-like halos, electron-dense membranes comparable to viral envelopes, and heterogeneous interiors representing viral RNA (Fig. 3 E, F, H and I). The MHV-3 viral-like particles could be clearly distinguished from other subcellular structures such as multi-vesicular bodies (Fig. 3 G), clathrin-coated vesicles with multiple electron-dense specks (Fig. 3 D), homogeneous interior and smaller sizes than MHV-3 particles. The

MHV-3-infected cells had a higher number of heterogeneous lipofuscin granules, a sign associated with recent subcellular damage, compared to the mock cells (Fig. 3 J–L). In addition, the MHV-3 MNs but not the mock cells depicted more membrane spirals (Fig. 3 N), a feature that has been assigned to organelle degeneration. We also observed that MHV-3-infected, but not mock, MNs often presented Golgi apparatus with dilated cisternae (Fig. 3 A–C), a finding associated with cellular stress.

To confirm the presence of MHV-3 particles in the spinal cord, qPCR assay and immunohistochemistry for the replicative viral RNA were performed. The cervical and lumbar spinal cord segments from the MHV-3 mice showed significant expression of the viral genetic material, as well as in the whole brain (Fig. 3 O). No viral gene expression was found in the cervical or lumbar spinal cord of mock mice. The immunostaining for the replicative viral RNA (dsRNA) showed positive MNs for dsRNA in the lumbar segment (Fig. 3 P and P') and in the cervical segment of the spinal cord (image not shown) of MHV-3-infected mice, but not in mock (Fig. 3 Q and Q'). Collectively, our results revealed that the MHV-3 virus invaded (Fig. 3 E, F, H, and I), and induced early signs of MNs degeneration.

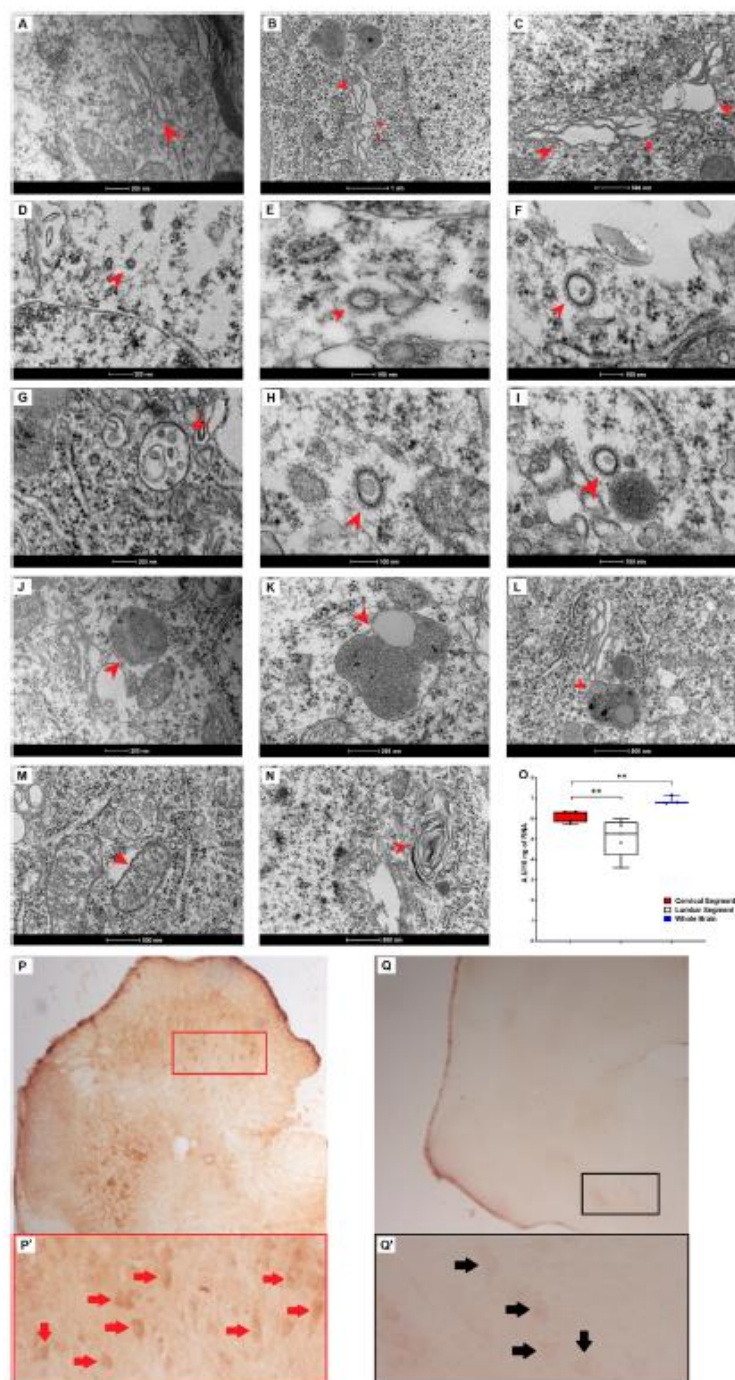


Fig. 3. MHV-3-infected mice present signs of MNs degeneration and particle-like virus at the ultrastructure level. (A-C) Representative image of dilated Golgi apparatus cisternae. (D) Representative image of clathrin-coated vesicles. (E, F, H and I) Representative image of the viral-like particle. (G) Representative image of multi-vesicular bodies. (J-L) Representative images of lipofuscin granules. (M) Representative image of altered mitochondria with warped external and internal membranes. (N) Representative image of membrane spirals. (O) qPCR for MHV-3 genetic material in cervical and lumbar segments of the spinal cord and in the brain. (P and P') Representative images of dsRNA immunostaining in spinal cord (red arrows highlight MN positive for replicative RNA for MHV-3-infected mice. (Q and Q') Representative images of dsRNA immunostaining in spinal cord (black arrows highlight MN negative for dsRNA for mock mice. $n = 5$ mice per group for TEM, $n = 5$ mice per group for cervical and lumbar segments of spinal cord and $n = 3$ mice for brain for qPCR, and $n = 4$ mice per group in immunohistochemistry for replicative RNA.

3.4. MHV-3 infected mice exhibit different signs of NMJ degeneration

Motoneurons communicate with skeletal muscles by their nerve endings named neuromuscular junctions (NMJs). To assess whether MNs changes in MHV-3 infected mice are followed by NMJs degeneration, we analyzed their features in various muscles with different biochemical properties and innervated by motor neurons (MNs) from different spinal cord segments. Therefore, we analyzed the following muscles: I- fast-twitching fibers EDL (extensor digitorum longus); II- the slow-twitching fibers SOL (soleus); III- and the hybrid fast-twitching fibers STM (sternomastoides) from both MHV-3 infected (5 dpi) and control mice.

We observed that neither the fast EDL (MOCK pre-synaptic: $410.5 \pm 205.3 \mu\text{m}^2$, MHV-3 pre-synaptic: $417.4 \pm 171.1 \mu\text{m}^2$, $p = 0.5932$; MOCK post-synaptic: $397.7 \pm 201.2 \mu\text{m}^2$, MHV-3 post-synaptic: $420.0 \pm 173.8 \mu\text{m}^2$, $p = 0.8781$) nor the mixed STM (MOCK pre-synaptic: $330.2 \pm 169.7 \mu\text{m}^2$, MHV-3: $379.1 \pm 178.4 \mu\text{m}^2$, $p = 0.2947$; MOCK post-synaptic: $351.5 \pm 157 \mu\text{m}^2$, MHV-3 post-synaptic: $390.5 \pm 203.5 \mu\text{m}^2$, $p = 0.3583$) muscles showed signs of NMJs atrophy in MHV-3 infected mice compared to mock counterparts (Fig. 4 A-B'' and 4 E-F'', respectively). In contrast, NMJs in SOL muscles showed significant alterations when comparing MHV-3 infected and mock mice in the area of pre (MOCK: $343.0 \pm 130.0 \mu\text{m}^2$, MHV-3: $297.0 \pm 143.9 \mu\text{m}^2$, $p = 0.0273$) and postsynaptic (MOCK: $348.4 \pm 111.0 \mu\text{m}^2$, MHV-3: $313.4 \pm 127.3 \mu\text{m}^2$, $p = 0.0326$) elements (Fig. 4 C-C'' and 4 D-D'', respectively).

The colocalization index between the pre- and postsynaptic elements was found to be different between MHV-3 and mock mice in the EDL but not in the other muscles (SOL and STM). For example, the EDL pre- and postsynaptic elements were significantly less colocalized than those from mock animals (MOCK: $96.97 \pm 5.61\%$, MHV-3: $94.19 \pm 6.97\%$, $p = 0.0004$) (Fig. 4 G).

The NMJs fragmentation index was assessed by measuring the α -bungarotoxin staining of nicotinic acetylcholine receptor (nAChR) in MHV-3 and mock mice. We found that in the NMJs from EDL muscle, α -bungarotoxin staining showed signs of discontinuity forming islands of fluorescent staining and were significantly more fragmented in the MHV-3 mice than in the mock ones (MOCK: $3.550 \pm 5.02\%$, MHV-3: $37.06 \pm 9.35\%$, $p = 0.0052$) (Fig. 5 A-A''). Similar findings were also observed in NMJs from STM of MHV-3 versus mock mice (MOCK: $1.385 \pm 1.95\%$, MHV-3: $20.03 \pm 8.75\%$, $p = 0.0480$) (Fig. 5 C-C''). On the contrary, NMJs from SOL (MOCK: $2.133 \pm 3.69\%$, MHV-3: $15.26 \pm 11.75\%$, $p = 0.1171$) (Fig. 5 B-B'') muscles did not show signs of fragmentation in both MHV-3 and mock mice. Altogether, these analyses showed significant NMJ degeneration in the MHV-3 infected animals, similar to those present in others neuromuscular diseases.

3.5. Morphometric analyses show a reduction in the cross-section area of fast and slow-twitching muscles in MHV-3 infected mice

Changes in skeletal muscle myofiber size and of myosin heavy chain isoforms expression are frequently observed in several neuromuscular diseases. Given our observations in MHV-3 infected mice signs of motoneuron degeneration in lumbar spinal cord and NMJs fragmentation, we next asked whether these alterations could cause any skeletal muscle atrophy. We measured muscle fiber sizes (cross-sectional area, CSA) of EDL, STM and SOL muscles stained with WGA (see Methods).

EDL (Fig. 6 A-A'') and SOL (Fig. 6 B-B'') myofibers from MHV-3 infected mice depicted significantly reduced CSAs when compared to the mock mice (EDL CSA: MOCK: $873.9 \pm 114.3 \mu\text{m}^2$, MHV-3: $457.3 \pm 113.3 \mu\text{m}^2$, $p = 0.0049$; SOL CSA: MOCK: $1102 \pm 197 \mu\text{m}^2$, MHV-3: $836.6 \pm 81.26 \mu\text{m}^2$, $p = 0.0471$). The STM muscle, on the other hand, did not show significant changes in the CSAs from MHV-3 infected mice at 5 dpi with respect to control (data not shown).

3.6. MHV-3 infection changes myofiber composition in EDL and SOL muscles

We then investigated whether muscle fiber type switching could also contribute to the decay in motor function observed in MHV-3 infected mice. We focused on EDL and SOL muscles to investigate fiber type switching because of their well-known cellular architecture (Bloemberg and Quadrilatero, 2012), and importantly, because both muscles showed significant alterations at the NMJ level and MNs in the MHV-3 infected mice. Thus, using antibody labeling against specific muscle fibers (see Methods), we compared the overall percentages of type I, IIA, IIX, and IIB muscle fibers among MHV-3 infected and mock mice.

The muscle fiber type-specific labeling showed that the EDL muscle from MHV-3 infected mice presented a significant switch towards slow muscle fibers, with a reduced number of type IIB fibers while increasing the percentage of type IIA cells (type IIA %: MOCK: 28.78 ± 4.3 , MHV-3: 56.63 ± 6.18 , $p = 0.0031$; type IIB %: MOCK: 30.77 ± 14.23 , MHV-3: 2.03 ± 1.48 , $p = 0.0253$) (Fig. 6 D). Interestingly, an opposite switch occurred in the SOL muscle from the MHV-3 mice with respect to control (type I %: MOCK: 49.33 ± 7.41 , MHV-3: 19.76 ± 10.19 , $p = 0.0153$; type IIB %: MOCK: 0.00 ± 0.00 , MHV-3: 12.41 ± 5.95 , $p = 0.0230$), a significant reduction in the percentage of type I fibers while the number of type IIB fibers increased (Fig. 6 G). In summary, EDL, a fast-twitching muscle, switched its fiber composition toward a slow-twitching profile while SOL, a slow-twitching muscle, switched its fiber composition toward a fast-twitching profile.

The next step was to examine whether the CSA of the specific fiber types in each muscle EDL and SOL differed between MHV-3 infected and mock mice. Fiber type I, IIA and IIX from MHV-3 mice EDL muscle showed a significant reduction in CSA when compared to mock fibers (type I CSA: MOCK: $417.5 \pm 282.9 \mu\text{m}^2$, MHV-3: $246.0 \pm 379.4 \mu\text{m}^2$, $p = 0.0002$; type IIA CSA: MOCK: $720.5 \pm 309.5 \mu\text{m}^2$, MHV-3: $202.0 \pm 238.3 \mu\text{m}^2$, $p < 0.0001$; type IIX CSA: MOCK: $567.0 \pm 419.0 \mu\text{m}^2$, MHV-3: $241.0 \pm 132.7 \mu\text{m}^2$, $p < 0.0001$) (Fig. 6 E). There was no alteration in IIB fiber CSA among MHV-3 and mock mice, although the total number of IIB positive fibers in the EDL from MHV-3 mice was almost negligible, unusual for a muscle like the EDL (Bloemberg and Quadrilatero, 2012). In the SOL muscle, the CSAs of type IIA and IIX fibers from MHV-3 mice were also reduced when compared to mock mice (type IIA CSA: MOCK: $1284.0 \pm 623.4 \mu\text{m}^2$, MHV-3: $473.0 \pm 420.5 \mu\text{m}^2$, $p < 0.0001$; type IIX CSA: MOCK: $834.0 \pm 210.3 \mu\text{m}^2$, MHV-3: $670.0 \pm 347.1 \mu\text{m}^2$, $p = 0.0003$; type IIB CSA: MOCK: $0.00 \pm 0.00 \mu\text{m}^2$, MHV-3: $1137.0 \pm 313.18 \mu\text{m}^2$, $p = 0.0002$) (Fig. 6 H). As expected, the number of type IIB fibers present in the mock mice was minimal, but in the MHV-3 mice the number of type IIB fibers was significantly high; again, unusual for a muscle like SOL (Bloemberg and Quadrilatero, 2012).

3.7. MHV-3 infection altered IL-6 myokine expression in a muscle-type specific manner

To determine whether signaling factors produced by skeletal muscle fibers (myokines) may contribute to muscle atrophy observed in MHV-3 infected mice, we performed ELISA assays for classical factors such as IL-6, BDNF, and irisin.

We observed no significant changes in the expression levels of BDNF and irisin in both EDL and SOL muscles from either MHV-3 infected or mock mice (data not shown). On the other hand, higher levels of IL-6 were found in the EDL muscles of MHV-3 infected mice compared to mock animals (MOCK: $54.33 \pm 17.18 \text{ pg/mg of tissue}$, MHV-3: $103.2 \pm 45.10 \text{ pg/mg of tissue}$, $p = 0.0356$) (Fig. 6 F). Conversely, a significant decrease in IL-6 concentration was observed in the SOL muscles of MHV-3 infected mice (MOCK: $61.41 \pm 11.74 \text{ pg/mg of tissue}$, MHV-3: $47.37 \pm 6.52 \text{ pg/mg of tissue}$, $p = 0.0226$) (Fig. 6 I). These results suggest that changes in IL-6 levels in skeletal muscles from MHV-3 infected mice are altered depending on the biochemical properties of the muscle.

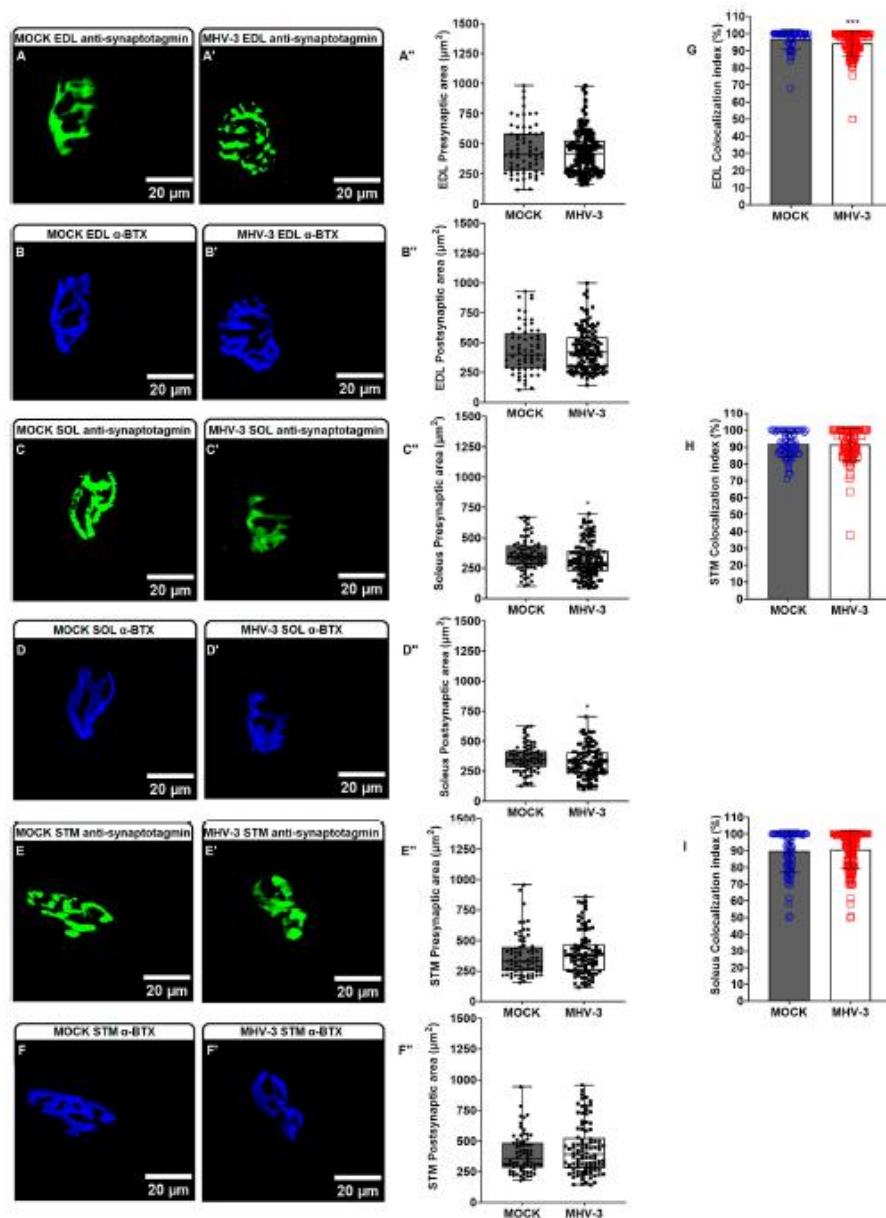


Fig. 4. MHV-3-infected mice showed alterations in the NMJ area and synaptic elements colocalization. (A-A') Representative images of EDL mock and MHV-3 pre-synaptic elements. (A'') Quantification of pre-synaptic element area from EDL muscle. (B-B') Representative images of EDL mock and MHV-3 post-synaptic elements. (B'') Quantification of post-synaptic element area. (C-C') Representative images of SOL mock and MHV-3 pre-synaptic elements. (C'') Quantification of pre-synaptic element area from SOL muscle. (D-D') Representative images of SOL mock and MHV-3 post-synaptic elements. (D'') Quantification of post-synaptic element area from SOL muscle. (E-E') Representative images of STM mock and MHV-3 pre-synaptic elements. (E'') Quantification of pre-synaptic element area from STM muscle. (F-F') Representative images of STM mock and MHV-3 post-synaptic elements. (F'') Quantification of post-synaptic element area from STM muscle. (G-I) Quantification of synaptic elements colocalization from EDL, SOL and STM, respectively. $n = 4$ animals for mock and $n = 6$ animals for MHV-3 and a minimum of 20 NMJ per animal were analyzed. Values were compared between groups by Mann-Whitney test (median $\pm$ SD). For MHV-3 compared to mock group * $p < 0.05$; *** $p < 0.0005$.

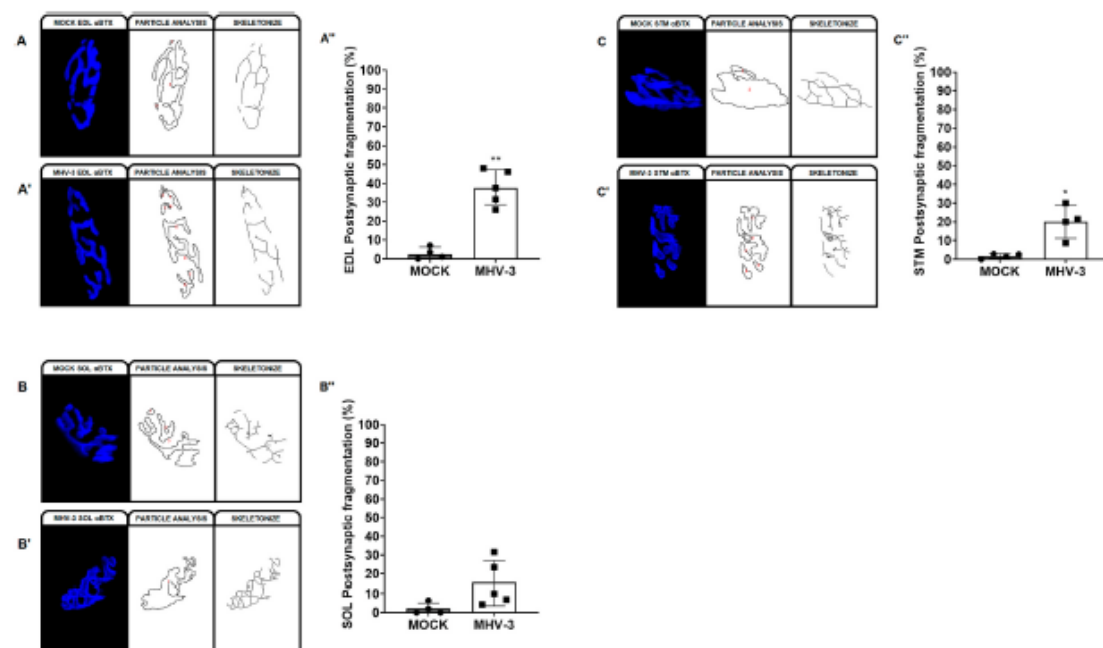


Fig. 5. NMJs from MHV-3-infected mice showed signs of fragmentation. (A-A') Representative images of EDL mock and MHV-3 NMJ fragmentation islands. (A'') Fragmentation index quantification. (B-B') Representative images of SOL mock and MHV-3 NMJ fragmentation islands. (B'') Fragmentation index quantification. (C-C') Representative images of STM mock and MHV-3 NMJ fragmentation islands. (C'') Fragmentation index quantification. $n = 4$ animals for mock and $n = 6$ animals for MHV-3 for EDL and SOL and $n = 4$ animals for mock and MHV-3 for STM. A minimum of 20 NMJ per animal were analyzed. Values were compared between groups by unpaired Student's *t*-test and results are presented as mean $\pm$ SD. For MHV-3 compared to mock group * $p < 0.05$; ** $p < 0.005$.

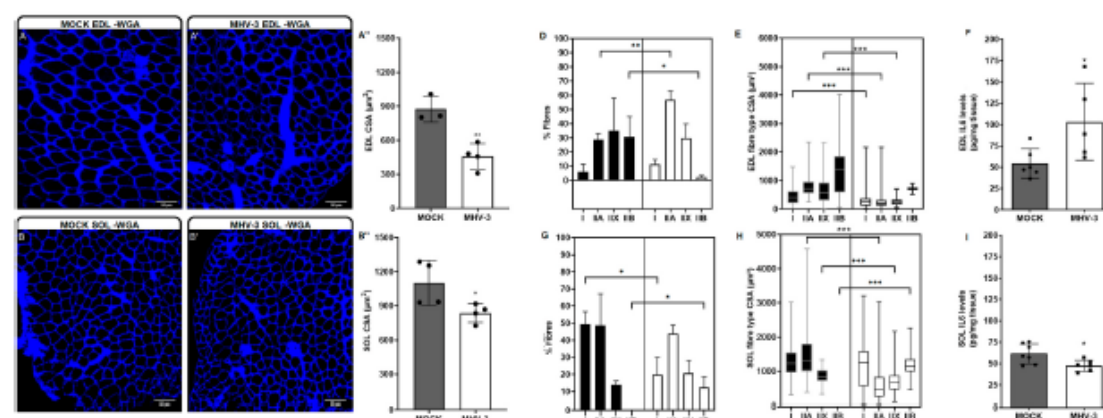


Fig. 6. MHV-3-infected mice presented muscle atrophy and fiber type switch that are accompanied by changes in muscle chemokines. (A-A') Representative images of EDL mock and MHV-3 muscle CSA. (A'') Quantification of CSA from EDL muscle ($n = 3$ animals for mock and $n = 4$ animals for MHV-3. Bars represent the mean of 1908 muscle fibers analyzed per group). (B-B') Representative images of SOL mock and MHV-3 muscle CSA. (B'') Quantification of CSA from SOL muscle ($n = 4$ animals for mock and MHV-3. Bars represent the mean of 1223 muscle fibers analyzed for mock and 1825 muscle fibers analyzed for MHV-3). (D and E) EDL % of fibers and CSA of specific fiber type, respectively. (F) EDL quantification of IL6 levels ($n = 6$ animals for mock and $n = 5$ animals for MHV-3). (G and H) SOL % of fibers and CSA of specific fiber type, respectively. (I) SOL quantification of IL6 levels ($n = 7$ animals for mock and 6 animals for MHV-3). Values were compared between groups by unpaired Student's *t*-test (mean $\pm$ SD) or Mann-Whitney test (median $\pm$ SD). For MHV-3 compared to mock group * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$.

4. Discussion

COVID-19 is now recognized as a complex disorder involving many systems in the human body. Here, using MHV-3-infected mice, a mouse model for COVID-19 (Andrade et al., 2021), we showed that this virus causes significant reduction in motor function as early as 3 dpi. The MHV-3 motor dysfunction can be explained, at least in part, by MNs and NMJ degeneration accompanied by muscle atrophy. Our results provide original evidence at the cellular structure level that contribute to a better understanding of typical symptoms and signs – i.e., muscle weakness, fatigue, myalgia, and muscle injury – observed in many patients infected with SARS-CoV-2 (Wu et al., 2020; Sun et al., 2020; Lahiri et al., 2020; Mao et al., 2020). For instance, Mao et al. (2020) showed that of 214 patients with COVID-19 (Wuhan, China), 23 presented skeletal muscle injury (17 classified as severe cases). Andrade-Junior et al. (2021) followed 32 critically ill patients for 10 days and found that nine showed a significant reduction in the handgrip test when comparing the first and tenth day of the trial. It is important to mention that 23 patients could not complete the task because of sedation or assisted ventilation, suggesting that the number of patients with muscle strength alterations may be even higher. In another clinical study with 41 patients, 56% and 73% of them showed quadriceps and biceps weakness, respectively (Paneroni et al., 2021).

In this study, we examined the motor capacity of MHV-3 infected mice using open field, wire hang, and grip force tests. Infected animals performed much worse at 5 dpi than at 3 dpi in all motor behavior tests. It is important to mention that this is not associated with sickness-behavior, once we used locomotor activity to measure it, and found no early alterations in the open field test but a reduction in the forelimb grip strength at 3 dpi. We, thus, hypothesized that changes in the motor unit components could be related to motor impairment. Next, we investigated MNs from both the cervical and lumbar segment of the spinal cord and found a reduction in their number in the lumbar segment from MHV-3-infected mice.

Fractalkine (CX3CL1) is expressed predominantly on neurons and mediates microglia activation through its receptor (CX3CR1). CX3CL1 is reported to have neuroprotective and anti-inflammatory effects in several animal models of neurodegenerative diseases, probably due to its key role in the neuron-glia crosstalk (Sheridan and Murphy 2013; Subbarayan et al., 2022). For example, in traumatic brain and spinal cord injury, disruption in CX3CL1/CX3CR1 signaling affects neuronal survival contributing to the pathogenesis of these and other CNS-related disorders (Poniatowski et al., 2017; Subbarayan et al., 2022; Pottorf et al., 2022). Because we observed here a significant reduction in both MNs number and fractalkine levels in spinal cords from MHV-3 infected mice, we can speculate that this could be related to an attempt to stimulate microglial activation, enhancing secretion of inflammatory mediators, leading to neuronal loss (Poniatowski et al., 2017).

Because SARS-CoV-1, SARS-CoV-2 and MHV-3 neuroinvasion has been reported previously (McCray et al., 2007; Netland et al., 2008; Song et al., 2021) we wondered if MHV-3 is also able to infect the CNS, especially the spinal cord ventral horn where MNs are located. Previous report has shown that MHV-3 viruses are able to cross the blood brain barrier and infect the brain at the optical microscopy level (Bleau et al., 2015). Here, we confirmed the presence of the MHV-3 virus RNA in the brains and spinal cords of infected mice by qPCR, replicative virus RNA using immunohistochemistry, and we identified virus-like structures inside spinal cord MNs at the TEM level. We also found organelles alterations in MHV-3 infected MNs such as Golgi cisternae dilation, lipofuscin granules, membrane spirals, that were not described before and that may be related to consequences of direct viral invasion of the CNS.

Considering that the MNs connect to skeletal muscle fibers by their final portion, the motor end plates (NMJs), we asked if those structures were also affected in a set of muscles from MHV-3 infected mice. We have previously shown that MHV-3 infected mice showed structural changes such as reduction in the NMJs sizes and an increase in the

fragmentation index in the diaphragm, a muscle essential for breathing (Andrade et al., 2021). Here, we found alterations in the NMJs area from SOL, co-localization index from EDL and fragmentation on EDL and STM neuromuscular junctions that could also interfere with neuromuscular communication in these muscles. Because MNs and their NMJs regulate skeletal muscle contraction, we analyzed some myofiber features such as CSA and fiber type in limb muscles from MHV-3 infected mice. We found EDL and SOL muscles atrophy and fiber type switching in MHV-3 infected mice compared to control. Patients with COVID-19 may present a decrease in CSA of muscles, such as rectus femoris (Andrade-Junior et al., 2021). Indeed, Suh et al. (2021) showed that post-mortem samples from human patients presented type 2 fiber atrophy in 32 out of 35 patients. To our knowledge, our study is the first to address systematically the fiber type composition and individual fiber CSA in a COVID-19 mice model. Fiber type switch is present in several neuromuscular diseases (Glaser and Suzuki 2018; Molotky et al., 2022), consisting of a common compensatory muscle response. Improving muscle function plays a significant role in the recovery from critical illness either by increasing muscle mass or strength (Wolfe, 2006). In addition, it highly correlates with the length of stay in the hospital in cases of moderate to severe COVID-19 (Gill et al., 2021).

In addition to muscle fiber type switching and atrophy, we also found alterations in IL-6 expression in EDL and SOL muscles. It is already known that different muscles with different biochemical properties, for example, EDL and SOL, have different IL-6 levels, with slow-oxidative muscles producing more IL-6 than fast-glycolytic (Liang et al., 2018). Once we found fiber type switching in EDL and SOL, this can be one explanation for IL-6 changes in the same muscles, with EDL shifting towards slow-oxidative profile and SOL shifting towards fast-glycolytic profile. Moreover, in sepsis animal models, IL-6 plays an important role in muscle atrophy and KO mice for IL-6 have attenuated muscle atrophy (Yang et al., 2022; Zanders et al., 2022). Other molecular determinants could also be related to muscle function impairment and muscle atrophy (Disser et al., 2020).

In conclusion, to our knowledge, this is the first study providing evidence of significant alterations in the motor behavior accompanied by motor unit changes induced by MHV-3 infection, a mice model for COVID-19, that might help to explain some motor alterations seen in COVID-19 patients. We cannot assure which component of the motor unit is altered first, whether the damage starts at the MNs side and continues toward the NMJs and muscle or if it starts at the muscle levels progressing towards the spinal cord. Further experiments are necessary to identify cellular and molecular mechanisms involved in neuromuscular degeneration induced by MHV-3.

Author statement

Leonardo Rossi: Conceptualization, Experimentation, Analysis, Writing; Kivia B.S. Santos: Experimentation, Analysis; Barbara I.S. Mota: Experimentation, Analysis; Jordane Pimenta: Experimentation; Bruna Oliveira: Experimentation, Analysis; Caroline A. Machado: Experimentation, Analysis; Heliana B. Fernandes: Experimentation; Leticia A. Barbosa: Experimentation; Hermann A. Rodriguez: Experimentation, Analysis; Gabriel H.M. Teixeira: Experimentation; Gabriel A. Gomes-Martins: Experimentation; Gabriel F. Chaimowicz: Experimentation, Writing; Celso Martins Queiroz-Junior: Experimentation; Ian Chaves: Experimentation; Juan C. Tapia: Analysis and Writing; Mauro M. Teixeira: Writing; Vivian V. Costa: Conceptualization, Experimentation, Analysis, Writing, Reviewing; Aline S. Miranda: Conceptualization, Experimentation, Analysis, Writing, Reviewing; Cristina Guatimosim: Conceptualization, Experimentation, Analysis, Writing, Reviewing.

Declaration of competing interest

We have no conflict of interest to declare.

Data availability

Data will be made available on request.

Acknowledgements

The authors would like to acknowledge the Center of Acquisition and Processing of Images (CAPI) – ICB – Universidade Federal de Minas Gerais and Microscopy Center at Universidade Federal de Minas Gerais for providing the equipment and technical support for experiments involving confocal and electron microscopy respectively. The authors thank Dr. Nathalia Augusta de Oliveira for her assistance in statistical analysis of the qPCR assay. This work was supported by grants from CNPq (# 402567/2021-3) FAPEMIG (APQ-00092-13), International Society for Neurochemistry and Chan Zuckerberg Initiative. This work also received financial support from the National Institute of Science and Technology in Dengue and Host-microorganism Interaction (INCT dengue), a program funded by The Brazilian National Science Council (CNPq, Brazil process 465425/2014-3) and Minas Gerais Foundation for Science (FAPEMIG, Brazil process 25036/2014-3) and by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil) – process 88881.507175/2020-01. The authors also thank the PhD scholarship provided by CAPES to JP and IC under project process 88881.507175/2020- n.

References

- Albuquerque, N., Baig, E., Ma, X., Zhang, J., He, W., Rowe, A., Habal, M., Liu, M., Shalev, I., Downey, G.P., Górczynski, R., Butany, J., Leibowitz, J., Weiss, S.R., McGilvray, I.D., Phillips, M.J., Fish, E.N., Levy, G.A., 2006. Murine hepatitis virus strain 1 produces a clinically relevant model of severe acute respiratory syndrome in A/J mice. *J. Virol.* 80, 10382–10394. <https://doi.org/10.1128/JVI.00747-06>.
- Andrade, A.C.S.P., Campolina-Silva, G.H., Queiroz-Junior, C.M., de Oliveira, L.C., Lacerda, L. de S.B., Gaggino, J.C.P., de Souza, F.R.O., de Mello, L., Passos, I. B., Teixeira, D.C., Elitencourt-Silva, P.G., Valadão, P.A.C., Rossi-Oliveira, L., Antunes, M.M., Figueiredo, A.F.A., Wnuk, N.T., Temerozo, J.R., Ferreira, A.C., Cramer, A., Oliveira, C.A., Durães-Carvalho, R., Weis, A.M., Guimarães, P.P.G., Costa, G.M.J., de Menezes, G.B., Guatimosim, C., da Silva, G.S.F., Souza, T.M.L., Barrioni, R.R., Pereira, M. de M., de Sousa, L.P., Teixeira, M.M., Costa, V.V., 2021. A Biosafety Level 2 Mouse Model for Studying Betacoronavirus-Induced Acute Lung Damage and Systemic Manifestations. *J. Virol.* 95. <https://doi.org/10.1128/JVI.01276-21>.
- Andrade-Junior, M.C. de, Salles, I.C.D. de, de Brito, C.M.M., Pastore-Junior, L., Righetti, R.F., Yamaguti, W.P., 2021. Skeletal muscle wasting and function impairment in intensive care patients with severe COVID-19. *Front. Physiol.* 12, 1–13. <https://doi.org/10.3389/fphys.2021.640973>.
- Bleau, C., Filloil, A., Samson, M., Lamontagne, L., 2015. Brain invasion by mouse hepatitis virus depends on impairment of tight junctions and beta interferon production in brain microvascular endothelial cells. *J. Virol.* 89, 9896–9908. <https://doi.org/10.1128/JVI.01501-15>.
- Bloembergen, D., Quadrilatero, J., 2012. Rapid determination of myosin heavy chain expression in rat, mouse, and human skeletal muscle using multicolor immunofluorescence analysis. *PLoS One* 7, e35273. <https://doi.org/10.1371/journal.pone.0035273>.
- Bourgonje, A.R., Abdulle, A.E., Timens, W., Hillebrands, J., Navis, G.J., Gordijn, S.J., Bolling, M.C., Dijkstra, G., Voors, A.A., Osterhaus, A.D.M.E., Voort, P.H., Mulder, D. J., Goor, H., 2020. Angiotensin-converting enzyme 2 (ACE2), SARS-CoV-2 and the pathophysiology of coronavirus disease 2019 (COVID-19). *J. Pathol.* 251, 228–248. <https://doi.org/10.1002/path.5471>.
- Del Valle, D.M., Kim-Schulze, S., Huang, H.-H., Beckmann, N.D., Nirenberg, S., Wang, B., Lavin, Y., Swartz, T.H., Madduri, D., Stock, A., Marron, T.U., Xie, H., Patel, M., Tuballes, K., Van Oekelen, O., Rahman, A., Kovatch, P., Aberg, J.A., Schadt, E., Jagannath, S., Mazumdar, M., Charney, A.W., Firpo-Betancourt, A., Mendu, D.R., Jhang, J., Reich, D., Sigel, K., Cordon-Cardo, C., Feldmann, M., Parekh, S., Merad, M., Gnjatic, S., 2020. An inflammatory cytokine signature predicts COVID-19 severity and survival. *Nat. Med.* 26, 1636–1643. <https://doi.org/10.1038/s41591-020-1051-9>.
- Dinzer, N.P., De Micheli, A.J., Schonk, M.M., Konnaris, M.A., Piacentini, A.N., Edon, D.L., Torresdahl, B.G., Rodeo, S.A., Casey, E.K., Mendias, C.L., 2020. Musculoskeletal consequences of COVID-19. *J. Bone Jt Surg* 102, 1197–1204. <https://doi.org/10.2106/JBJS.20.00847>.
- Drey, M., Krieger, B., Sieber, C.G., Bauer, J.M., Hettwer, S., Bertsch, T., Dahinden, P., Mäder, A., Vrijbloed, J.W., Schuster, G., Zollinger, S., Beeler, C., Unterer, T., 2014. Motoneuron loss is associated with sarcopenia. *J. Am. Med. Dir. Assoc.* 15, 435–439. <https://doi.org/10.1016/j.jamda.2014.02.002>.
- Gil, S., Jacob Filho, W., Shinjo, S.K., Ferrioli, E., Busse, A.L., Avelino-Silva, T.J., Longobardi, I., Oliveira Júnior, G.N., Swinton, P., Gualano, E., Roschel, H., 2021. Muscle strength and muscle mass as predictors of hospital length of stay in patients with moderate to severe COVID-19: a prospective observational study. *J. Cachexia Sarcopenia Muscle* 12, 1871–1878. <https://doi.org/10.1002/jcsm.12789>.
- Glaser, J., Suzuki, M., 2018. Skeletal muscle fiber types in neuromuscular diseases. In: *Muscle Cell and Tissue - Current Status of Research Field*. InTech. <https://doi.org/10.5772/intechopen.79474>.
- Głowacka, A., Ji, E., Szczepankiewicz, A.A., Skup, M., Gajewska-Woźniak, O., 2022. BDNF spinal overexpression after spinal cord injury partially protects soleus neuromuscular junction from disintegration, increasing VACHT and AChE transcripts in soleus but not tibialis anterior motoneurons. *Biomedicines* 10, 2851. <https://doi.org/10.3390/biomedicines10112851>.
- Graff Reis, J., Dai Prá, I., Michelon, W., Viancelli, A., Piedrahita Marquez, D.G., Schmitz, C., Maraschin, M., Moura, S., Thaís Silva, I., de Oliveira Costa, G., Tiziani, T., Sandjo, L.P., Rodríguez-Lázaro, D., Fongaro, G., 2022. Characterization of planktochlorella nurekii extracts and virucidal activity against a coronavirus model, the murine coronavirus 3. *Int. J. Environ. Res. Publ. Health* 19, 15823. <https://doi.org/10.3390/ijerph192315823>.
- Gransce, H.M., Mantilla, C.B., Steck, G.C., 2015. Respiratory muscle plasticity. In: *Comprehensive Physiology*. Wiley, pp. 1441–1462. <https://doi.org/10.1002/cphy.c110050>.
- Gurumurthy, C.B., Quadros, R.M., Richardson, G.P., Poluektova, L.Y., Mansour, S.L., Ohtsuka, M., 2020. Genetically modified mouse models to help fight COVID-19. *Nat. Protoc.* 15, 3777–3787. <https://doi.org/10.1038/s41596-020-00403-2>.
- Kolinsky, B., Guzman, M.S., Raulic, S., Fan, J., Magalhães, A.C., Feng, G., Gros, R., Prado, V.F., Prado, M.A.M., 2013. ChAT-ChR2-EYFP Mice Have Enhanced Motor Endurance But Show Deficits in Attention and Several Additional Cognitive Domains. *J. Neurosci.* 33, 10427–10438. <https://doi.org/10.1523/JNEUROSCI.0395-13.2013>.
- Körner, R., Majjouti, M., Alcazar, M., Mahabir, E., 2020. Of mice and men: the coronavirus MHV and mouse models as a translational approach to understand SARS-CoV-2. *Viruses* 12, 880. <https://doi.org/10.3390/v12080880>.
- Lahlri, D., Mondal, R., Deb, S., Bandyopadhyay, D., Shome, G., Sarkar, S., Biswas, S.C., 2020. Neuroinvasive potential of a primary respiratory pathogen SARS-CoV2: summarizing the evidences. *Diabetes Metab Syndr Clin Res Rev* 14, 1053–1060. <https://doi.org/10.1016/j.dsx.2020.06.062>.
- Liang, A.P., Drazick, A.T., Gao, H., Li, Y., 2018. Skeletal muscle secretion of IL-6 is muscle type specific: ex vivo evidence. *Biochem. Biophys. Res. Commun.* 505, 146–150. <https://doi.org/10.1016/j.bbrc.2018.09.042>.
- Lu, R., Zhao, X., Li, J., Niu, P., Yang, B., Wu, H., Wang, W., Song, H., Huang, B., Zhu, N., Bi, Y., Ma, X., Zhan, F., Wang, L., Hu, T., Zhou, H., Hu, Z., Zhou, W., Zhao, L., Chen, J., Meng, Y., Wang, J., Lin, Y., Yuan, J., Xie, Z., Ma, J., Liu, W.J., Wang, D., Xu, W., Holmes, E.C., Gao, G.F., Wu, G., Chen, W., Shi, W., Tan, W., 2020. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet* 395, 565–574. [https://doi.org/10.1016/S0140-6736\(20\)30251-8](https://doi.org/10.1016/S0140-6736(20)30251-8).
- Mao, L., Jin, H., Wang, M., Hu, Y., Chen, S., He, Q., Chang, J., Hong, C., Zhou, Y., Wang, D., Miao, X., Li, Y., Hu, B., 2020. Neurologic manifestations of hospitalized patients with coronavirus disease 2019 in Wuhan, China. *JAMA Neurol.* 77, 683. <https://doi.org/10.1001/jamaneurol.2020.1127>.
- McCrack, P.B., Pewe, L., Wohlford-Lenane, C., Hickey, M., Manzel, L., Shi, L., Netland, J., Jia, H., Halabi, C., Sigmund, C.D., Meyerholz, D.K., Kirby, P., Look, D.C., Perlman, S., 2007. Lethal infection of K18-hACE2 mice infected with severe acute respiratory syndrome coronavirus. *J. Virol.* 81, 813–821. <https://doi.org/10.1128/JVI.02012-06>.
- Molotsky, E., Liu, Y., Lieberman, A.P., Merry, D.E., 2022. Neuromuscular junction pathology is correlated with differential motor unit vulnerability in spinal and bulbar muscular atrophy. *Acta Neuropathol Commun* 10, 97. <https://doi.org/10.1186/s40478-022-01402-y>.
- Motta-Santos, D., dos Santos, R.A.S., Oliveira, M., Qadri, F., Foglitsch, M., Mosienko, V., Kappes Becker, L., Campagnole-Santos, M.J., M Penninger, J., Alenina, N., Bader, M., 2016. Effects of ACE2 deficiency on physical performance and physiological adaptations of cardiac and skeletal muscle to exercise. *Hypertens. Res.* 39, 506–512. <https://doi.org/10.1038/hr.2016.28>.
- Netland, J., Meyerholz, D.K., Moore, S., Cassell, M., Perlman, S., 2008. Severe acute respiratory syndrome coronavirus infection causes neuronal death in the absence of encephalitis in mice transgenic for human ACE2. *J. Virol.* 82, 7264–7275. <https://doi.org/10.1128/JVI.00737-08>.
- Paneroni, M., Simonelli, C., Saleri, M., Bertacchini, L., Venturini, M., Troosters, T., Ambrosino, N., Vitacca, M., 2021. Muscle strength and physical performance in patients without previous disabilities recovering from COVID-19 pneumonia. *Am. J. Phys. Med. Rehabil.* 100, 105–109. <https://doi.org/10.1097/PHM.0000000000001641>.
- Pawelec, P., Ziemka-Nalecz, M., Sypecka, J., Zalewska, T., 2020. The impact of the CX3CL1/CX3CR1 Axis in neurological disorders. *Cells* 9, 2277. <https://doi.org/10.3390/cells9102277>.
- Poniatowski, L.A., Woźniakiewicz, P., Krawczyk, M., Szukiewicz, D., Gasik, R., Kubaszewski, I., Kurkowska-Jastrzębska, I., 2017. Analysis of the role of CX3CL1 (fractalkine) and its receptor CX3CR1 in traumatic brain and spinal cord injury: insight into recent advances in actions of neurochemokine agents. *Mol. Neurobiol.* 54, 2167–2188. <https://doi.org/10.1007/s12035-016-9787-4>.
- Potter, T.S., Rotterman, T.M., McCallum, W.M., Haley-Johnson, Z.A., Alvarez, F.J., 2022. The role of microglia in neuroinflammation of the spinal cord after peripheral nerve injury. *Cells* 11, 2083. <https://doi.org/10.3390/cells11132083>.
- Rockx, B., Kuiken, T., Herfst, S., Bestebroer, T., Lamers, M.M., Oude Munnink, B.B., de Meulder, D., van Amerongen, G., van den Brand, J., Okba, N.M.A., Schipper, D., van Run, P., Leijten, L., Sikkema, R., Verschoor, E., Verstrepen, E., Rogers, W., Langermans, J., Drosten, C., Fentener van Vlissingen, M., Fouchier, R., de Swart, R.,

- Koopmans, M., Haagmans, B.L., 2020. Comparative pathogenesis of COVID-19, MERS, and SARS in a nonhuman primate model. *Science* (80-) 368, 1012–1015. <https://doi.org/10.1126/science.abb7314>.
- Rodríguez, H.A., Fonseca, M.D.C., Camargo, W.L., Lima, P.M.A., Martinelli, P.M., Naves, L.A., Prado, V.F., Prado, M.A.M., Guatimosim, C., 2013. Reduced expression of the vesicular acetylcholine transporter and neurotransmitter content affects synaptic vesicle distribution and shape in mouse neuromuscular junction. *PLoS One* 8, e78342. <https://doi.org/10.1371/journal.pone.0078342>.
- Sheridan, G.K., Murphy, K.J., 2013. Neuron-glia crosstalk in health and disease: fractalkine and CX3CR1 take centre stage. *Open Biol* 3, 130181. <https://doi.org/10.1098/rsob.130181>.
- Sia, S.F., Yan, L.-M., Chin, A.W.H., Pung, K., Choy, K.-T., Wong, A.Y.L., Kaeuwpreedee, P., Perera, R.A.P.M., Poon, L.L.M., Nicholls, J.M., Feiris, M., Yen, H.-L., 2020. Pathogenesis and transmission of SARS-CoV-2 in golden hamsters. *Nature* 583, 834–838. <https://doi.org/10.1038/s41586-020-2342-5>.
- Song, E., Zhang, C., Israelow, B., Lu-Culligan, A., Prado, A.V., Skirshane, S., Lu, P., Weizman, O.E., Liu, F., Dai, Y., Szegedi-Buck, K., Yasumoto, Y., Wang, G., Castaldi, C., Helke, J., Ng, E., Wheeler, J., Alfajaro, M.M., Levavasseur, E., Fontes, B., Ravindra, N.G., Van Dijk, D., Mane, S., Gunel, M., Ring, A., Kazmi, S.A.J., Zhang, K., Wilen, C.B., Horvath, T.L., Piu, L., Haik, S., Thomas, J.-L., Louvi, A., Farhadian, S.F., Huttner, A., Seilhean, D., Renier, N., Bilguvar, K., Iwasaki, A., 2021. Neuroinvasion of SARS-CoV-2 in human and mouse brain. *J. Exp. Med.* 218, 2013–2015. <https://doi.org/10.1084/jem.20202135>.
- Subbarayan, M.S., Joly-Amado, A., Bickford, P.C., Nash, K.R., 2022. CX3CL1/CX3CR1 signaling targets for the treatment of neurodegenerative diseases. *Pharmacol. Ther.* 231, 107989. <https://doi.org/10.1016/j.pharmthera.2021.107989>.
- Suh, J., Mukerji, S.S., Collins, S.J., Padera, R.F., Pinkus, G.S., Amato, A.A., Solomon, I.H., 2021. Skeletal muscle and peripheral nerve histopathology in COVID-19. *Neurology* 97, e849–e858. <https://doi.org/10.1212/WNL.0000000000002344>.
- Sun, P., Lu, X., Xu, C., Sun, W., Pan, B., 2020. Understanding of COVID-19 based on current evidence. *J. Med. Virol.* 92, 548–551. <https://doi.org/10.1002/jmv.25722>.
- Takahashi, A., Kato, K., Makino, J., Shirosaki, T., Koide, T., 2006. Multivariate analysis of temporal descriptions of open-field behavior in wild-derived mouse strains. *Behav. Genet.* 36, 763–774. <https://doi.org/10.1007/s10519-005-9038-3>.
- Tanaka, H., Ma, J., Tanaka, K.F., Takao, K., Komada, M., Tada, K., Suzuki, A., Ishibashi, T., Baba, H., Ito, T., Shigemoto, R., Ono, K., Miyakawa, T., Ikenaka, K., 2009. Mice with altered myelin proteolipid protein gene expression display cognitive deficits accompanied by abnormal neuron-glia interactions and decreased conduction velocities. *J. Neurosci.* 29, 8363–8371. <https://doi.org/10.1523/JNEUROSCI.3216-08.2009>.
- Tejeda, G.S., Esteban-Ortega, G.M., San Antonio, E., Vidaurre, Ó.G., Díaz-Guerra, M., 2019. Prevention of excitotoxicity-induced processing of BDNF receptor TrkB-FL leads to stroke neuroprotection. *EMBO Mol. Med.* 11, 1–20. <https://doi.org/10.15252/emmm.201809950>.
- Valadão, P.A.C., de Aragão, B.C., Andrade, J.N., Magalhães-Gomes, M.P.S., Foureau, G., Joviano-Santos, J.V., Nogueira, J.C., Ribeiro, F.M., Tapia, J.C., Guatimosim, C., 2017. Muscle atrophy is associated with cervical spinal motoneuron loss in EACHD mouse model for Huntington's disease. *Eur. J. Neurosci.* 45, 785–796. <https://doi.org/10.1111/ejn.13510>.
- Weiss, S.R., Leibowitz, J.L., 2011. Coronavirus pathogenesis. In: *Advances in Virus Research*. Elsevier Inc., pp. 85–164. <https://doi.org/10.1016/B978-0-12-385885-6.00009-2>.
- Winkler, E.S., Bailey, A.L., Kafai, N.M., Nair, S., McCune, B.T., Yu, J., Fox, J.M., Chen, R., Earnest, J.T., Keeler, S.P., Ritter, J.H., Kang, L.-I., Dort, S., Robichaud, A., Head, R., Holtzman, M.J., Diamond, M.S., 2020. SARS-CoV-2 infection of human ACE2-transgenic mice causes severe lung inflammation and impaired function. *Nat. Immunol.* 21, 1327–1335. <https://doi.org/10.1038/s41590-020-0778-2>.
- Wolfe, R.R., 2006. The underappreciated role of muscle in health and disease. *Am. J. Clin. Nutr.* 84, 475–482. <https://doi.org/10.1093/ajcn/84.3.475>.
- Wu, J., Liu, J., Zhao, X., Liu, C., Wang, W., Wang, D., Xu, W., Zhang, C., Yu, J., Jiang, B., Cao, H., Li, L., 2020. Clinical characteristics of imported cases of coronavirus disease 2019 (COVID-19) in Jiangsu province: a multicenter descriptive study. *Clin. Infect. Dis.* 71, 706–712. <https://doi.org/10.1093/cid/ciaa199>.
- Yang, B., Yang, X., Sun, X., Shi, J., Shen, Y., Chen, R., 2022. IL-6 deficiency attenuates skeletal muscle atrophy by inhibiting mitochondrial ROS production through the upregulation of PGC-1 α in septic mice, 2022 *Oxid. Med. Cell. Longev.* <https://doi.org/10.1155/2022/9148246>.
- Yang, Z., Du, J., Chen, G., Zhao, J., Yang, X., Su, L., Cheng, G., Tang, H., 2014. Coronavirus MHV-A59 infects the lung and causes severe pneumonia in C57BL/6 mice. *Virol. Sin.* 29, 393–402. <https://doi.org/10.1007/s12250-014-3530-y>.
- Zanders, L., Kny, M., Hahn, A., Schmidt, S., Wundersitz, S., Todiras, M., Lahmann, L., Bandyopadhyay, A., Wollensheim, T., Kaderali, L., Luft, F.C., Birckmeier, C., Weber-Carstens, S., Fielitz, J., 2022. Sepsis induces interleukin 6, gp130/JAK2/STAT3, and muscle wasting. *J. Cachexia Sarcopenia Muscle* 13, 713–727. <https://doi.org/10.1002/jcsm.12867>.

8.4 Artigo 4: A Biosafety Level 2 Mouse Model for Studying Betacoronavirus-Induced Acute Lung Damage and Systemic Manifestations.



Journal of
Virology®

PATHOGENESIS AND IMMUNITY



A Biosafety Level 2 Mouse Model for Studying Betacoronavirus-Induced Acute Lung Damage and Systemic Manifestations

Ana Cláudia dos Santos Pereira Andrade,^a Gabriel Henrique Campolina-Silva,^b Celso Martins Queiroz-Junior,^a Leonardo Camilo de Oliveira,^a Larisse de Souza Barbosa Lacerda,^a Jordane C. Pimenta,^a Filipe Resende Oliveira de Souza,^a Ian de Meira Chaves,^a Ingridy Beatriz Passos,^c Danielle Cunha Teixeira,^a Paloma Grazielle Bittencourt-Silva,^d Priscila Aparecida Costa Valadão,^a Leonardo Rossi-Oliveira,^a Maisa Mota Antunes,^a André Felipe Almeida Figueiredo,^a Natália Teixeira Wnuk,^a Jairo R. Temerozo,^{a,f} André Costa Ferreira,^{a,h,i} Allysson Cramer,^b Cleida Aparecida Oliveira,^a Ricardo Durães-Carvalho,^j Clarice Weis Arns,^j Pedro Pires Goulart Guimarães,^d Guilherme Mattos Jardim Costa,^a Gustavo Batista de Menezes,^a Cristina Guatimosim,^a Glauber Santos Ferreira da Silva,^d Thiago Moreno L. Souza,^{a,h} Breno Rocha Barrioni,^k Marivalda de Magalhães Pereira,^k Lirlândia Pires de Sousa,^l Mauro Martins Teixeira,^b Vivian Vasconcelos Costa^{a,b}

^aDepartment of Morphology, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

^bDepartment of Biochemistry and Immunology, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

^cDepartment of Microbiology, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

^dDepartment of Physiology and Biophysics, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

^eLaboratory on Thymus Research, Oswaldo Cruz Institute, Fiocruz, Rio de Janeiro, RJ, Brazil

^fNational Institute for Science and Technology on Neuroimmunomodulation, Oswaldo Cruz Foundation (Fiocruz), Rio de Janeiro, RJ, Brazil

^gNational Institute for Science and Technology on Innovation on Diseases of Neglected Populations (INCT/IDNP), Center for Technological Development in Health (CDTS), Oswaldo Cruz Foundation (Fiocruz), Rio de Janeiro, RJ, Brazil

^hImmunopharmacology Laboratory, Oswaldo Cruz Foundation (Fiocruz), Rio de Janeiro, RJ, Brazil

ⁱLaboratório de Pesquisas Pré-clínicas, Universidade Iguaçu (UNIG), Rio de Janeiro, RJ, Brazil

^jLaboratory of Virology, Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brazil

^kDepartment of Metallurgical Engineering and Materials, Federal University of Minas Gerais, School of Engineering, Belo Horizonte, Brazil

^lDepartment of Clinical and Toxicological Analysis, Faculty of Pharmacy, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

Ana Cláudia dos Santos Pereira Andrade and Gabriel Henrique Campolina-Silva contributed equally to this work. Co-first authorship order was determined based on who initiated the study.

ABSTRACT The emergence of life-threatening zoonotic diseases caused by betacoronaviruses, including the ongoing coronavirus disease 19 (COVID-19) pandemic, has highlighted the need for developing preclinical models mirroring respiratory and systemic pathophysiological manifestations seen in infected humans. Here, we showed that C57BL/6J wild-type mice intranasally inoculated with the murine betacoronavirus murine hepatitis coronavirus 3 (MHV-3) develop a robust inflammatory response leading to acute lung injuries, including alveolar edema, hemorrhage, and fibrin thrombi. Although such histopathological changes seemed to resolve as the infection advanced, they efficiently impaired respiratory function, as the infected mice displayed restricted lung distention and increased respiratory frequency and ventilation. Following respiratory manifestation, the MHV-3 infection became systemic, and a high virus burden could be detected in multiple organs along with morphological changes. The systemic manifestation of MHV-3 infection was also marked by a sharp drop in the number of circulating platelets and lymphocytes, besides the augmented concentration of the proinflammatory cytokines interleukin 1 beta (IL-1 β), IL-6, IL-12, gamma interferon (IFN- γ), and tumor necrosis factor (TNF), thereby mirroring some clinical features observed in moderate and severe cases of COVID-19. Importantly, both respiratory and systemic changes triggered by MHV-3 infection were greatly prevented by blocking TNF signaling, either via genetic or pharmacologic approaches. In line with this, TNF blockage also diminished the infection-mediated release of proinflammatory cytokines and virus replication of human

Citation Andrade ACS, Campolina-Silva GH, Queiroz-Junior CM, de Oliveira LC, Lacerda LDSB, Pimenta JC, de Souza FRQ, de Meira Chaves I, Passos IB, Teixeira DC, Bittencourt-Silva PG, Valadão PAC, Rossi-Oliveira L, Antunes MM, Figueiredo AFA, Wnuk NT, Temerozo JR, Ferreira AC, Cramer A, Oliveira CA, Durães-Carvalho R, Weis Arns C, Guimarães PPG, Costa GMJ, de Menezes GB, Guatimosim C, da Silva GSF, Souza TML, Barrioni BR, Pereira MDM, de Sousa LP, Teixeira MM, Costa VV. 2021. A biosafety level 2 mouse model for studying betacoronavirus-induced acute lung damage and systemic manifestations. *J Virol* 95:e01276-21. <https://doi.org/10.1128/JVI.01276-21>.

Editor Stacey Schultz-Cherry, St. Jude Children's Research Hospital

Copyright © 2021 American Society for Microbiology. All Rights Reserved.

Address correspondence to Vivian Vasconcelos Costa, vivianvcosta@ufmg.br.

Received 29 July 2021

Accepted 1 September 2021

Accepted manuscript posted online 8 September 2021

Published 27 October 2021

epithelial lung cells infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Collectively, results show that MHV-3 respiratory infection leads to a large range of clinical manifestations in mice and may constitute an attractive, lower-cost, biosafety level 2 (BSL2) *in vivo* platform for evaluating the respiratory and multiorgan involvement of betacoronavirus infections.

IMPORTANCE Mouse models have long been used as valuable *in vivo* platforms to investigate the pathogenesis of viral infections and effective countermeasures. The natural resistance of mice to the novel betacoronavirus SARS-CoV-2, the causative agent of COVID-19, has launched a race toward the characterization of SARS-CoV-2 infection in other animals (e.g., hamsters, cats, ferrets, bats, and monkeys), as well as adaptation of the mouse model, by modifying either the host or the virus. In the present study, we utilized a natural pathogen of mice, MHV, as a prototype to model betacoronavirus-induced acute lung injury and multiorgan involvement under biosafety level 2 conditions. We showed that C57BL/6J mice intranasally inoculated with MHV-3 develops severe disease, which includes acute lung damage and respiratory distress that precede systemic inflammation and death. Accordingly, the proposed animal model may provide a useful tool for studies regarding betacoronavirus respiratory infection and related diseases.

KEYWORDS betacoronavirus, MHV-3, lung infection, COVID-19, animal model, tumor necrosis factor

The *Betacoronavirus* genus belongs to the *Coronaviridae* family and encompasses positive-stranded enveloped RNA viruses. This viral group is broadly distributed among humans and other mammals, including rodents, bats, pigs, and ruminants (1–3). The high prevalence of betacoronaviruses all over the world, combined with their great genetic diversity and the increased human occupation of isolated ecosystems, makes the periodic emergence of novel coronaviruses in zoonotic outbreaks highly probable after host escape events (1). In fact, over the last 2 decades, there were three isolated zoonotic outbreaks of severe coronavirus infection in humans, including the currently circulating severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is responsible for the ongoing coronavirus disease 2019 (COVID-19) pandemic (4). Due to their epidemiological importance, a deeper understanding of viral biology, pathogenesis, and the discovery of therapeutic options against betacoronaviruses is imperative.

COVID-19 has a broad spectrum of clinical manifestations. While in most cases, SARS-CoV-2 respiratory infection is silent or triggers only mild respiratory symptoms, in others it leads to severe conditions, including acute respiratory distress syndrome (ARDS), systemic inflammation, multiorgan failure, and death (5, 6). Several animal models have been proposed as preclinical platforms to study the pathogenesis of SARS-CoV-2, including mice, ferrets, Syrian hamsters, and primates. (7–12). The inherent resistance of wild-type mice to SARS-CoV-2 infection led to the establishment of strategies to adapt these rodents to infection by modifying either the host or the virus (9, 13, 14). One of the most common strategies has been the infection of transgenic mice expressing human ACE2 (hACE2) using SARS-CoV-2 strains. However, these animal models in general fail to reproduce the prolonged pneumonia with severe manifestations and multiorgan impairment, besides displaying a high rate of SARS-CoV-2 replication in nontarget tissues (15, 16). Due to interspecies differences, a set of diverse models is necessary for a broader understanding of viral tropism, replication, dissemination, clinical signs, pathogenesis, and immune response caused by SARS-CoV-2 (17).

The use of other betacoronaviruses, such as the murine coronavirus, has been suggested as a strategy to emulate many of the key aspects of human coronavirus infection (18, 19). Among murine coronaviruses, the group known as murine hepatitis coronavirus (MHV) is the prototype of this genus and a natural pathogen of the *Mus musculus* species (20). MHV is capable of inducing a severe and lethal disease in mice,

and some of its variants, such as MHV-1 and MHV-A59, may have an initial pulmonary tropism when inoculated intranasally, before systemic dissemination. Thus, targeting MHV respiratory infection may constitute a promising preclinical platform to mirror betacoronavirus-induced pathophysiological features seen in humans, including severe acute respiratory syndrome (18, 19, 21). An important advantage of using this murine model is the requirement for biosafety level 2 (BSL2), which makes this model significantly less costly and safer for screening compounds of therapeutic interest.

The murine hepatitis virus strain 3 (MHV-3) has classically been described as a causative agent of severe hepatitis in several strains of mice (20). Due to its hepatotropism, the potential of this MHV strain to also cause respiratory disease during an intranasal infection has been poorly investigated. In this work, we showed that intranasal infection of wild-type mice with MHV-3 produced a transient respiratory disease with pulmonary functional impairment before leading to systemic inflammation, multiorgan injury, and death. We also showed that the MHV-3-induced pulmonary and extrapulmonary manifestations were dependent on tumor necrosis factor (TNF) signaling.

RESULTS

Intranasally delivered MHV-3 favors pulmonary viral replication in C57BL/6J mice. To characterize a mouse model of coronavirus-induced acute respiratory infection inside a BSL2 facility, 6- to 7-week-old C57BL/6J mice were inoculated via the intranasal route with 10^3 PFU of MHV-3 and monitored daily for signs of illness (Fig. 1a). Following infection, mice underwent significant weight loss, which started on the third day postinfection (dpi), and they became moribund and died by 6 dpi (Fig. 1b and c). Male and female animals succumbed similarly to the disease, as their median survival times upon MHV-3 challenge were 5.5 and 6 days, respectively. MHV-3 infection was also lethal at a lower dose (10^2 PFU); however, there was a modest delay in weight loss and death compared to that observed at a higher inoculum (Fig. 1b and c).

Intranasal infection of mice with 10^3 PFU led to progressive thrombocytopenia (Fig. 1d). The number of circulating leukocytes also decreased as the infection progressed and was mostly driven by the sharp lymphopenia observed from 3 dpi onwards (Fig. 1d and e). Thermal response to viral infection was also observed. As depicted in Fig. 1f, body temperature began to rise slowly 24 h after virus inoculation. At 2 and 3 dpi, mice reached $37.4^\circ\text{C} \pm 0.3^\circ\text{C}$ and $38.1^\circ\text{C} \pm 0.2^\circ\text{C}$, respectively, both of which were significantly higher than the average temperature recorded over 3 days before infection ($36.3^\circ\text{C} \pm 0.1^\circ\text{C}$; $P < 0.05$) (Fig. 1f and g). It is worth noting that major changes in body temperature occurred during the light phase, which is the resting period for nocturnal habit animals (Fig. 1g).

To ascertain whether intranasal delivered MHV-3 favors viral replication in the respiratory system, viral load was assessed in the lung at 1, 3, and 5 dpi and compared with that found in the plasma, liver, spleen, heart, kidney, brain, and testes. Results confirmed the lungs as the initial replication site of MHV-3, as assessed by the presence of infectious virus in this tissue, with viral loads significantly increasing from 1 dpi to 5 dpi (Fig. 1h). Conversely, viruses were recovered from 3 dpi onwards in other organs, with viremia being more stably detected at 5 dpi (Fig. 1h and i). Together, these data show that C57BL/6J mice are highly susceptible to intranasal inoculation of MHV-3 and that lungs are likely the primary site of viral infection and replication.

MHV-3 triggers inflammation-associated lung damage and respiratory dysfunction. We next investigated whether MHV-3 infection would affect lung morphology and trigger inflammation-associated tissue damage (Fig. 2a). Compared to mock controls, a higher number of cells stained for the panleukocyte marker CD45 was found in the lung sections of infected mice after 1 day and especially 3 days after inoculation (Fig. 3b). Flow cytometry results concurred with the recruitment of leukocytes to the lung tissue early during infection, showing a significant rise in the percentage of neutrophils and macrophages/monocytes. On the other hand, we found that the percentage of both CD4⁺ and CD8⁺ T cells in the lung gradually decreased as the infection progressed (Fig. 2c). The histopathologic examination of lung sections revealed that

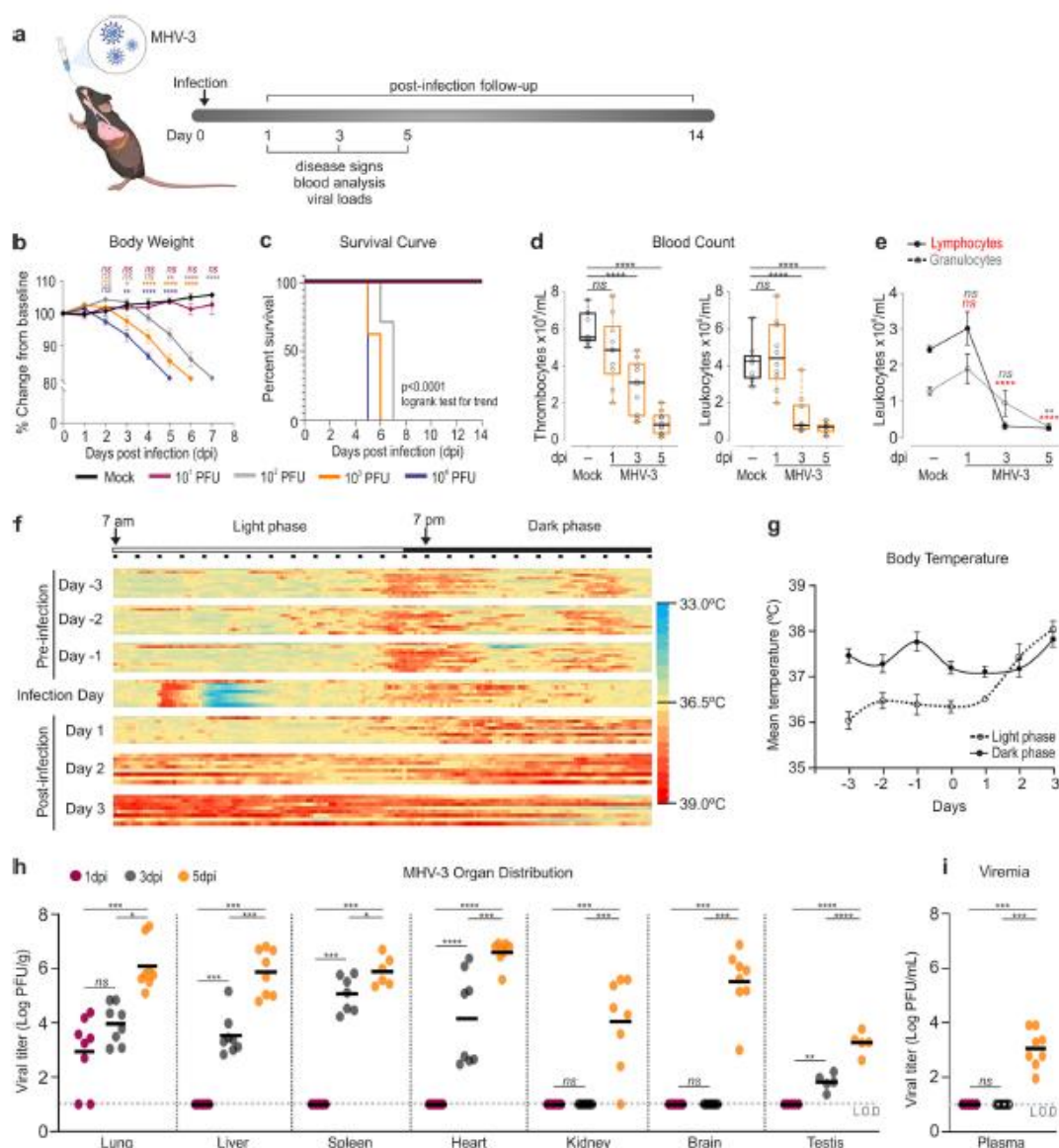


FIG 1 Intranasally inoculated MHV-3 triggers lethal disease in C57BL/6J mice. (a) Experimental design. (b) Body weight change upon infection assessed by two-way repeated-measures analysis of variance (ANOVA) plus Sidak's multiple-comparison test (mean $\pm$ standard error of the mean [SEM]; $n = 8$). (c) Kaplan-Meier survival curve of infected mice versus mock controls ($n = 8$). (d) Changes in the number of circulating thrombocytes and leukocytes over 5 days postinoculation (dpi), represented as box plots. The whiskers go from the first and third quartiles to the minimum or maximum value. Data from each infection time were compared with those from the mock group by one-way ANOVA plus Dunnett's multiple-comparison test ($n = 10$). (e) Differential blood count highlighting the sharp infection-related drop of lymphocyte counts that likely guided leukopenia noticed from 3 dpi onwards. Differences between infection groups and the mock control were assessed by one-way ANOVA plus Dunnett's multiple-comparison test (mean $\pm$ SEM; $n = 6$). (f) Heatmap showing the body temperatures recorded in a group of 10 mice over 3 days pre- and postinfection. Note an increase in the mean temperature on 2 and 3 dpi, especially in the light phase. (g) Means $\pm$ SEM of body temperatures depicted in panel f according to the time of the day. (h, i) Viral load determined in organ extracts and plasma of MHV-3-infected mice by plaque assay. The results are presented as $\log_{10}$ PFU per gram of tissue or milliliter of plasma. Differences among groups were assessed by Kruskal-Wallis plus Dunn's post hoc test ($n = 5$ to 8). LOD, limit of detection. ns, not significant ($P > 0.05$); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

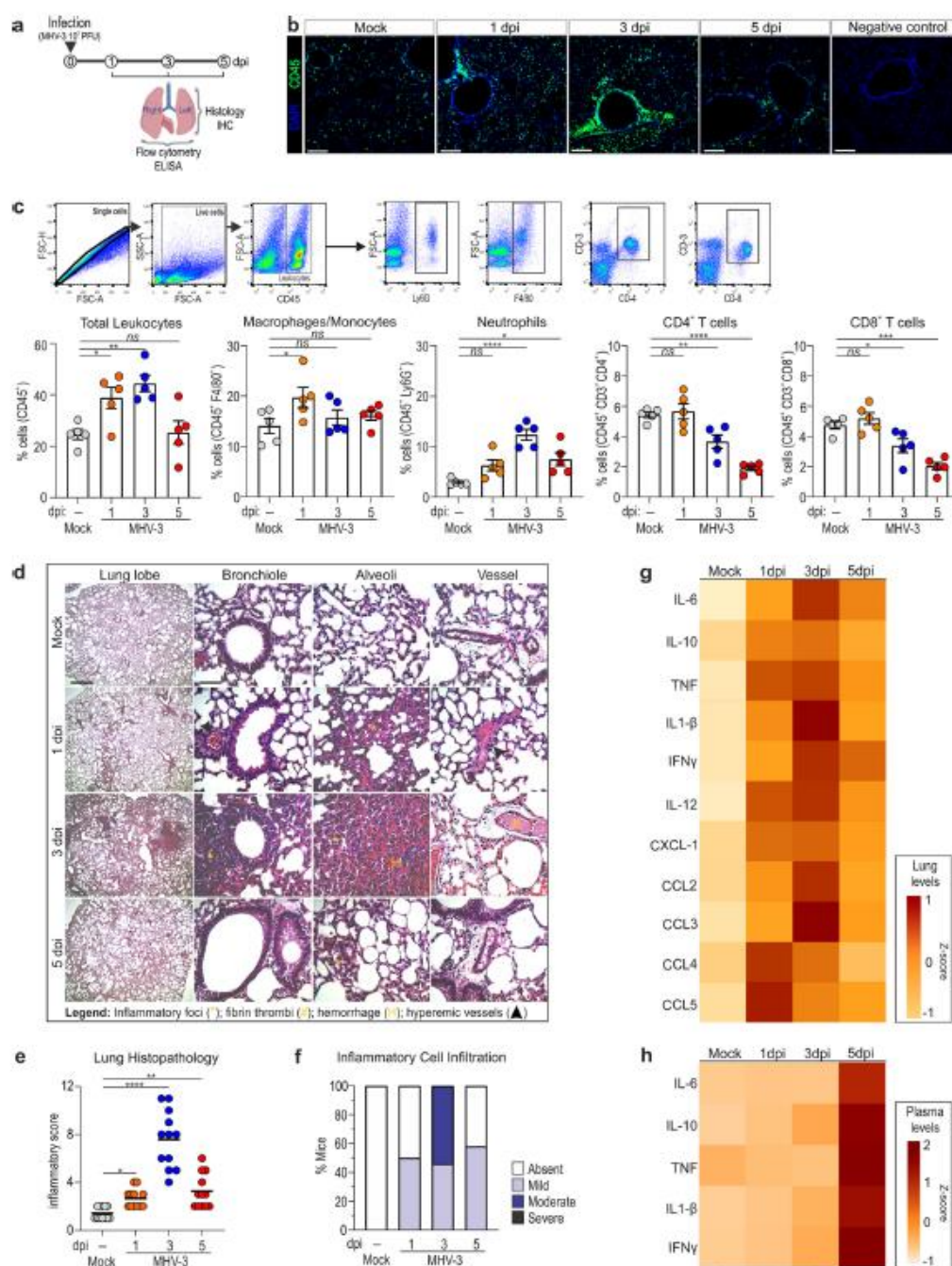


FIG 2 MHV-3 infection triggers inflammation-associated lung injury. (a) Experimental design. (b) Representative confocal three-dimensional (3D) image showing high abundance of CD45⁺ leukocytes (green staining) in the lungs of mice at 1 and 3 dpi. (c) Flow cytometry analyses showing the (Continued on next page)

MHV-3 infection triggered transient inflammation-associated lung injury. As such, approximately 50% of mice had discrete inflammatory cell infiltration in close association with areas of alveolar edema and hyperemic vessels at 1 day after MHV-3 infection (Fig. 2d to f). At 3 dpi, the inflammatory infiltrate became robust and widespread throughout the lung tissue, leading to greater histopathological changes. Inflammation foci were frequently seen in the vicinity of bronchioles, in perivascular areas, and in the lumen of hyperemic vessels (Fig. 2b, d, e, and f). Some blood vessels presented eosinophilic fibrillar material adherent to the vascular wall, which is characteristic of fibrin thrombi. In some samples, areas of necrosis and hemorrhage were also observed (Fig. 2d). Conversely, the inflammation-associated lung damage in the mouse lung at 5 dpi was milder and encompassed the changes portrayed here for day 1 after infection (Fig. 2d to f), suggesting that inflammation was resolving.

The intrapulmonary concentration of major chemokines (CCL2, CCL3, CCL4, and CCL5) and cytokines (TNF, interleukin 6 [IL-6], IL-1 β , IL-12, and gamma interferon [IFN- γ]) was markedly increased at 1 dpi and/or 3 dpi and decreased thereafter (Fig. 2g). In contrast, high levels of the inflammatory mediators (IL-6, IL-10, TNF, IL-1 β , and IFN- γ) were only detected in the blood at 5 dpi (Fig. 2h). These data suggest that the current infection model exhibits transient pulmonary pneumonia followed by systemic inflammation.

Next, we asked whether these histopathological changes could impact lung function. By using the whole-body plethysmography method, two control preinfection measurements of ventilatory parameters were performed in freely moving mice and compared with that recorded on 3 dpi (Fig. 3a). Interestingly, there was an increase in respiratory frequency in MHV-3-infected animals (Fig. 3b) that was characterized by shortened respiratory cycles (expiration/inhalation event; see Fig. 3b). In addition, the mechanics of the respiratory system were evaluated invasively in another group of mice at 3 dpi and compared to those of mock controls (Fig. 3a). There was restricted lung distention after MHV-3 infection, as determined by the lower static compliance of the respiratory system (Fig. 3c). There was also a decrease in vital capacity of the infected group (Fig. 3c). These restrictive aspects of pulmonary function are likely secondary to the pulmonary inflammation and damage. These data suggest that intranasal inoculation of MHV-3 in C57BL/6J mice triggers dysfunction of the respiratory system.

Given the pivotal role of diaphragm contraction for breathing and the recent evidence that SARS-CoV-2 might trigger diaphragm myopathy (22), we next checked the integrity of diaphragm neuromuscular junctions (NMJs) in the current infection model. By combining whole-mount confocal microscopy and computer-assisted image analysis, we showed that NMJs from MHV-3-infected mice at 3 and 5 dpi were significantly smaller and more fragmented at both pre- and postsynaptic endings compared to those of mock controls (Fig. 4). Although this deterioration of the diaphragm synaptic apparatus may not be sufficient to disrupt pulmonary ventilation in infected mice, we do not rule out the possibility that coronavirus-induced denervation at diaphragm NMJs could account for respiratory failure in the long term, which could be of particular interest for COVID-19 patients under mechanical ventilatory support.

MHV-3 induces extrapulmonary damage. MHV-3 is a murine coronavirus well-known for its hepatotropism (20). Overall, intranasally inoculated MHV-3 triggered hepatitis and liver necrosis, especially at 5 dpi (Fig. 5), the period that preceded systemic

FIG 2 Legend (Continued)

percentages among groups of total leukocytes, macrophages/monocytes, neutrophils, and CD4⁺ and CD8⁺ T cells. The percentage of each cell type at 1, 3, and 5 dpi was estimated following the representative gating scheme (upper panels) and compared with that found in the mock group by one-way ANOVA plus Dunnett's multiple-comparison test (mean $\pm$ SEM; $n = 5$). (d) Hematoxylin and eosin (H&E) staining of lung sections showing notorious signs of inflammatory injury in infected mice. Bars, 450 μ m (low magnification) and 50 μ m (high magnification). (e) Histopathological assessment in relation to overall inflammatory score. Comparisons between mock and infection groups were carried out by Kruskal-Wallis plus Dunn's post hoc test ($n = 12$). (f) Percentages of mice according to the grade of inflammatory cell infiltration. (g) Heatmap evidencing changes in the levels of cytokines and chemokines measured by enzyme-linked immunosorbent assay (ELISA) in the lung of mock controls and MHV-3-infected mice ($n = 5$). (h) Heatmap showing greatly higher levels of IL-6, IL-10, TNF, IL-1 β , and IFN- γ in the plasma of infected mice at 5 dpi ($n = 5$). ns, not significant ($P > 0.05$); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

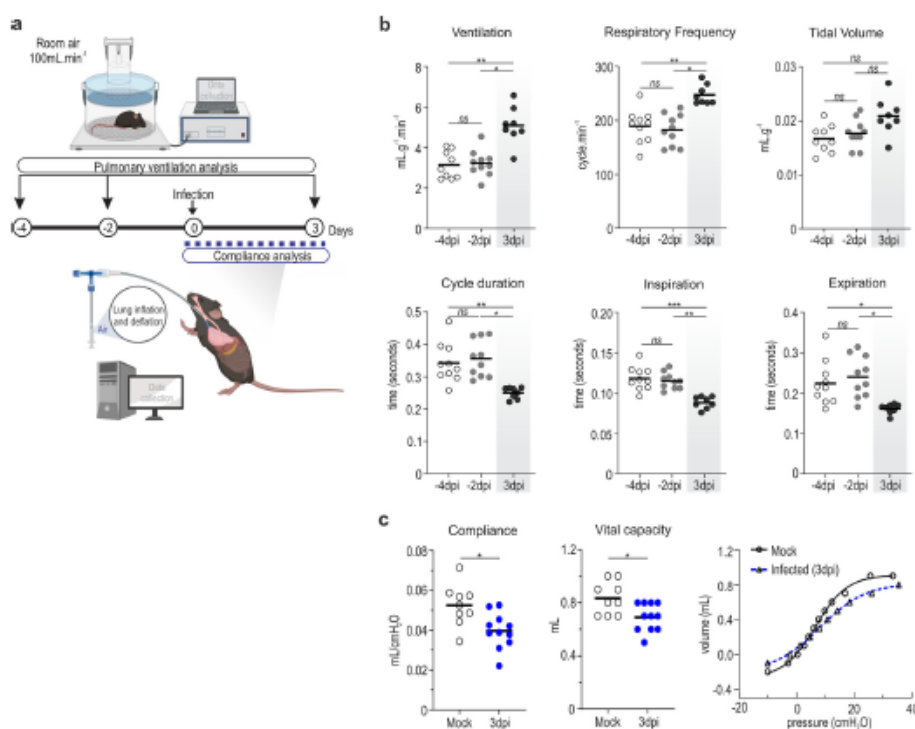


FIG 3 Intranasal infection of MHV-3 impairs lung function. (a) Experimental design. (b) Pairwise comparison of ventilatory parameters recorded at 4 and 2 days before infection (–4 and –2 dpi) and at 3 dpi. Note that mice showed a significant increase in ventilation along with shortened respiratory cycles upon MHV-3 challenge. One-way repeated measures ANOVA plus Tukey's *post hoc* test were applied to assess differences between preinfection and postinfection periods ($n = 8$). (c) Analysis of pulmonary compliance showing reduced compliance and vital capacity in infected mice compared to that in mock-infected mice (unpaired *t* test; $n = 9$ to 11). Static compliance was calculated from the steepest point of the deflation part of the pressure-volume curve (right). ns, not significant ($P > 0.05$); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

clinical manifestations and death of the animals (as shown in Fig. 1b and c). Consistent with the liver damage, liver function was heavily impaired at 5 dpi, as seen by the high serum levels of alanine aminotransferase (ALT) and the reduced hepatic ability to metabolize indocyanine dye at this time point (Fig. 5). Other organs, such as the brain, small intestine, and colon, had minimal to mild leukocyte infiltration at 3 and 5 dpi and were much less affected than the liver (Fig. 6).

Furthermore, in view of recent evidence pointing to the high susceptibility of the testes to both SARS-CoV and SARS-CoV-2 infection (23, 24), we evaluated whether MHV-3 could trigger testicular damage. Interestingly, the percentage of altered seminiferous tubules increased significantly after inoculation. The altered sites displayed epithelium sloughing, elevated germ cell apoptosis, and retention of residual bodies (Fig. 6c and d). Also, there appeared to be an increase in the volumetric density of blood vessels and lymph space in the testis interstitium of infected mice (Fig. 6d to f), although such changes did not attain statistical significance. Together, these results indicate that intranasal inoculation of MHV-3 triggers multisystem changes beyond the transient lung damage.

TNF signaling abrogation significantly reduces lung injury and improves survival in MHV-3-infected mice. Increasing concentrations of TNF have previously been associated with tissue damages triggered by MHV and human coronaviruses and are currently thought to aggravate COVID-19 severity (6, 25–27). Once this cytokine was

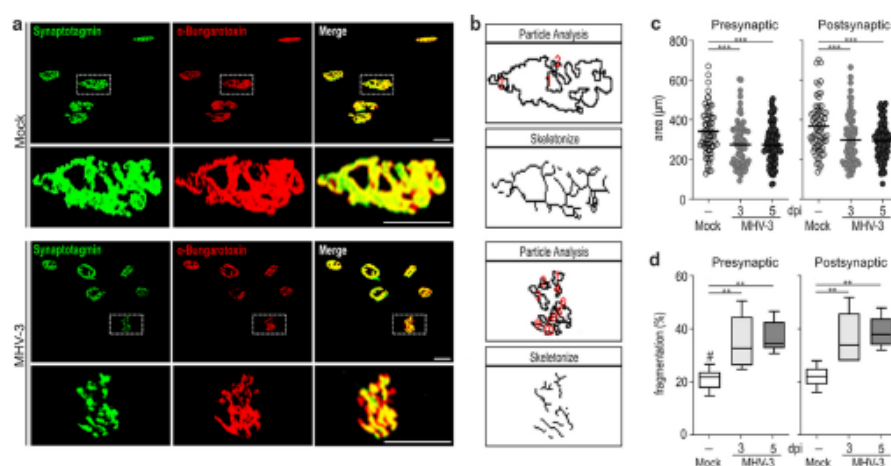


FIG 4 Neuromuscular junctions (NMJs) from diaphragm muscle are smaller and more fragmented in MHV-3-infected mice. (a) Three-dimensional confocal images from whole-mount diaphragm showing presynaptic (green) and postsynaptic terminals (red) at NMJs following 3 days of infection. Bar, 50 μm. (b) Representative images from skeletonization and particle analysis of the NMJ highlighted in figure a by dotted squares. Red numbers indicate fragmentation points. (c) Quantification of the area occupied by presynaptic and postsynaptic terminals. In total, 125 NMJs per group were considered in this analysis, and the values from infected animals were compared with those from mock controls by one-way ANOVA plus Dunnett's multiple-comparison test. (d) Quantification of the fragmentation percentages found in presynaptic and postsynaptic endings identified by particle analysis. Assessed by one-way ANOVA plus Dunnett's multiple-comparison test ($n = 5$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

found here along with other proinflammatory mediators at higher levels within the lung of infected mice (Fig. 2g), we next interrogated the contribution of TNF signaling to the respiratory pathogenesis of MHV-3 infection. To this end, we intranasally infected mice genetically deficient for TNF receptor type 1 (TNFR1, also known as p55) with 10^3 PFU of MHV-3 and compared them with wild type (WT) mice. Strikingly, TNFR1 knockout (KO) mice were protected from abrupt weight loss and lethality, with 100% of mice surviving by the end of the 14-day follow-up period (Fig. 7a and b). The progressive leukopenia and thrombocytopenia phenotype observed in infected WT mice were not found in TNFR1 KO mice (Fig. 7c). Moreover, compared to the WT group, mice in the TNFR1 KO group had significantly lower viral loads (Fig. 7d), inflammation, and injury in their lungs (Fig. 7e to g).

To verify whether such a protective profile against MHV-3 could also be achieved using pharmacological approaches, WT mice were first infected intranasally with 10^3 PFU and then treated with etanercept, a selective TNF inhibitor (Fig. 8a). Regardless of the treatment route, etanercept did not prevent, but delayed, body weight loss (Fig. 8b). Similar findings were seen when survival rates were compared among groups, with etanercept-treated mice living 3 days longer on average than the untreated ones (vehicle group) (Fig. 8c). It is worth noting that both the local and systemic treatments with etanercept were capable of inhibiting MHV-3 replication in the lungs, with infectious viruses no longer detected in the majority of animals (Fig. 8d). Consistently, the inflammation-associated lung damage and overproduction of proinflammatory cytokines triggered by MHV-3 infection were substantially prevented with etanercept treatment, irrespective of the route of administration (Fig. 8e to g). Taken together, these findings reinforce an important contribution of the TNF pathway to the pathogenesis of respiratory coronavirus infection in mice.

Blocking TNF decreases SARS-CoV-2 replication and related cellular damage and proinflammatory cytokine production in human lung cells. We next evaluated the effects of etanercept treatment upon SARS-CoV-2 infection *in vitro*. For this, the human epithelial lung cell line Calu-3 was infected with SARS-CoV-2 and then treated

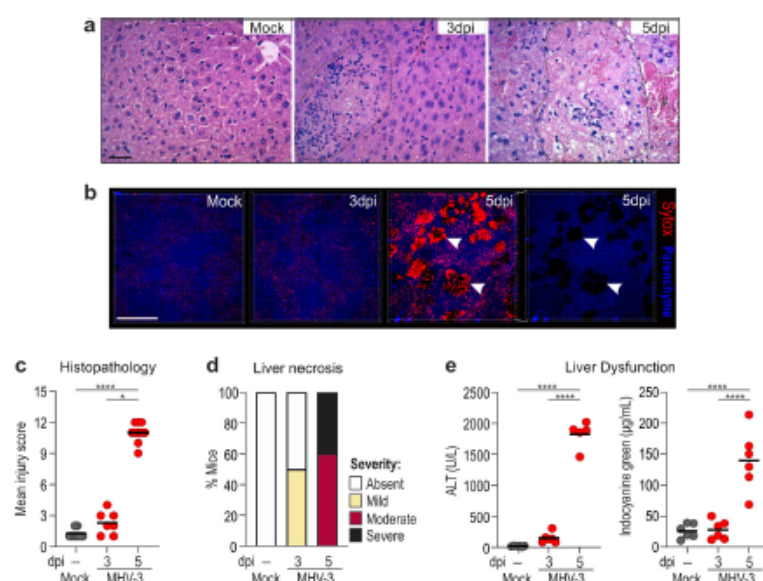


FIG 5 MHV-3 triggers liver dysfunction following 5 days of infection. (a) H&E staining of liver sections showing significant signs of tissue damage at 3 dpi and especially at 5 dpi. Areas morphologically compatible with necrosis were indicated by dotted circles. #, hemorrhage. Bar, 50 μ m. (b) Z-stack reconstruction showing extracellular DNA deposition in necrotic areas (arrowheads) identified by Sytox staining (in red). Autofluorescence (blue) was used to highlight the liver parenchyma. Bar, 300 μ m. (c) Mean injury scores compared among groups by Kruskal-Wallis plus Dunn's *post hoc* test. (d) Percentage of mice in each group according to the necrosis severity ($n = 7$ to 10). (e) Estimation of liver dysfunction analyzed by serum concentration of alanine aminotransferase (ALT) and indocyanine green (ICG) dye. Statistical differences among groups were assessed by one-way ANOVA plus Tukey's *post hoc* test ($n = 5$ or 6). ns, not significant ($P > 0.05$); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

with etanercept at different concentrations (0.5, 1, 5, and 10 ng/ml) (Fig. 9a). The results showed that etanercept treatment reduced the SARS-CoV-2-mediated cellular damage in a dose-dependent manner (Fig. 9b). Etanercept at 5 and 10 ng/ml had also a slight but statistically inhibitory effect on SARS-CoV-2 (Fig. 9c). Moreover, the overproduction of TNF, IL-8, and IL-18 triggered by SARS-CoV-2 infection was significantly reduced in Calu-3 cells treated with etanercept (Fig. 9d). These results suggest that TNF blocking by pharmacologic approaches might also be beneficial against SARS-CoV-2 infection in human lung epithelial cells.

DISCUSSION

The recent emergence and continuing spread of SARS-CoV-2 all over the world have launched a race toward the establishment of suitable *in vivo* platforms for evaluating vaccines and antiviral agents against human betacoronavirus (17). Here, we showed that the murine betacoronavirus MHV-3, despite its hepatotropism, may serve as a prototype to recapitulate, in wild-type mice, the many aspects of the respiratory disease and systemic alterations seen in patients with moderate or severe COVID-19. MHV-3 efficiently replicated in the lungs of C57BL/6J mice and triggered a robust inflammatory response, including accumulation of neutrophils and macrophages/monocytes accompanied by augmented tissue concentrations of proinflammatory cytokines and chemokines.

MHV-3 induced major lung histopathological changes, including alveolar edema, hemorrhage, and fibrin thrombi, which translated to functional alterations. Others betacoronaviruses might trigger transient pneumonia in wild-type mice, including the murine strains MHV-1, MHV-A59, and MHV-S (20, 21), as well as mouse-adapted SARS-CoV-2

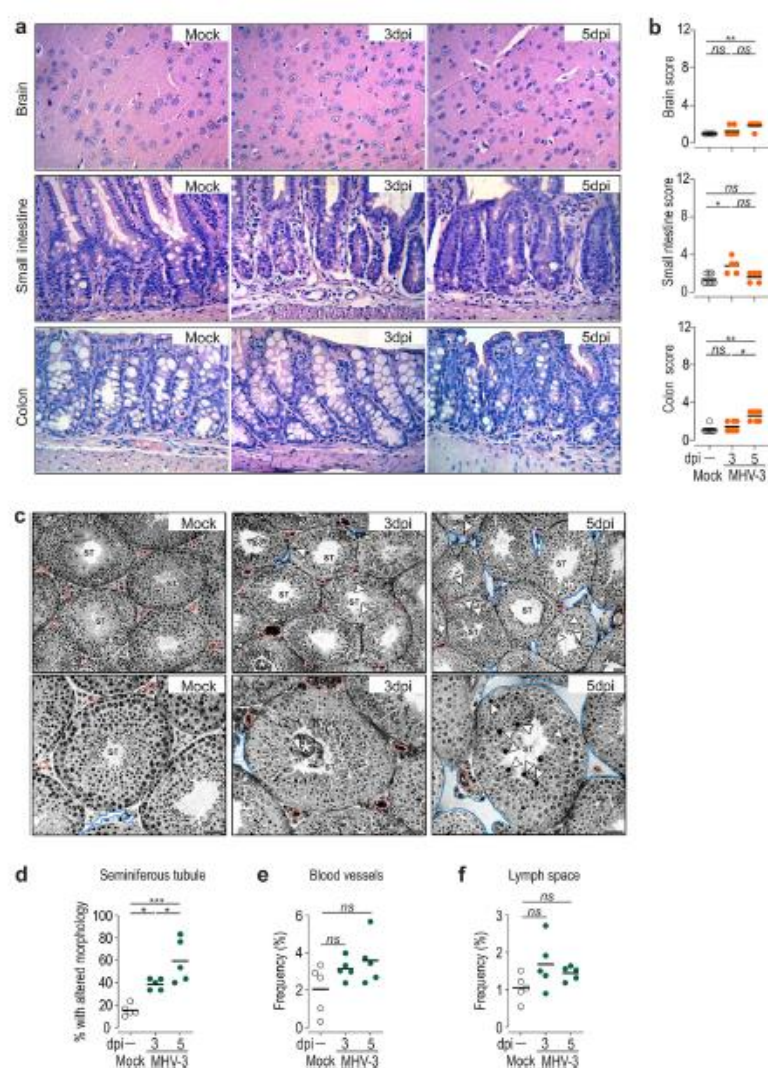


FIG 6 Extrapulmonary changes triggered by MHV-3 inoculated via the intranasal route. (a) H&E staining of the brain, small intestine, and colon sections showing mild signs of inflammatory injury in infected mice. Original magnification, $\times 400$. (b) Mean injury scores compared among groups by Kruskal-Wallis plus Dunn's *post hoc* test ($n = 5$ or 6). (c) Toluidine blue staining of testicular cross sections showing morphological alterations in infection groups. ST, seminiferous tubule; IC, intertubular compartment; *, epithelium sloughing. Arrowheads indicate germ cell apoptosis, reddish lines indicate blood vessels, and bluish lines indicate lymph space. Bar, $50 \mu\text{m}$. (d and e) Comparative morphometric analysis conducted in the tubular (d) and intratubular (e and f) compartments of mock versus MHV-infected mice. Assessed by one-way ANOVA plus Tukey's *post hoc* test (mean $\pm$ SEM; $n = 5$). ns, not significant ($P > 0.05$); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

strains (15, 16); however, their impact on respiratory mechanics has scarcely been evaluated. Here, we showed that pulmonary ventilation was increased in MHV-3-infected mice supported by an augmented respiratory frequency (i.e., tachypnea) without significant alteration in tidal volume. The augmented ventilation may be a result of the hypoxemia-induced ventilatory reflex response, mediated by chemoreceptors (28). Although

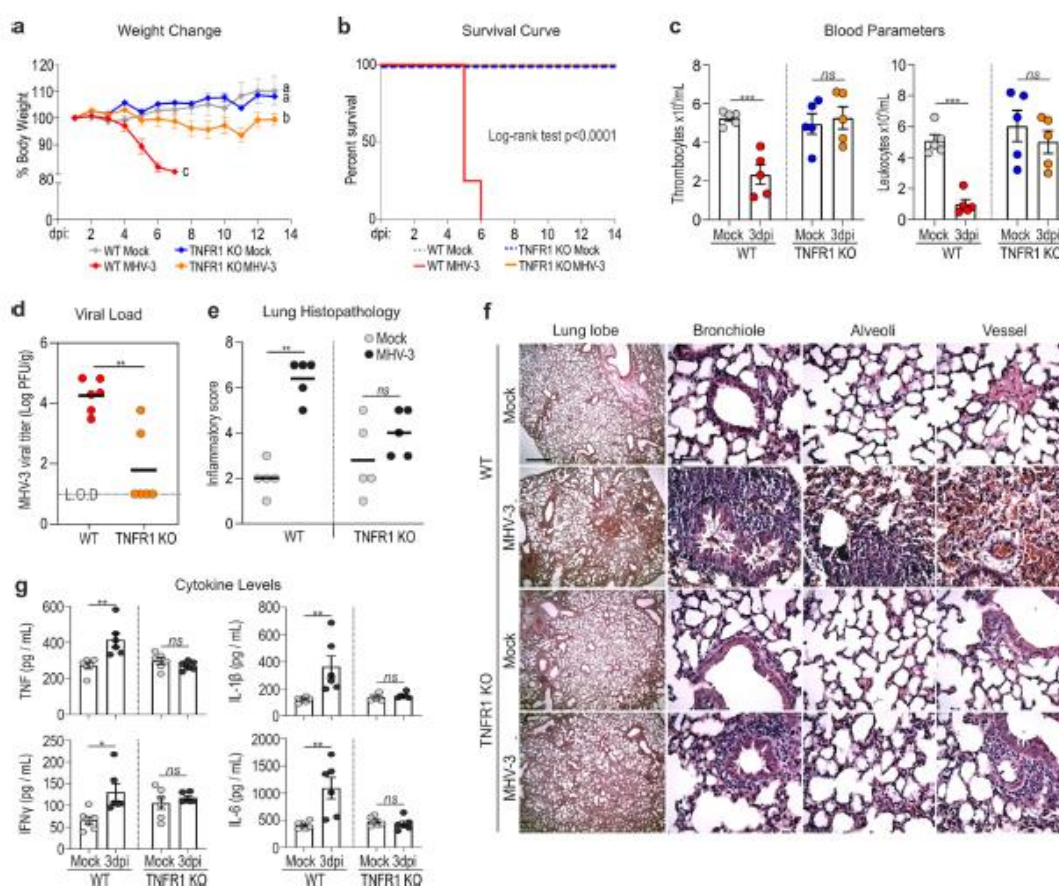


FIG 7 Genetic depletion of TNFR1 prevents MHV-3-mediated lung damage and death in mice. (a) Profile of body weight change in wild-type (WT) and TNFR1 knockout (KO) mice following 14 days of MHV-3 inoculation. Different letters represent statistically significant differences ($P < 0.05$) among groups assessed by two-way repeated-measures ANOVA plus Sidak's multiple-comparison test (mean $\pm$ SEM; $n = 6$ to 8). (b) Kaplan-Meier survival curve of WT and TNFR1 KO mice upon infection ($n = 6$ to 8). (c) Blood analysis showing that the MHV-mediated thrombocytopenia and leukopenia in the WT do not occur in TNFR1 KO mice. Mean $\pm$ SEM assessed by unpaired t test ($n = 5$). (d) Comparison of the viral load determined by plaque assay in WT versus TNFR1 KO mice at 3 days after infection. The data were presented as log₁₀ PFU per gram of tissue and assessed by Mann-Whitney test ($n = 6$). LOD, limit of detection. (e, f) Comparative histopathology of lungs from mock and infection groups (at 3 dpi) in relation to the mouse genotype. Statistical comparison among groups was made using the Mann-Whitney test ($n = 5$). Bars, 450 μ m (low magnification) and 50 μ m (high magnification). (g) Intrapulmonary concentration of TNF, IFN- γ , IL-6, and IL-1 β cytokines determined ELISA. Note that TNFR1 KO mice were prevented from the MHV-related overproduction of proinflammatory cytokines. Comparisons between mock versus infected mice were assessed by unpaired t test (mean $\pm$ SEM; $n = 5$ or 6). ns, not significant ($P > 0.05$); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

we have not directly measured arterial blood gases, the lung pathologies of infected mice (alveolar edema, hyperemic vessels, and the presence of vessels with thrombi) strongly suggest deficits in gas exchange, provoked by dysfunctional diffusion and ventilation-to-perfusion ratio. The breathing pattern found in the infected mice, i.e., tachypnea-mediated increased ventilation, was well correlated with alterations of the mechanical properties of the respiratory system. Infected mice presented lower respiratory compliance that is suggestive of restrictive lung dysfunction. This sort of alteration is also described at least in some COVID-19 patients (29). From the viewpoint of respiratory work, a stiffened system (low compliance) augments the elastic work of breathing, and hence, increasing ventilation by a large tidal volume is energetically expensive and tachypnea is the prevailing breathing pattern in these circumstances (30, 31).

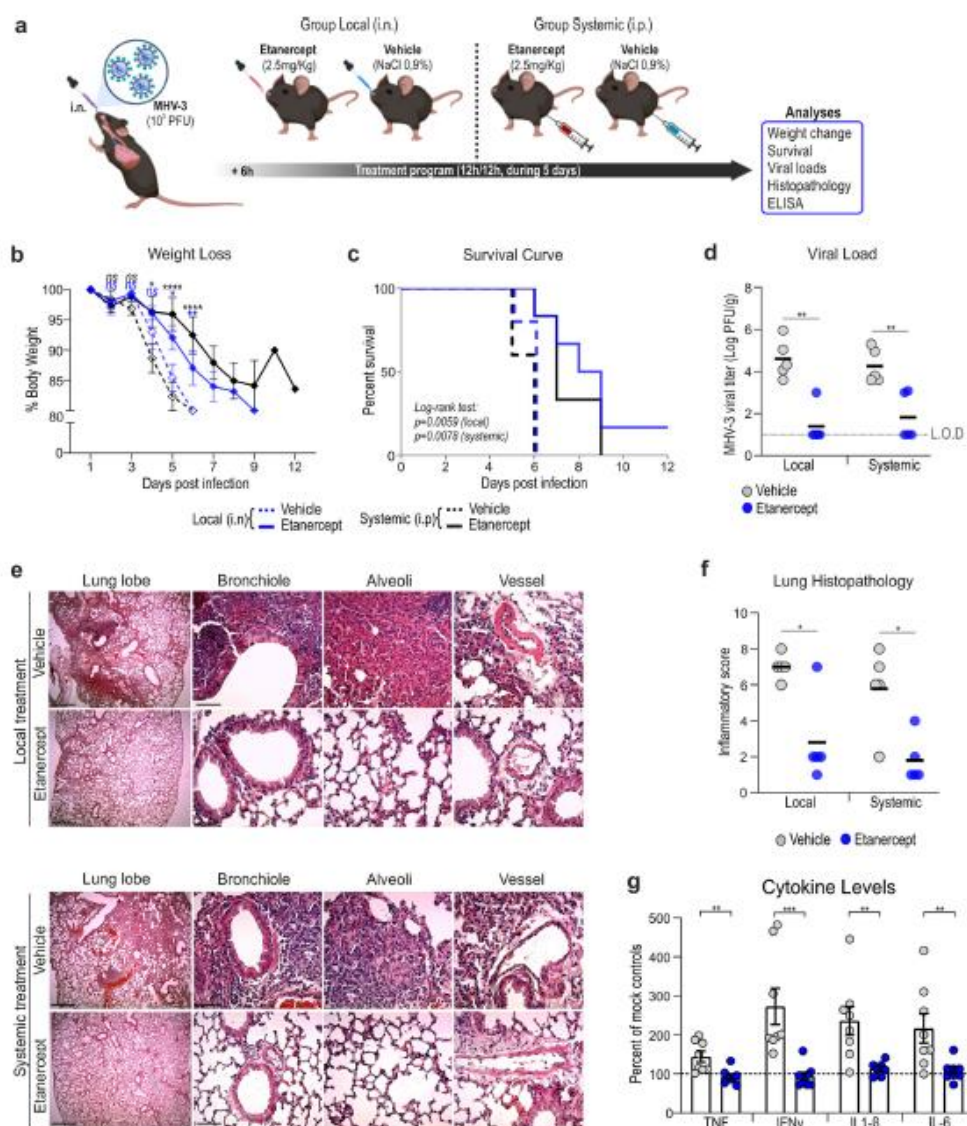


FIG 8 Pharmacologic blockade of TNF cytokine inhibits MHV-3 replication and inflammation-associated injury in the lungs of WT mice. (a) Experiment design. Following 24 h of MHV-3 inoculation, the animals received 2 mg/kg of etanercept or saline (vehicle) twice daily via the intranasal (i.n.; local treatment) or intraperitoneal (i.p.; systemic treatment) route. Treatment schedule was kept for 10 days in a group of animals to assess body weight change and lethality, whereas in another group, samples were collected at 3 dpi. (b) Profile of body weight change among groups. Differences between vehicle and etanercept groups were assessed according to the treatment route by two-way repeated measures ANOVA plus Sidak's multiple-comparison test (mean $\pm$ SEM; $n = 8$). (c) Kaplan-Meier survival analysis of MHV-3-infected mice treated with etanercept versus vehicle controls ($n = 8$). (d) Viral load determined by plaque assay at 3 dpi in the lung of MHV-3-infected mice treated or not with etanercept. Statistical differences were assessed using the Mann-Whitney test ($n = 5$). LOD, limit of detection. (e) H&E staining of lung sections showing reduction of inflammation-associated injury signs in etanercept group versus vehicle controls at 3 dpi. Bar, 50 μ m. (f) Comparative histopathology of lungs according to groups (Mann-Whitney test; $n = 5$). (g) Concentrations of TNF, IFN- γ , IL-1 β , and IL-6 measured by ELISA in the lung of MHV-3-infected mice treated or not with etanercept. The values were normalized to the mock group and presented as percentage of mock controls (mean $\pm$ SEM). Assessed by Mann-Whitney test. $n = 7$ or 8. ns, not significant ($P > 0.05$); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

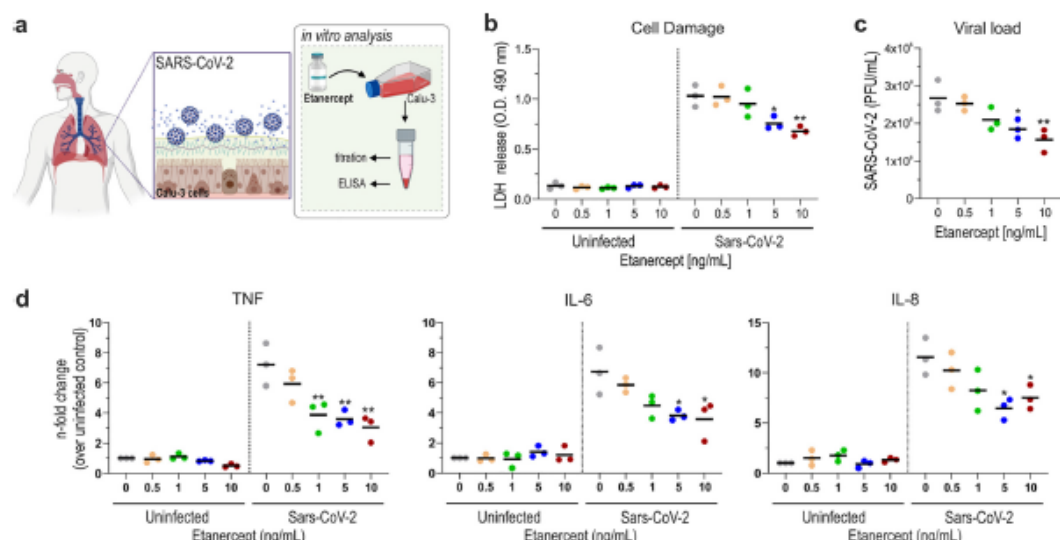


FIG 9 TNF blocking decreases SARS-CoV-2 replication and related cellular damage in human lung epithelial cells. (a) Experiment design. (b) Measurement of cell damage by dosing lactate dehydrogenase (LDH) levels released in cell supernatant of Calu-3 cells treated or not with different concentration of etanercept. (c) Changes in SARS-CoV-2 viral load determined in Calu-3 cells supernatants after treatment with increasing doses of etanercept. (d) ELISA showing lower concentrations of TNF, IL-6, and IL-8 in infected Calu-3 cells following etanercept treatment. Values from each dose group were compared to those from untreated controls by one-way ANOVA plus Dunnett's multiple-comparison test. $n = 3$ independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Moreover, the infection-elicited symptoms in mice described here, fever and tachypnea, recapitulate symptoms described in some COVID-19 patients (32, 33).

It is noteworthy that after inducing pulmonary changes, MHV-3 spread to multiple organs and triggered a systemic and lethal disease. Although the host receptor used for virus entry differs between murine and human coronaviruses (34, 35), the dissemination of MHV-3 to extrapulmonary sites and the subsequent systemic hyperinflammation with high circulating levels of TNF, IFN- γ , IL-1 β , and IL-6 may provide a substantial platform to understand the mechanisms underlying the systemic inflammatory response syndrome (SIRS) and multiple organ failure (14, 36).

TNF has an important role in the coordination and development of most overexuberant inflammatory responses (6, 25, 26), and its inhibition can alleviate acute lung injury caused by severe respiratory syncytial virus and influenza virus (37, 38). During betacoronavirus infection, the overt TNF release can act synergistically with IFN- γ and trigger robust inflammatory cell death (39, 40). Increasing studies in both MHV and SARS-CoV-2 have demonstrated that overt proinflammatory release, including TNF, is mediated by the host Toll-like receptor (TLR2)-Myd88 cascade (26, 41). Therefore, targeting either TNF or TLR2 blockade may provide substantial protection against the pathogenesis of coronavirus infection (39, 41). TNF has already been described as an important cytokine involved in inducing liver damage after intraperitoneal MHV-3 infection in mice (27). In this work, TNF was also associated with the induction of lung damage after intranasal infection with MHV-3. We showed that TNFR1 KO infected mice were fully protected from lung tissue injury and death. In line with this, treating infected wild-type mice with etanercept, a selective TNF inhibitor, mitigated the MHV-3-induced cytokine release and tissue damage in lungs. Moreover, we have shown that etanercept can also significantly decrease the cellular damage and proinflammatory cytokine release triggered by SARS-CoV-2 infection, *in vitro*. Although this preliminary result needs to be further confirmed by distinct *in vitro* and *in vivo* models of SARS-CoV-2 infection, it suggests that TNF blocking may alleviate the pathogenesis of betacoronavirus infection.

In summary, MHV-3 intranasal infection produced pathological features of severe acute respiratory syndrome in wild-type mice and translated into efficient virus replication in the lungs, induction of inflammation-associated tissue damage, and functional impairment of the respiratory function. Moreover, following respiratory manifestations, MHV-3 spread to distant organs and led to systemic inflammation and death, which enable the use of this mouse model for studying betacoronavirus-induced systemic alterations. Akin to other animal models already proposed for COVID-19 study, the MHV-3 model has limitations. One of them is the difference in the host cell receptor used for viral entry. All MHV strains uses the CEACAM-1 receptor (34), whereas SARS-CoV-2 uses ACE2 (35), which may preclude studies of viral entry or drugs that act on this stage of the replication cycle. Another important limitation is the strong viral tropism to extrapulmonary sites following 3 days of infection, which shortens the time window to assess unique pulmonary changes caused by MHV infection. In particular, the liver was severely affected at 5 dpi, at which necrotic areas could be frequently seen in the parenchyma. Although signs of liver injury (e.g., elevated levels of aminotransferases) have frequently been reported for COVID-19 patients and may be associated with disease severity (42), the MHV-induced hepatic changes seem to be greater in magnitude than those triggered by SARS-CoV-2. Thus, caution must be given to using the proposed model to understand the gastrointestinal manifestations of human betacoronavirus infections. Similarly, drugs that rely on the metabolism by hepatic enzymes should have their action limited at late time points of MHV-3 infection.

MATERIALS AND METHODS

Cells, viruses, and plaque assay. L929 (ATCC CCL-1), Vero E6 (ATCC CRL-1586), and Calu-3 (ATCC HTB-55) cells were cultured under a controlled atmosphere (37°C and 5% CO₂) in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Vero and L929) or minimal essential medium (MEM) (Calu-3) supplemented with 7% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. The MHV-3 strain was provided and sequenced (GenBank accession no. MW620427.1; see reference 43) by Clarice Weis Arris and Ricardo Durães-Carvalho from the Universidade Estadual de Campinas (UNICAMP, Brazil), and propagated in L929 cells. SARS-CoV-2 was expanded in Vero E6 cells from an isolate contained on a nasopharyngeal swab obtained from a confirmed case of COVID-19 in Rio de Janeiro, Brazil (GenBank accession no. MT710714), according to WHO guidelines. For viral titration, 100 µl of serially diluted virus suspension, plasma samples, and tissue homogenates (1:9 tissue to DMEM) were inoculated onto a confluent monolayer of L929 cells (for MHV-3) or Vero E6 cells (for SARS-CoV-2) grown in 24-well plates. After gentle shaking for 1 h (4 × 15 min), samples were removed and replaced with the overlay medium (DMEM containing 0.8% carboxymethylcellulose, 2% FBS, and 1% penicillin-streptomycin-glutamine) and kept for 2 days (for MHV-3) or 3 days (for SARS-CoV-2), at 37°C and 5% CO₂. Then, cells were fixed with 10% neutral buffered formalin (NBF) for 1 h and stained with 0.1% crystal violet. Virus titers were determined as PFU.

Mouse strains. Animal experimental procedures were carried out with mixed groups (males and females) of mice aged 6 to 7 weeks and received the approval of the Ethical Committee for Animal Experimentation of the Universidade Federal de Minas Gerais (UFMG) (process no. 190/2020). Wild-type C57BL/6 (Central Animal House of the UFMG) and TNF receptor knockout mice (TNFRp55^{-/-}, stock no. 002818; Jackson Laboratories) were housed in individually ventilated cages placed in an animal care facility at 24°C ± 2°C on a 12-h light/12-h dark cycle, receiving *ad libitum* access to water and food.

MHV-3 infection. Mice were lightly anesthetized (ketamine [50 mg/kg]-xylazine [5 mg/kg], intraperitoneally [i.p.]) and received an intranasal inoculation of 30 µl sterile saline solution, loaded or not (mock controls) with MHV-3 at different concentrations (3 × 10¹ to 3 × 10⁴ PFU). Signs of disease, including ruffled fur, back arching, weight loss, facial edema, and lack of activity were monitored daily for up to 14 days postinoculation (dpi).

Sample collection. Following anesthesia (ketamine [80 mg/kg]-xylazine [10 mg/kg] i.p.), the animals were euthanized by cervical dislocation, and blood samples were collected from the abdominal vena cava and analyzed. The lungs were then harvested, and the right lobes were snap-frozen in liquid nitrogen, whereas the left lobes were fixed in 10% neutral buffered formalin, unless otherwise mentioned. Formalin-fixed and frozen fragments of other target tissues were also collected for additional analyses.

Hematological evaluation. The number of circulating thrombocytes and leukocytes was determined in blood samples using the a Celltac MEK-6500K hemocytometer (Nihon Kohden).

Histopathology. Formalin-fixed and paraffin-embedded (FFPE) tissues were sectioned into 5-µm-thickness slices, stained with hematoxylin and eosin (H&E), and examined under light microscopy. The inflammation-mediated injury in mouse lungs was determined by a pathologist (C.M.Q.-J.) who was blind to the experimental conditions, employing a scoring system encompassing (i) airway inflammation (up to 4 points), (ii) vascular inflammation (up to 4 points), (iii) parenchyma inflammation (up to 5 points), and general neutrophil infiltration (up to 5 points) (44). Histopathological assessments were also performed in FFPE samples of liver, brain, small intestine, and colon, following previously established

criteria (45–48). For testis analysis, the tissue was fixed by immersion in Bouin's solution, embedded in glycol methacrylate resin, sectioned at 3- μ m thickness, and then stained with toluidine blue prior to stereological and morphometric assessments, as described previously (49).

Cytokine and chemokine dosage. Mouse lung homogenates were acquired by homogenizing 40 mg of frozen tissue in 400 μ l of chilled cytokine extraction buffer (100 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, and 1% protease inhibitor cocktail). After centrifugation (14,000 $\times$ g, 15 min, 4°C), the supernatant was collected and submitted to a dosage of TNF, IFN- γ , IL-1 α , IL-1 β , IL-12 p70, CXCL-1, CCL-2, CCL-3, CCL-4, or CCL-5 using the mouse DuoSet enzyme-limited immunosorbent assay (ELISA) system (R&D Systems). Plasma concentrations of TNF, IFN- γ , IL-10, and IL-6 were also determined.

Immunofluorescence. Lung tissues were flushed with OCT/4% paraformaldehyde solution and processed for OCT embedding. The cryosections (15- μ m thickness) were permeabilized with phosphate-buffered saline (PBS)/0.5% Triton X-100, incubated for 1 h in the blocking solution (PBS containing 5% goat serum and 5 μ g/ml mouse BD Fc Block) and then labeled overnight at 4°C with the allophycocyanin (APC)-conjugated rat anti-mouse CD45 antibody (1:100 dilution, catalog no. 559864; BD Pharmingen). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; 1 μ g/ml, catalog no. D9542; Sigma-Aldrich). Fluorescent signals were evaluated by confocal microscopy using an inverted Nikon Eclipse Ti microscope coupled to an A1 scanning head.

Flow cytometry. Lungs were minced into small pieces and allowed to digest with gentle agitation (80 rpm, 37°C, 45 min) onto aeration tubes containing 5 ml of digestion buffer (0.5 mg/ml collagenase and 20 μ g/ml DNase diluted in RPMI medium). After, the cell suspension was passed through the cell strainer (pore size, 70 μ m; BD Biosciences, San Jose, CA), and the remaining erythrocytes were lysed with ACK buffer (Invitrogen, Carlsbad, CA). Cells (1×10^6) were blocked with mouse BD Fc Block (5 μ g/ml, catalog no. 553141; BD Pharmingen) and then stained using fluorescent-labeled monoclonal antibodies, as follows: Ly6G-BV421 (1:200, catalog no. 127627; BioLegend); CD45-fluorescein isothiocyanate (FITC) (1:200, catalog no. 5530801; BD Pharmingen); F4/80-phycoerythrin (PE)-Cyanine 7 (1:100, catalog no. 25-4801-82; Invitrogen); CD4-PE (1:100, catalog no. 100408; BioLegend); CD3-PerCP-Cyanine 5.5 (1:100, catalog no. 100218; BioLegend); CD8-allophycocyanin (APC) (1:100, catalog no. 17-0081-82; Invitrogen). The acquisition was carried out in a BD FACSCanto II cell analyzer and analyzed using FlowJo software (Tree Star, Ashland, OR).

Liver function analyses. Serum concentrations of alanine aminotransferase (ALT) and indocyanine green (ICG) were used to estimate the liver function status, as previously described (50). In addition, liver necrosis was evaluated by intravital microscopy after labeling free DNA. To this end, 20 min before *in vivo* imaging, mice received an intravenous injection of 1 mg Sytox orange diluted in 0.1 ml of sterile saline solution (51).

Measurement of body temperature. Ten days before MHV-3 inoculation, a group of 10 mice underwent surgery to implant a temperature probe (mini dataloggers; SubCue, Calgary, AB, Canada) into the abdominal cavity. The probes were programmed to start recording data at 7 a.m. on the fifth day after surgery. Upon starting, each animal's abdominal temperature was monitored every 10 min (sample rate) throughout the days that comprised pre- and postinfection procedures. On the fourth day postinfection, mice were euthanized, the dataloggers were collected, and the body temperature readings of each animal were downloaded and analyzed (SubCue Datalogger Software; Calgary, AB, Canada).

Pulmonary ventilation. The whole-body plethysmography method was employed to assess pulmonary ventilation (*in vivo*, freely moving animals) at 4 and 2 days before infection, as well as at 3 dpi. Mice were placed in a cylindrical plethysmography chamber flushed with room air (1,000 ml $\cdot$ min $^{-1}$) and allowed to move freely and acclimate for at least 30 min. To measure pulmonary ventilation, the chamber was totally closed and airflow was stopped for a short period (~2 min) while the breathing-related pressure oscillations were detected using a differential pressure transducer, amplified (MLT141 spirometer, PowerLab; ADInstruments, NSW, Australia), and recorded with LabChart v7 software (ADInstruments, NSW, Australia). The ventilation was calculated as the product of the tidal volume (ml body temperature and pressure, saturated [BTPS] $\cdot$ g $^{-1}$; see reference 52) and respiratory frequency (breath cycles $\cdot$ min $^{-1}$), corrected by the body weight (g). From the ventilatory recording traces, the inspiration and expiration time, as well as total respiratory cycle duration, were also calculated.

Respiratory mechanics. Mice were divided into two groups (mock $n = 9$ and infected $n = 11$) and 3 days postinfection animals were deeply anesthetized until respiratory arrest. Mice were tracheostomized and a polyethylene tube (P50) was inserted into the trachea. The pressure-volume curve was made by injecting air volume in a stepwise manner (using the 3-ml glass syringe), with 0.1-ml increments until intratracheal pressure peaked at approximately 35 cmH $_2$ O. In the deflation limb, the system was deflated in the same volume steps until the pressure reached approximately -15 cmH $_2$ O and finally inflated again to resting lung volume. Signals were acquired and recorded on the PowerLab software (LabChart v. 7; ADInstruments, NSW, Australia). The vital capacity was determined by maximal inflation (lung volume at 35 cmH $_2$ O), and the static compliance of the total respiratory system (expressed as ml/cmH $_2$ O) was measured at the steepest point of the deflation limb of the pressure-volume curve.

Neuromuscular junction analysis of diaphragm muscle. Whole-mount diaphragms were used to evaluate changes in neuromuscular junctions (NMJs) of MHV-3-infected mice and mock controls. Sample processing and labeling of NMJs were performed based on a previous protocol (53). The presynaptic and postsynaptic terminals were stained using the monoclonal anti-synaptotagmin antibody (1:250 dilution, catalog no. 3H2 2D7; Developmental Studies Hybridoma Bank) and the tetramethylrhodamine-conjugated α -bungarotoxin (1:1,000 dilution, catalog no. T1175; Invitrogen), respectively. After Z-stack imaging in a Zeiss LSM 880 confocal microscope, 25 NMJs per animal were assessed by particle analysis to estimate the fragmentation index at pre- and postsynaptic endings (54).

Etanercept treatment. For *in vivo* TNF inhibition, C57BL/6J WT mice were intranasally inoculated with 1×10^3 PFU of MHV and received 2.5 mg/kg of etanercept (Enbrel; Pfizer) twice a day delivered in 30 μ l sterile saline solution via the nasal route (local treatment scheme) or intraperitoneally (systemic treatment scheme). Treatments started following 6 h after infection and lasted until 6 dpi. The vehicle groups received only saline solution.

TNF inhibition was also tested *in vitro* against SARS-CoV-2. To this end, Calu-3 cells were seeded onto 24-well plates and infected with SARS-CoV-2 (multiplicity of infection [MOI] 0.1). The control group was kept uninfected. After 1 h of viral adsorption, the medium containing virus was replaced for fresh medium loaded with increasing concentrations of Etanercept (0.5, 1, 5, and 10 ng/ml). Following 48 h of treatment, the Calu-3 supernatant was collected for measuring virus load as well as the concentration of target cytokines and lactate dehydrogenase (LDH).

Statistical analyses. Prism 8.0 software (GraphPad) was used for statistical analysis. First, data distribution was assessed by the Shapiro-Wilk test and Q-Q plots. Parametric comparisons between two or more groups were done using Student's *t* test or one-way analysis of variance (ANOVA), respectively, or by using a Mann-Whitney or Kruskal-Wallis test to assess differences between two or more nonparametric data sets. Survival rates among groups were determined by Kaplan-Meier survival analysis. Finally, the body weight changes triggered by MHV-3 were compared among groups by two-way repeated-measures ANOVA. Data are presented as mean $\pm$ standard error of the mean (SEM). Differences with a *P* value of <0.05 were considered statistically significant.

ACKNOWLEDGMENTS

This work was financially supported by grants from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-CAPES/Brazil (grant 88887.507173/2020-00 to M.M.T.), JBS-INVTARE/Brazil (grant 27991 to M.M.T.), Fundação de Amparo à Pesquisa do Estado de São Paulo-FAPESP/Brazil (grant 2019/01255-9 to R.D.-C.), a Ferring COVID-19 Investigational Grant (grant FIN0042393 to G.M.J.C.), and the L'Oréal-Unesco-ABC For Women in Science Program (grant to V.V.C.). This work also received support from the National Institute of Science and Technology in Dengue and Host-Microorganism Interaction (INCT em Dengue), sponsored by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; Brazil) and the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG; Brazil).

We also acknowledge Leda Quercia Vieira for the donation of the TNFR1 KO mice and Ilma Marçal de Souza for her technical assistance with experiments.

Author contributions were as follows. Study design: A.C.D.S.P.A., G.H.C.-S., C.M.Q.-J., L.C.D.O., L.P.D.S., M.M.T., G.S.F.D.S., and V.V.C. Performed experiments: A.C.D.S.P.A., G.H.C.-S., C.M.Q.-J., L.C.D.O., L.D.S.B.L., J.C.P., F.R.O.D.S., I.D.M.C., L.B.P., D.C.T., P.G.B.-S., P.A.C.V., L.R.-O., M.M.A., A.F.A.F., N.T.W., A.C.F., J.R.T., and A.C. Data analysis: A.C.D.S.P.A., G.H.C.S., L.P.D.S., G.S.F.D.S., G.M.J.C., C.G., T.M.L.S., R.D.-C., and V.V.C. Intellectual, technical and resource support: M.M.T., C.A.D., C.W.A., P.P.G.G., G.B.D.M., T.M.L.S., M.D.M.P., and B.R.B. Wrote the first draft of the paper: A.C.D.S.P.A., G.H.C.-S., C.M.Q.-J., L.C.D.O., L.P.D.S., G.S.F.D.S., C.G., and V.V.C. All authors reviewed and approved the final version of the manuscript.

We declare no competing financial interest in relation to this work.

REFERENCES

- Cui J, Li F, Shi ZL. 2019. Origin and evolution of pathogenic coronaviruses. *Nat Rev Microbiol* 17:181–192. <https://doi.org/10.1038/s41579-018-0118-9>.
- Boni MF, Lemey P, Jiang X, Lam TTY, Perry BW, Castoe TA, Rambaut A, Robertson DL. 2020. Evolutionary origins of the SARS-CoV-2 sarbecovirus lineage responsible for the COVID-19 pandemic. *Nat Microbiol* 5:1408–1417. <https://doi.org/10.1038/s41564-020-0771-4>.
- Wang W, Lin XD, Zhang HL, Wang MR, Guan XQ, Holmes EC, Zhang YZ. 2020. Extensive genetic diversity and host range of rodent-borne coronaviruses. *Virus Evol* 6:veaa078. <https://doi.org/10.1093/ve/veaa078>.
- Lu R, Zhao X, Li J, Niu P, Yang B, Wu H, Wang W, Song H, Huang B, Zhu N, Bi Y, Ma X, Zhan F, Wang L, Hu T, Zhou H, Hu Z, Zhou W, Zhao L, Chen J, Meng Y, Wang J, Lin Y, Yuan J, Xie Z, Ma J, Liu WJ, Wang D, Xu W, Holmes EC, Gao GF, Wu G, Chen W, Shi W, Tan W. 2020. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet* 395:565–574. [https://doi.org/10.1016/S0140-6736\(20\)30251-8](https://doi.org/10.1016/S0140-6736(20)30251-8).
- Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, Wei Y, Wu W, Xie X, Yin W, Li H, Liu M, Xiao Y, Gao H, Guo L, Xie J, Wang G, Jiang R, Gao Z, Jin Q, Wang J, Cao B. 2020. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 395:497–506. [https://doi.org/10.1016/S0140-6736\(20\)30183-5](https://doi.org/10.1016/S0140-6736(20)30183-5).
- Del Valle DM, Kim-Schulze S, Huang HH, Beckmann ND, Nirenberg S, Wang B, Lavin Y, Swartz TH, Madduri D, Stock A, Marron TU, Xie H, Patel M, Tuballes K, Van Oekelen O, Rahman A, Kovatch P, Aberg JA, Schadt E, Jagannath S, Mazumdar M, Chamey AW, Firpo-Betancourt A, Mendu DR, Jhang J, Reich D, Sigel K, Cordon-Cardo C, Feldmann M, Parekh S, Merad M, Grnatic S. 2020. An inflammatory cytokine signature predicts COVID-19 severity and survival. *Nat Med* 26:1636–1643. <https://doi.org/10.1038/s41591-020-1051-9>.
- Gurumurthy CB, Quadros RM, Richardson GP, Poluektova LY, Mansour SL, Ohtsuka M. 2020. Genetically modified mouse models to help fight COVID-19. *Nat Protoc* 15:3777–3787. <https://doi.org/10.1038/s41596-020-00403-2>.
- Winkler ES, Bailey AL, Kafai NM, Nair S, McCune BT, Yu J, Fox JM, Chen RE, Earnest JT, Keeler SP, Ritter JH, Kang LI, Cort S, Robichaud A, Head R, Holtzman MJ, Diamond MS. 2020. SARS-CoV-2 infection of human ACE2-transgenic mice causes severe lung inflammation and impaired function. *Nat Immunol* 21:1327–1335. <https://doi.org/10.1038/s41590-020-0778-2>.

9. Dinnon KH, Leist SR, Schäfer A, Edwards CE, Martinez DR, Montgomery SA, West A, Yount BL, Hou YJ, Adams LE, Gully KL, Brown AJ, Huang E, Bryant MD, Choong IC, Glenn JS, Gralinski LE, Sheahan TP, Baric RS. 2020. A mouse-adapted model of SARS-CoV-2 to test COVID-19 countermeasures. *Nature* 586:560–566. <https://doi.org/10.1038/s41586-020-2708-8>.
10. Shi J, Wen Z, Zhong G, Yang H, Wang C, Huang B, Liu R, He X, Shuai L, Sun Z, Zhao Y, Liu P, Liang L, Cui P, Wang J, Zhang X, Guan Y, Tan W, Wu G, Chen H, Bu Z, Bu Z. 2020. Susceptibility of ferrets, cats, dogs, and other domesticated animals to SARS-coronavirus 2. *Science* 368:1016–1020. <https://doi.org/10.1126/science.abb7015>.
11. Rodx B, Kuiken T, Herfst S, Bestebroer T, Lamers MM, Munnink BBO, De Meulder D, Van Amerongen G, Van Den Brand J, Okba NMA, Schipper D, Van Run P, Leijten L, Sikkema R, Verschoor E, Vestrepen B, Bogers W, Langermans J, Langermans J, Drosten C, Van Vlietingsen MF, Fouchier R, De Swart R, Koopmans M, Haagmans BL. 2020. Comparative pathogenesis of COVID-19, MERS, and SARS in a nonhuman primate model. *Science* 368:1012–1015. <https://doi.org/10.1126/science.abb7314>.
12. Sia SF, Yan LM, Chin AWH, Fung K, Choy KT, Wong AY, Kaewpreedee P, Perera RAPM, Poon LLM, Nicholls JM, Peiris M, Yen HL. 2020. Pathogenesis and transmission of SARS-CoV-2 in golden hamsters. *Nature* 583:834–838. <https://doi.org/10.1038/s41586-020-2342-5>.
13. Gu H, Chen Q, Yang G, He L, Fan H, Deng YQ, Wang Y, Teng Y, Zhao Z, Cui Y, Li Y, Li XF, Li J, Zhang NN, Yang X, Chen S, Guo Y, Zhao G, Wang X, Luo DY, Wang H, Yang X, Li Y, Han G, He Y, Zhou X, Geng S, Sheng X, Jiang S, Sun S, Qin CF, Zhou Y. 2020. Adaptation of SARS-CoV-2 in BALB/c mice for testing vaccine efficacy. *Science* 369:1603–1607. <https://doi.org/10.1126/science.abc4730>.
14. Wang J, Shuai L, Wang C, Liu R, He X, Zhang X, Sun Z, Shan D, Ge J, Wang X, Hua R, Zhong G, Wen Z, Bu Z. 2020. Mouse-adapted SARS-CoV-2 replicates efficiently in the upper and lower respiratory tract of BALB/c and C57BL/6J mice. *Protein Cell* 11:776–782. <https://doi.org/10.1007/s13238-020-00767-x>.
15. Bao L, Deng W, Huang B, Gao H, Liu J, Ren L, Wei Q, Yu P, Xu Y, Qi F, Qu Y, Li F, Lv Q, Wang W, Xue J, Gong S, Liu M, Wang G, Wang S, Song Z, Zhao L, Liu P, Zhao L, Ye F, Wang H, Zhou W, Zhu N, Zhen W, Yu H, Zhang X, Guo L, Chen L, Wang C, Wang Y, Wang X, Xiao Y, Sun Q, Liu H, Zhu F, Ma C, Yan L, Yang M, Han J, Xu W, Tan W, Peng X, Jin Q, Wu G, Qin C. 2020. The pathogenicity of SARS-CoV-2 in hACE2 transgenic mice. *Nature* 583:830–833. <https://doi.org/10.1038/s41586-020-2312-y>.
16. Ehsaie SN, Abdullah ML, Abuyassin B, Bouchama A. 2020. Evidence of a wide gap between COVID-19 in humans and animal models: a systematic review. *Crit Care* 24:–594. <https://doi.org/10.1186/s13054-020-03304-8>.
17. Muñoz-Fontela C, Dowling WE, Funnell SGP, Gsell PS, Riveros-Balta AX, Albrecht RA, Andersen H, Baric RS, Carroll MW, Cavaleri M, Qin C, Croder I, Dallmeier K, de Waal L, de Wit E, Delang L, Dolm E, Duprex WP, Falzarano D, Finch CL, Frieman MB, Graham BS, Gralinski LE, Guilloyle K, Haagmans BL, Hamilton GA, Hartman AL, Herfst S, Kaptein SJF, Kinnema WB, Knezevic I, Krause PR, Kuhn JH, Le Gend R, Lewis MG, Liu WC, Maisonneuve P, McBryoy AK, Munster V, Oreshkova N, Rasmussen AL, Roda-Pereira J, Rodx B, Rodriguez E, Rogers TF, Salguero FJ, Schotseert M, Stillebaer KJ, Thibaut HJ, Te Tseng C, Vergara-Alert J, et al. 2020. Animal models for COVID-19. *Nature* 586:509–515. <https://doi.org/10.1038/s41586-020-2787-6>.
18. De Albuquerque N, Baig E, Ma X, Zhang J, He W, Rowe A, Habal M, Liu M, Shalev I, Downey GP, Gorczynski R, Butany J, Leibowitz J, Weiss SR, McGilvray ID, Phillips MJ, Fish EN, Levy GA. 2006. Murine hepatitis virus strain 1 produces a clinically relevant model of severe acute respiratory syndrome in A/J mice. *J Virol* 80:10382–10394. <https://doi.org/10.1128/JVI.00747-06>.
19. Yang Z, Du J, Chen G, Zhao J, Yang X, Su L, Cheng G, Tang H. 2014. Coronavirus MHV-A59 infects the lung and causes severe pneumonia in C57BL/6 mice. *Viral Sin* 29:393–402. <https://doi.org/10.1007/s12250-014-3530-y>.
20. Weiss SR, Leibowitz JL. 2011. Coronavirus pathogenesis, p 85–164. In *Advances in virus research*, 1st ed. Academic Press, Cambridge, MA.
21. Körner RW, Majjouti M, Alejandro Alcazar MA, Mahabir E. 2020. Of mice and men: the coronavirus MHV and mouse models as a translational approach to understand SARS-CoV-2. *Viruses* 12:880. <https://doi.org/10.3390/v12080880>.
22. Shi Z, de Vries HJ, Vlaar APJ, van der Hoeven J, Boon RA, Heunks LMA, Ottenheim CAC, Dutch COVID-19 Diaphragm Investigators. 2021. Diaphragm pathology in critically ill patients with COVID-19 and postmortem findings from 3 medical centers. *JAMA Intern Med* 181:122–124. <https://doi.org/10.1001/jamainternmed.2020.6278>.
23. Chen L, Huang X, Yi Z, Deng Q, Jiang N, Feng C, Zhou Q, Sun B, Chen W, Guo R. 2021. Ultrasound imaging findings of acute testicular infection in patients with coronavirus disease 2019. *J Ultrasound Med* 40:1787–1788. <https://doi.org/10.1002/jum.15558>.
24. Ma X, Guan C, Chen R, Wang Y, Feng S, Wang R, Qu G, Zhao S, Wang F, Wang X, Zhang D, Liu L, Liao A, Yuan S. 2021. Pathological and molecular examinations of postmortem testis biopsies reveal SARS-CoV-2 infection in the testis and spermatogenesis damage in COVID-19 patients. *Cell Mol Immunol* 18:487–489. <https://doi.org/10.1038/s41423-020-00604-5>.
25. Hega S, Yamamoto N, Nakai-Murakami C, Osawa Y, Tokunaga K, Sata T, Yamamoto N, Sasaaki T, Ishizaka Y. 2008. Modulation of TNF- α -converting enzyme by the spike protein of SARS-CoV and ACE2 induces TNF- α production and facilitates viral entry. *Proc Natl Acad Sci U S A* 105:7809–7814. <https://doi.org/10.1073/pnas.0711241105>.
26. Jacques A, Bleau C, Turbide C, Beauchemin N, Lamontagne L. 2009. Macrophage interleukin-6 and tumor necrosis factor- α are induced by coronavirus fixation to Toll-like receptor 2/heparan sulphate receptors but not carcinoembryonic cell adhesion antigen 1a. *Immunology* 128:e181–e192. <https://doi.org/10.1111/j.1365-2567.2008.02946.x>.
27. Xu H, Li H, Cao D, Wu Y, Chen Y. 2014. Tumor necrosis factor α (TNF- α) receptor-1 is required for TNF- α -mediated fulminant virus hepatitis caused by murine hepatitis virus strain-3 infection. *Immunol Lett* 158:25–32. <https://doi.org/10.1016/j.imlet.2013.11.008>.
28. Vaporioti K, Akoumianaki E, Telias I, Goligher EC, Brochard L, Georgopoulos D. 2020. Respiratory drive in critically ill patients: pathophysiology and clinical implications. *Am J Respir Crit Care Med* 201:20–32. <https://doi.org/10.1164/rccm.201903-0595O>.
29. Gattinoni L, Chiumello D, Caironi P, Busana M, Romitti F, Brazzi L, Camporota L. 2020. COVID-19 pneumonia: different respiratory treatments for different phenotypes? *Intensive Care Med* 46:1099–1102. <https://doi.org/10.1007/s00134-020-06033-2>.
30. Mortola JP. 2019. How to breathe? Respiratory mechanics and breathing pattern. *Respir Physiol Neurobiol* 261:48–54. <https://doi.org/10.1016/j.resp.2018.12.005>.
31. Robichaud A, Fereydoonad L, Limjunyawong N, Rabold R, Allard B, Benedetti A, Martin JG, Mitzner W. 2017. Automated full-range pressure-volume curves in mice and rats. *J Appl Physiol* (1985) 123:746–756. <https://doi.org/10.1152/jappphysiol.00856.2016>.
32. Zhao Y, Shang Y, Song W, Li Q, Xie H, Xu Q, Jia J, Li L, Mao H, Zhou X, Luo H, Gao Y, Xu A. 2020. Follow-up study of the pulmonary function and related physiological characteristics of COVID-19 survivors three months after recovery. *EclinicalMedicine* 25:100463. <https://doi.org/10.1016/j.eclinm.2020.100463>.
33. Poor HD, Ventetulo CE, Tolbert T, Chun G, Serrao G, Zeidman A, Dengayad NS, Olin J, Kohli-Seth R, Powell CA. 2020. COVID-19 critical illness pathophysiology driven by diffuse pulmonary thrombi and pulmonary endothelial dysfunction responsive to thrombolysis. *Clin Transl Med* 10:3–7.
34. Blau DM, Turbide C, Tremblay M, Olson M, Lévesque S, Michalyszyn E, Jothy S, Holmes KV, Beauchemin N. 2001. Targeted disruption of the Ccam1 (MHVR) gene leads to reduced susceptibility of mice to mouse hepatitis virus infection. *J Virol* 75:8173–8186. <https://doi.org/10.1128/JVI.75.17.8173-8186.2001>.
35. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, Schiergens TS, Henler G, Wu NH, Nitsche A, Müller MA, Drosten C, Pöhlmann S. 2020. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell* 181:271–280.e8. <https://doi.org/10.1016/j.cell.2020.02.052>.
36. Lai CC, Ko WC, Lee PJ, Jean SS, Hsueh PR. 2020. Extra-respiratory manifestations of COVID-19. *Int J Antimicrob Agents* 56:106024. <https://doi.org/10.1016/j.ijantimicag.2020.106024>.
37. Huxell T, Pennycook A, Openshaw PJM. 2001. Inhibition of tumor necrosis factor reduces the severity of virus-specific lung immunopathology. *Eur J Immunol* 31:2566–2573. [https://doi.org/10.1002/1521-4141\(200109\)31:9<2566::AID-IMMU2566>3.0.CO;2-L](https://doi.org/10.1002/1521-4141(200109)31:9<2566::AID-IMMU2566>3.0.CO;2-L).
38. Shi X, Zhou W, Huang H, Zhu H, Zhou P, Zhu H, Ju D. 2013. Inhibition of the inflammatory cytokine tumor necrosis factor- α with etanercept provides protection against lethal H1N1 influenza infection in mice. *Crit Care* 17:R301. <https://doi.org/10.1186/cc13171>.
39. Zheng M, Williams EP, Subbarao Malireddi RK, Karki R, Baneth B, Burton A, Webby R, Channappanavar R, Jonsson CB, Kanneganti TD. 2020. Impaired NLRP3 inflammasome activation/pyroptosis leads to robust inflammatory cell death via caspase-8/RIPK3 during coronavirus infection. *J Biol Chem* 295:14040–14052. <https://doi.org/10.1074/jbc.RA120.015036>.

40. Karki R, Sharma BR, Tuladhar S, Williams EP, Zalduendo L, Samir P, Zheng M, Sundaram B, Banoth B, Malireddi RKS, Schreiner P, Neale G, Vogel P, Webb R, Jonsson CB, Kanneganti TD. 2021. Synergism of TNF- α and IFN- γ triggers inflammatory cell death, tissue damage, and mortality in SARS-CoV-2 infection and cytokine shock syndromes. *Cell* 184:149–168.e17. <https://doi.org/10.1016/j.cell.2020.11.025>.
41. Zheng M, Karki R, Williams EP, Yang D, Fitzpatrick E, Vogel P, Jonsson CB, Kanneganti TD. 2021. TLR2 senses the SARS-CoV-2 envelope protein to produce inflammatory cytokines. *Nat Immunol* 22:829–838. <https://doi.org/10.1038/s41590-021-00937-x>.
42. Wang Y, Liu S, Liu H, Li W, Lin F, Jiang L, Li X, Xu P, Zhang L, Zhao L, Cao Y, Kang J, Yang J, Li L, Liu X, Li Y, Nie R, Mu J, Lu F, Zhao S, Lu J, Zhao J. 2020. SARS-CoV-2 infection of the liver directly contributes to hepatic impairment in patients with COVID-19. *J Hepatol* 73:807–816. <https://doi.org/10.1016/j.jhep.2020.05.002>.
43. Garcia AB, De Moraes P, Rodrigues DM, Gilioli R, de Oliveira-Filho EF, Durães-Carvalho R, Weis Arns C. 2021. Coding-complete genome sequence of murine hepatitis virus strain 3 from Brazil. *Microbiol Resour Announc* 10:e00248-21. <https://doi.org/10.1128/MRA.00248-21>.
44. Horvat JC, Beagley KW, Wade MA, Preston JA, Hansbro NG, Hickey DK, Kaiko GE, Gibson PG, Foster PS, Hansbro PM. 2007. Neonatal chlamydial infection induces mixed T-cell responses that drive allergic airway disease. *Am J Respir Crit Care Med* 176:556–564. <https://doi.org/10.1164/rccm.200607-1005OC>.
45. Costa VV, Fagundes CT, Valadão DF, Cisalpino D, Dias ACF, Silveira KD, Kangussu LM, Ávila TV, Bonfim MRQ, Bonaventura D, Silva TA, Sousa LP, Rachid MA, Vieira LQ, Menezes GB, de Paula AM, Atscheshevska A, Ignatyev G, Teixeira MM, Souza DG. 2012. A model of DENV-3 infection that recapitulates severe disease and highlights the importance of IFN- γ in host resistance to infection. *PLoS Negl Trop Dis* 6:e1663. <https://doi.org/10.1371/journal.pntd.0001663>.
46. Costa VV, Del Sarto JL, Rocha RF, Silva FR, Doria JG, Olmo IG, Marques RE, Queiroz-Junior CM, Foureau G, Araújo JMS, Cramer A, Real ALCV, Ribeiro LS, Sardi SI, Ferreir AJ, Machado FS, De Oliveira AC, Teixeira AL, Nakaya HI, Souza DG, Ribeiro FM, Teixeira MM. 2017. N-Methyl-D-aspartate (NMDA) receptor blockade prevents neuronal death induced by Zika virus infection. *mBio* 8:e00350-17. <https://doi.org/10.1128/mBio.00350-17>.
47. Arifa RDN, Madeira MFM, De Paula TP, Lima RL, Tavares LD, Menezes-Garcia Z, Fagundes CT, Radhi MA, Ryffel B, Zamboni DS, Teixeira MM, Souza DG. 2014. Inflammasome activation is reactive oxygen species dependent and mediates irinotecan-induced mucositis through IL-1 β and IL-18 in mice. *Am J Pathol* 184:2023–2034. <https://doi.org/10.1016/j.ajpath.2014.03.012>.
48. MacPherson BR, Pfeiffer CJ. 1978. Experimental production of diffuse colitis in rats. *Digestion* 17:135–150. <https://doi.org/10.1159/000198104>.
49. Costa GMJ, Lacerda SMSN, Figueiredo AFA, Leal MC, Rezende-Neto JV, França LR. 2018. Higher environmental temperatures promote acceleration of spermatogenesis *in vivo* in mice (*Mus musculus*). *J Therm Biol* 77:14–23. <https://doi.org/10.1016/j.jtherbio.2018.07.010>.
50. Nakagaki BN, Mafra K, de Carvalho É, Lopes ME, Carvalho-Gontijo R, de Castro-Oliveira HM, Campolina-Silva GH, de Miranda CDM, Antunes MM, Silva ACC, Diniz AB, Alvarenga DM, Lopes MAF, de Souza Lacerda VA, Mattos MS, Araújo AM, Vidigal PVT, Lima CX, Mahecha GAB, Madeira MFM, Fernandes GR, Nogueira RF, Moreira TG, David BA, Rezende RM, Menezes GB. 2018. Immune and metabolic shifts during neonatal development reprogram liver identity and function. *J Hepatol* 69:1294–1307. <https://doi.org/10.1016/j.jhep.2018.08.018>.
51. Marques PE, Oliveira AG, Pereira RV, David BA, Gómes LF, Saraiva AM, Pires DA, Novaes JT, Patrício DO, Cisalpino D, Menezes-Garcia Z, Leevy WM, Chapman SE, Mahecha G, Marques RE, Guabiraba R, Martins VP, Souza DG, Mansur DS, Teixeira MM, Leite MF, Menezes GB. 2015. Hepatic DNA deposition drives drug-induced liver injury and inflammation in mice. *Hepatology* 61:348–360. <https://doi.org/10.1002/hep.27216>.
52. Mortola JP, Frappell PB. 2013. Measurements of air ventilation in small vertebrates. *Respir Physiol Neurobiol* 186:197–205. <https://doi.org/10.1016/j.resp.2013.02.001>.
53. Valadão PAC, Gomes MPSM, Aragão BC, Rodrigues HA, Andrade JN, Górcias R, Joviano-Santos JV, Luiz MA, Camargo WL, Naves LA, Kushmerick C, Cavalcante WLG, Gallacci M, de Jesus JCG, Guatimosim S, Guatimosim C. 2018. Neuromuscular synapse degeneration without muscle function loss in the diaphragm of a murine model for Huntington's disease. *Neurochem Int* 116:30–42. <https://doi.org/10.1016/j.neuint.2018.03.007>.
54. Valadão PAC, de Aragão BC, Andrade JN, Magalhães-Gomes MPS, Foureau G, Joviano-Santos JV, Nogueira JC, Ribeiro FM, Tapia JC, Guatimosim C. 2017. Muscle atrophy is associated with cervical spinal motoneuron loss in BACHD mouse model for Huntington's disease. *Eur J Neurosci* 45:785–796. <https://doi.org/10.1111/ejn.13510>.

8.5 Artigo 5: A suitable model to investigate acute neurological consequences of coronavirus infection.

Inflammation Research (2023) 72:2073–2088
<https://doi.org/10.1007/s00011-023-01798-w>

Inflammation Research

ORIGINAL RESEARCH PAPER



A suitable model to investigate acute neurological consequences of coronavirus infection

Jordane Pimenta¹ · Bruna Da Silva Oliveira¹ · Anna Luiza Diniz Lima³ · Caroline Amaral Machado¹ · Larisse De Souza Barbosa Lacerda¹ · Leonardo Rossi¹ · Celso Martins Queiroz-Junior¹ · Luiz Pedro De Souza-Costa⁵ · Ana Claudia Santos Pereira Andrade¹ · Matheus Rodrigues Gonçalves⁶ · Bárbara Mota¹ · Fernanda Martins Marim² · Renato Santana Aguiar² · Pedro Pires Goulart Guimarães³ · Antônio Lúcio Teixeira⁴ · Luciene Bruno Vieira³ · Cristina Guatimosim¹ · Mauro Martins Teixeira⁵ · Aline Silva De Miranda¹ · Vivian Vasconcelos Costa¹

Received: 2 June 2023 / Revised: 6 September 2023 / Accepted: 13 September 2023 / Published online: 14 October 2023
 © The Author(s), under exclusive licence to Springer Nature Switzerland AG 2023

Abstract

Objective and design The present study aimed to investigate the neurochemical and behavioral effects of the acute consequences after coronavirus infection through a murine model.

Material Wild-type C57BL/6 mice were infected intranasally (i.n) with the murine coronavirus 3 (MHV-3). **Methods:** Mice underwent behavioral tests. Euthanasia was performed on the fifth day after infection (5 dpi), and the brain tissue was subjected to plaque assays for viral titration, ELISA, histopathological, immunohistochemical and synaptosome analysis. **Results:** Increased viral titers and mild histological changes, including signs of neuronal degeneration, were observed in the cerebral cortex of infected mice. Importantly, MHV-3 infection induced an increase in cortical levels of glutamate and calcium, which is indicative of excitotoxicity, as well as increased levels of pro-inflammatory cytokines (IL-6, IFN- γ) and reduced levels of neuroprotective mediators (BDNF and CX3CL1) in the mice brain. Finally, behavioral analysis showed impaired motor, anhedonia-like and anxiety-like behaviors in animals infected with MHV-3.

Conclusions In conclusion, the data presented emulate many aspects of the acute neurological outcomes seen in patients with COVID-19. Therefore, this model may provide a preclinical platform to study acute neurological sequelae induced by coronavirus infection and test possible therapies.

Keywords MHV · SARS-CoV-2 · Acute neurological outcomes · Glutamatergic levels · COVID-19 · Animal behavior

Responsible Editor: Anatolii Kubyshev.

Jordane Pimenta and Bruna Da Silva Oliveira authors contributed equally to this work.

✉ Aline Silva De Miranda
 mirandas.aline@gmail.com

✉ Vivian Vasconcelos Costa
 vivianvcosta@gmail.com

¹ Department of Morphology, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Avenida Presidente Antônio Carlos, 6627, Belo Horizonte, MG 31270-901, Brazil

² Department of Genetics, Ecology and Evolution, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

³ Department of Physiology and Biophysics, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

⁴ Department of Psychiatry and Behavioral Sciences, McGovern Medical Houston, The University of Texas Health Science Center at Houston, Houston, TX, USA

⁵ Department of Biochemistry and Immunology, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

⁶ Department of Microbiology, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

Introduction

COVID-19 has resulted in more than 769 million cases and 6.9 million deaths worldwide [31]. Initially, COVID-19 was considered a respiratory disease, however, soon after it has been observed that the disease affects several organs and tissues, including the central nervous system (CNS) [1].

The first neurological symptom resulting from COVID-19 is typically gustatory and/or olfactory dysfunction [2]. In the most severe cases, peripheral neurological conditions such as Guillain-Barré syndrome, as well as conditions that indicate CNS involvement such as stroke, epilepsy/seizures, encephalitis, and others have been reported [2]. The GCS-NeuroCOVID study, which involved more than 3,500 patients, found that 80% of them reported neurological manifestations during COVID-19, with encephalopathy being the most prevalent [51].

Viral genetic material and SARS-CoV-2 (severe acute respiratory syndrome coronavirus-2) proteins have been identified in post-mortem brain tissue and cerebrospinal fluid (CSF) of patients affected by COVID-19 [3–7]. It is assumed that the olfactory nerve and olfactory bulb serve as a gateway to the CNS. Several viruses, including murine hepatitis coronavirus (MHV) [9, 10], have been shown to use this route to reach the CNS. SARS-CoV-2 RNA found in the CSF or CNS parenchyma of patients with COVID-19 may originate from the blood due to compromised blood–brain barrier (BBB) permeability [7], as well as from the migration of peripheral immune cells carrying the virus or viral fragments. COVID-19-related neurological symptoms are likely due to immune responses instead of a consequence of SARS-CoV-2 neurotropism [30]. The most accepted mechanism involved in the neurological and neuropsychiatric changes resulting from COVID-19 is the “cytokine storm”, in which various mediators that are peripherally produced can reach the CNS, triggering neuroinflammation [8]. Thus, SARS-CoV-2 may induce a loss of central homeostasis both through direct action and by a systemic inflammatory response [14]. Hypoxia resulting from SARS is also associated with disturbances in brain metabolism that can result in neurological manifestations [12].

Different mouse strains of CoVs induce hepatic, respiratory, and enteric diseases that are followed by neurological alterations [11]. Given the inherent limits of human studies on the kinetics and mechanisms related to the disease [7], experimental models may provide a better understanding of the disease and the development of therapeutic strategies. Recently, our research group standardized a murine model to study the acute lung injury and systemic manifestation induced by a betacoronavirus, the

MHV-3, which has the *Mus musculus* species as a natural host [13, 15]. When inoculated intranasally, infections induce severe lung inflammation which peaks at day 3 post-infection. This is followed by subsequent viral dissemination and systemic disease manifestations, which peak at day 5, when animals start to perish [13]. Recognizing the relationship between COVID-19 and the CNS, our objective was to investigate and characterize the effect of betacoronavirus infection in the CNS, using the intranasal route. We found that the MHV-3 infected mice displayed important inflammatory, neurochemical, and behavioral changes. Overall, this model may serve as a platform for studying possible acute neuropsychiatric alterations resulting from betacoronavirus infection, as well as for testing the efficacy of potential therapeutic strategies.

Materials and methods

Assay of cells, viruses, and plates

The cells used for viral propagation were L929 (ATCC CCL-1), a fibroblast cell line derived from a clone of normal subcutaneous connective tissue, areolar and adipose tissue of a male C3H/An mouse [65]. The cells were kept under a controlled atmosphere (37 °C and 5% CO₂) in Dulbecco's modified Eagle's medium (DMEM) with high content glucose content. The MHV-3 strain was granted by Clarice Weis Arns and Ricardo Durães-Carvalho of the State University of Campinas [(UNICAMP, Brazil; GenBank accession n°. MW620427.1; see reference [16])]. The propagated virus was titrated on L929 cells to determine the viral stock titer, which was found to be 1×10^6 PFU/mL. From the viral stock containing 1×10^6 PFU/mL, 100 µL of the viral stock was mixed with 900 µL of sterile saline solution to obtain 3×10^3 PFU in 30 µL. For viable virus detection, viral titration was performed from brain homogenates (1:9 tissue for DMEM). Cells were grown in 24-well plates and each well with a confluent monolayer of cells was inoculated with 100 µL of this homogenate. These plates were gently rotated for 1 h (4 × 15 min) to ensure effective viral adsorption. After 1 h the samples were removed from the wells, the overlay medium was added (DMEM containing 0.8% carboxymethylcellulose, 2% FBS and 1% penicillin–streptomycin–glutamine) and the plates were placed for 48 h at 37 °C and 5% CO₂. Subsequently, 10% neutral buffered formalin (NBF) was added for 1 h for cell fixation and 0.1% crystal violet was used for staining. The viral titer was expressed by counting the number of plaque forming units per gram of tissue (PFU/g tissue).

Mice

Animal experiments were carried out in WT C57BL/6, male and female, aged between 6 and 8 weeks, with approval from the Ethics Committee on Animal Experimentation of the UFMG (process n° 253/2020). The WT animals were obtained from the Central Animal Facility of UFMG maintained in micro-isolator cages at ABSL-2 under controlled conditions ($24 \pm 2^\circ\text{C}$; on a 12-h light/12-h dark cycle), with ad libitum access to water and food.

MHV-3 infection

Mice were smoothly anesthetized (ketamine solution [50 mg/kg] and xylazine [5 mg/kg], ip) and then inoculated i.n with 30 μL of sterile saline (mock), with MHV-3 at a concentration of 3×10^3 PFU/mL. Manifestations of morbidity, such as weight loss, dorsal arching, facial swelling, ruffled hair and prostration were monitored daily until the 5th dpi and the survival until the 14th dpi.

Sample

For sample collection, the simulated and infected animals were previously anesthetized (ketamine solution [50 mg/kg] and xylazine [5 mg/kg], ip) and after sedation they were euthanized by cervical dislocation, on the 5th dpi. Cervical dislocation without anesthesia was used only in experiments in which drug interference in the analyses is proven. The brain was harvested, the left hemisphere was placed in 10% formalin buffer, and the other hemisphere was frozen. Other tissues were collected and frozen for further analysis.

Histopathological analysis

Brains from mock and infected mice with MHV-3 were removed and processed for hematoxylin and eosin (H&E) staining according to the following works [17, 18]. The histopathological score was adapted from the cited works and the analyses performed by a pathologist in a blinded manner. The evaluation was performed in the cerebral cortex and in the hippocampus, following a point scale: 0, no lesion; 1 mild tissue injury and/or mild inflammation; 2 mild tissue injury and/or inflammation moderate; 3, definitive tissue damage (neuronal loss and parenchymal damage) and intense inflammation; 4, necrosis (complete loss of all tissue elements with the presence of cellular debris). Meningeal inflammation was assessed using a point scale (0 to 4) with 0 representing no inflammation and 1 to 4

corresponding to 1 to 4 layers affected by inflammation. The final score was the sum of the cerebral cortex and hippocampus scores plus the meningitis score, totaling 12 points.

Measurement of cytokine and chemokine concentration

Cerebral cortex homogenates were obtained by homogenizing frozen tissue in refrigerated cytokine extraction buffer (100 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 mM Triton X-100, 0.5% sodium deoxycholate and 1% protease inhibitor). This homogenate was subjected to centrifugation (14,000 g, 15 min, 4°C) and then the supernatant was collected for the determination of TNF, IFN- γ , IL-10, IL-6, BDNF and CX3CL1 using the immunosorbent assay system limited by mouse DuoSet enzymes (ELISA) (R&D Systems). According to the manufacturer, the sensitivity of the kit for each mediator: TNF (7.21 pg/mL), IFN- γ (2 pg/mL), IL-10 (5.22 pg/mL), IL-6 (1.8 pg/mL), BDNF (1.35 pg/mL) e CX3CL1 (0.32 ng/mL).

Immunohistochemistry IBA1, S100B, NeuN and cleaved Caspase-3

Sections of mouse cortex were evaluated for quantitative alteration of microglia, IBA1 (ionized calcium-binding adaptor molecule 1; antibody PA5-21,274, Invitrogen; 1:150), astrocytes (Anti-S100 beta antibody; ab41548, Abcam; 1:75), neurons (Anti-NeuN, MAB377, EMD Millipore; 1:200) and apoptosis (Anti-cleaved caspase-3; Asp175, Cell Signaling; 1:100,) according to the manufacturer's instructions (Vector Elite kit). Two independent experiments were carried out, each with $n = 5$ mice and both presented similar results. Representative photos of one of these experiments were used.

Transmission electron microscopy (TEM)

For ultrastructural analyzes, we used the protocol described by Rodrigues et al. [see reference [22]]. Briefly, mice were anesthetized with ketamine/xylazine and transcardially perfused with ice-cold modified Karnovsky fixative and maintained in this solution overnight at 4°C . The brain was removed, and the cortex was isolated were washed with cacodylate buffer (0.1 M), cut into several pieces, post-fixed in reduced osmium (1% osmium tetroxide containing 1.6% potassium ferrocyanide) and contrasted en bloc with uranyl acetate (2% uranyl acetate in deionized water). The samples were then dehydrated through an ascending series of ethanol solutions and embedded in EPON resin. Blocks were sectioned (50 nm) and collected on 200 or 300 mesh copper grids 8 and contrasted with lead citrate. Sections

were viewed with a Tecnai- G2-SpiritFEI/Quanta electron microscope (120 kV Philips) located at Microscopy Center – UFMG.

Purification of synaptosomes

Immediately after euthanasia, the cortex was removed and homogenized in a gradient solution containing: 320 mM sucrose, 0.25 mM dithiothreitol, 1 mM EDTA. Then, the homogenate was exposed to a low-speed centrifugation ($1000 \times g \times 10$ min). Synaptosomes were isolated from the supernatant by discontinuous Percoll density gradient centrifugation [according to reference [19]]. Isolated nerve terminals were resuspended in Krebs–Ringer–HEPES (KRH) solution containing: 124 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 10 mM glucose, 25 mM HEPES, with pH 7.4 and without addition of $CaCl_2$, at a concentration of approximately 10 mg/mL. For measurement of glutamate release and intrasynaptosomal calcium concentration, aliquots of 30 μ L were prepared and kept on ice until use.

Measurements of glutamate release

To measure continuous glutamate release, a fluorimetric assay was performed in a fluorimeter (Synergy TM2, Biotek®). Fluorescence emission was registered using an excitation wavelength of 360 nm and emission of 450 nm. Glutamate release was measured indirectly by following the increase in the fluorescence due to the production of NADPH in the presence of glutamate dehydrogenase type II and $NADP^+$. In brief, synaptosomes were incubated with 1 mM $CaCl_2$, 1 mM $NADP^+$ in KRH medium for 5 min. Glutamate dehydrogenase (50 units per well) was added to each well after 5 min. For depolarization, we used 33 mM KCl. Calibration curve was achieved by the addition of glutamate (5 nM/ μ L) to the reaction medium. Glutamate levels were normalized to the total amount of protein per well.

Measurements of intrasynaptosomal free calcium concentration

To measure intrasynaptosomal free calcium concentration, synaptosomes were preincubated with 5 μ M of Fura-2 pentakis (acetoxymethyl) Ester (FURA2-AM) probe for 30 min at 35.5 °C. Next, synaptosome was centrifuged ($3000 \times g \times 60$ s), resuspended in KRH without $CaCl_2$, and reincubated again during 30 min. After washout with KRH without $CaCl_2$ synaptosomes were immediately used for quantification of intracellular free calcium ($[Ca^{2+}]_i$). Fluorescence was recorded with an excitation wavelength of 340/380 nm and an emission of 510 nm. $CaCl_2$ (1 mmol/L, final concentration) was added in synaptosomal suspension before reading and 33 mM of KCl was added to evoke

calcium influx. Finally, we added 10% SDS to obtain R_{max} and tris-EGTA (3 mol/L Tris, 400 mmol/L EGTA, pH 8.6) to obtain R_{min} [as described in references 20 and 21].

Open field test

Locomotor activity and anxiety-like behavior were evaluated by the Open field test on the 3rd, 4th and 5th dpi [28]. Briefly, animals were videotaped by the Phenotyper apparatus (Noldus, Information Technology, Leesburg, VA, USA). There were four analysis cages in the room, which allowed for observation individually at the same time. Each cage (30 $\times$ 30 cm) contained a top unit with a digital video camera and infrared lights. Mice were placed in the center of the open field arena and were allowed to freely move and explore the environment for thirty minutes. Parameters such as locomotion activity and percentage of time spent at the center area (anxiety measure) were recorded and analyzed by a tracking software (EthoVision XT, Noldus Information Technology, Leesburg, VA, USA).

Marble buried test

Mice at 4th day post-MHV-3 infection were tested in the Marble buried test, which evaluates compulsive-like behavior. The test was previously described [see reference [26]]. Mice were tested in a rectangular cage (30 $\times$ 30 $\times$ 50 cm) with 20 cm of fresh beddings. 25 marbles were placed equidistant to each other. Briefly, the animal was placed in the middle of the cage and allowed to explore and bury for 30 min. At the end of the section, the animals were removed and measured the number of marbles buried. Only the balls buried by more than 2 cm were considered.

Forced swim test

Mice at 4th day post-MHV-3 infection were tested in the forced swimming test, which evaluates the depressive-like behavior. The test was performed as previously described [see reference [27]]. Mice were placed in a cylindrical tank (30 cm height $\times$ 20 cm diameters) and recorded for 6 min. The duration of immobility time was recorded during the last 4 min of the 6 min testing period, after a 2 min habituation period by the EthoVision XT (Noldus, Technology, Leesburg, VA, USA).

Elevated plus maze test

Mice at 4th day post-MHV-3 infection were tested in the elevated plus-maze test (EPM), which evaluates mice anxiety-like behavior. The EPM test was performed as previously described [see reference [30]]. Mice were placed in the EPM apparatus consisting of two open and two closed

arms, crossed in the middle perpendicularly to each other creating a center area. Briefly, mice were placed in the center of the apparatus and allowed to freely explore for 5 min. The apparatus was wiped clean with 70% alcohol and dried with paper towels between each trial. The percentage (%) of open arm entries and % of time spent on the open arms (anxiety-like behavior measure) were recorded and analyzed by EthoVision XT (Noldus, Technology, Leesburg, VA, USA).

Sucrose preference test

The sucrose preference test was employed to assess anhedonic-like behavior in mice at 3rd, 4th and 5th day post-MHV-3 infection as described elsewhere [29]. Briefly, at day 1 and 2 after MHV infection, i.e., the first two days of the test, the animals were habituated to two bottles of freshwater. On the following three days, corresponding to the 3rd, 4th, and 5th day post-MHV infection, the previous bottles were removed and replaced with a bottle filled with water and another one filled with 1% sucrose solution. The percentage of sucrose preference was calculated for each of the last three days of experiment.

Statistical analyses

All statistical analyses were performed, and all graphs were created in GraphPad Prism 8 (GraphPad Software). Data distribution was assessed by the Shapiro–Wilk test. Parametric comparisons between groups were performed using Student's *t*-test. The Mann–Whitney test was used to assess differences between nonparametric data. Data were presented as mean $\pm$ Standard Deviation (SD). Differences with a *p*-value < 0.05 were considered statistically significant.

Results

Characterization of cellular and histopathological alterations in the brain of mice infected with MHV-3

To evaluate potential brain alterations induced by the coronavirus, male and female C57BL/6 J mice, 6–8 weeks of age, were infected i.n. with 3×10^3 PFU/mL of MHV-3 (Fig. 1a). Analyses were performed on the fifth day after infection (5th dpi) when systemic manifestations peaked, as previously described [13]. Upon infection, MHV-3 replicates in the brain of female (Fig. 1b) and male mice (Fig. S1b). Histopathological analysis (H&E) of the cerebral cortex revealed the presence of hyperemic blood vessels, surrounded by few leukocytes, which translated to a mild histopathological score in a similar way in both sexes (Fig. 1c–d and S1c–d). Subsequently, we investigated whether this mild CNS damage was related to changes in the number of cells as

well as in the process of apoptosis. According to H&E data, no significant differences were found in the number of cells stained for NeuN (Fig. 1e), IBA1 (Fig. 1f), S100B (Fig. 1g), and caspase 3 (Fig. S1h) in the cerebral cortex of female MHV-3-infected mice compared with mock animals. Similar results were also found in male C57BL/6 mice (Fig. S1e–g, i). Overall, the presented data suggest that the mild histopathological damage induced by MHV-3 was not associated with neuronal or glial death.

Signs of neuronal degeneration were observed in the cerebral cortex of mice infected with MHV-3

To evaluate potential neurodegenerative signs caused by MHV-3 infection, we performed a qualitative ultrastructure analysis of female mouse cerebral cortex samples from infected and non-infected mice (Fig. 2). Our data showed signs of degeneration in neuronal cell bodies. For example, Fig. 2a (red arrowhead) shows a dark degenerating neuron with a shrunk cell body, darker staining (increased electron density), Golgi and endoplasmic reticulum dilation (Fig. 2a', arrows), and mitochondria with disrupted cristae (Fig. 2a'' and c'', arrows). We also observed typical neuronal cell bodies such as the profile shown in Fig. 2b but with prominent and complex lipofuscin granules (Fig. 2b', c' and c'' arrowheads). Both in control and infected mice (in addition to the ones described above), there were neuronal cell bodies with typical morphological features such normal cell size, typical nucleus with normal chromatin (Fig. 2d) and mitochondria with conserved cristae (Fig. 2d'). Eventually, we noticed occasional lipofuscin granules in control mice, such as the one on Fig. 2d, (blue arrowhead) but they were less complex than the ones observed in MHV-3 infected cerebral cortex.

MHV-3 infection induces glutamate release, intracellular calcium levels elevation and production of inflammatory mediators in the cortical brain of mice

Glutamate is an important excitatory neurotransmitter, and numerous studies have indicated that overstimulation of the glutamatergic system through activation of ionotropic glutamate receptors can lead to excitotoxicity, which may result in neuronal calcium overload and consequent neurodegeneration [18, 23, 24]. To investigate whether coronavirus has the potential to induce neurochemical changes in the cerebral cortex, we measured glutamate levels and intracellular calcium in cortical synaptosomes of female and male mice [25–27]. Indeed, MHV-3 infection induced a greater release of glutamate, as well as an increase in intracellular calcium levels, in the cerebral cortex of both male and female infected animals (Fig. 3b–e).

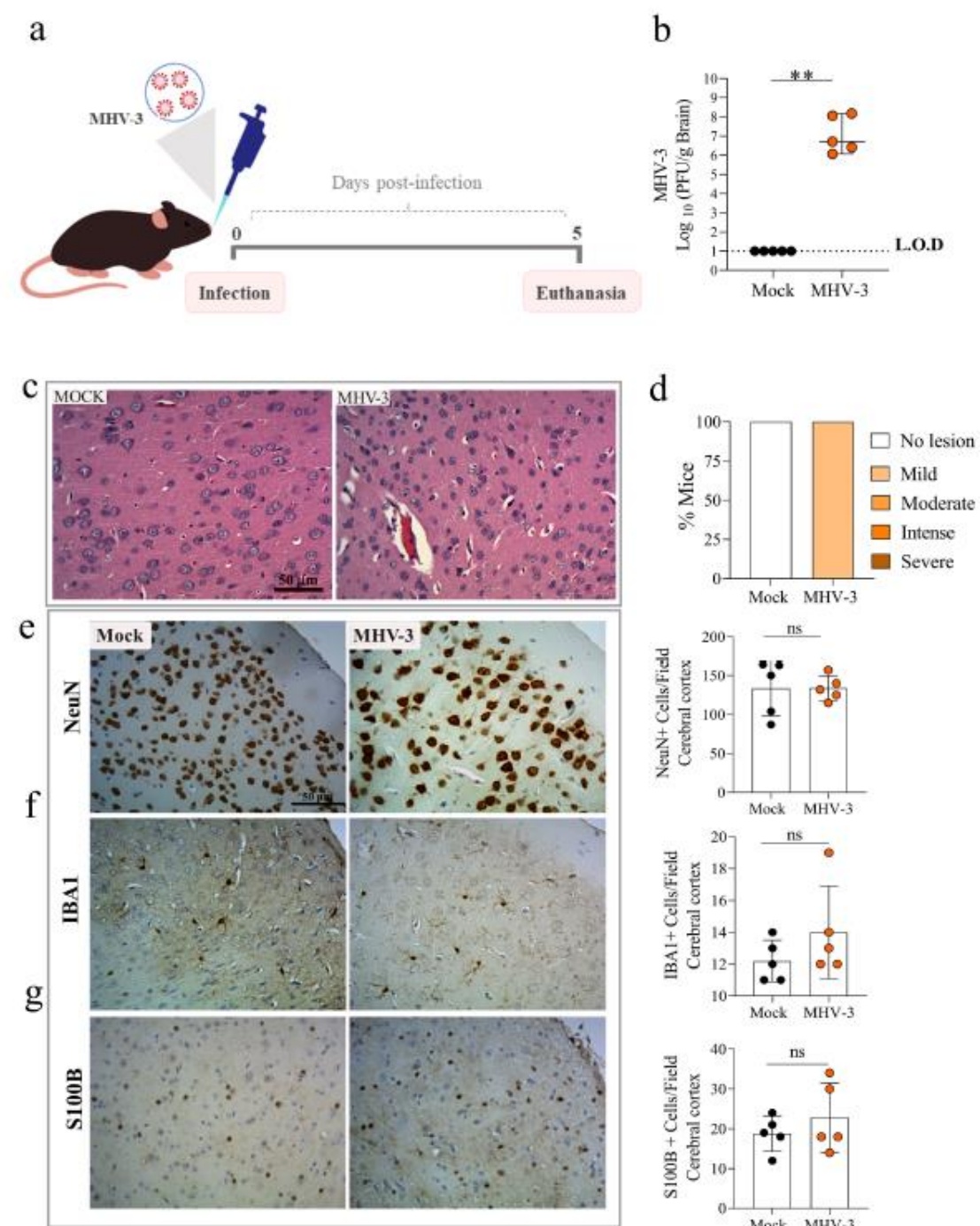


Fig. 1 Intranasal MHV-3 infection induces mild alterations in the cerebral cortex of mice. Female C57BL/6 mice ($n=5$ /group) were infected i.n. with 3×10^3 PFU/mL of MHV-3. Euthanasia was performed at 5 dpi. **a** Experimental design. **b** Viral titers quantified in brain extracts of MHV-3 infected mice by plaque assay. Results are demonstrated as $\log_{10}$ PFU per gram of tissue. Statistical comparison among groups were made using the Mann–Whitney test (median with 95% CI). LOD, limit of detection. **, $p < 0.01$. **c** Hematoxylin and eosin (H&E) staining of sections of the cerebral cortex showed mild histological damage in infected mice, 50 μ m bars. **d** Percentages of mice according to the degree of tissue damage in the brain. **e** Immunohistochemistry using antibodies anti-NeuN (count of neurons), **f** anti-IBA1 (microglia/macrophages count), **g** anti-S100B (astrocyte count). Representative images are on the left panels and the quantification of positive cells (ImageJ software) is presented on the right panels. Results are expressed as the mean of all cells counts per group and statistical comparison among groups was made using unpaired Student *t*-test (mean $\pm$ SD); ns, not significant ($p > 0.05$)

Next, we evaluated the levels of certain molecules involved in the host response induced by infections, such as cytokines (interleukin-6 [IL-6], interferon-gamma [IFN- γ], tumor necrosis factor [TNF], interleukin-10 [IL-10], brain-derived neurotrophic factor [BDNF]), and the chemokine CX3CL1 (fractalkine) in distinct brain areas of mice infected with MHV-3 and their control littermates. Cytokines IL-6 and IFN- γ showed an increase in the prefrontal cortex (PFC) of female mice infected with MHV-3 compared to mock animals (Fig. 4a and b). In male mice infected with MHV-3, there was an increase in IFN- γ levels in the PFC, while IL-6 was not altered (Fig. 4g and h). No significant differences were found in the concentration of TNF and IL-10 in both sexes (Fig. 4c–d and i–j). Interestingly, female mice infected with MHV-3 had a reduction in BDNF and CX3CL1 levels in the PFC compared with control mice, while male mice responded to the infection with an increase in CX3CL1 levels and no significant changes in BDNF concentrations in the PFC compared with control littermates (Fig. 4e–f and k–l, respectively). We measured the same mediators in the hippocampus and striatum of both sexes. Regarding the concentrations of IL-10, TNF, IFN- γ and CX3CL1, there were no significant changes in these regions in both sex (Figs. S2 and S3). IL-6, on the other hand, had its concentration increased in the hippocampus and striatum of females infected with MHV-3 compared with their controls, while no change was observed in males (Fig. S2a and S2g; S3a and S3g, respectively). Levels of the neurotrophic factor BDNF did not change in the hippocampus and striatum of infected female mice (Fig. S2e and S2k) but were increased in the hippocampus of male MHV-3 mice (Fig. S3e).

Overall, our results show that MHV-3 infection induces acute neurochemical alterations in the brain of mice, as demonstrated by increased levels of glutamate/calcium, suggesting the occurrence of excitotoxicity, and changes in the levels of inflammatory mediators. Furthermore, we observed that female mice seem to be more susceptible to MHV-3

infection and to the reduction of neuroprotective mediators compared to male mice.

MHV-3 infection promotes anxiety-like and anhedonia-like behaviors alongside motor dysfunction in mice

We evaluated whether MHV-3 infection is associated with acute behavioral changes in mice. No significant differences were observed in the total distance traveled in the Open Field Test (OFT) on days 3 (Fig. 5b and S4a) and 4 (Fig. 5c) post-infection. However, on day 5, when systemic disease symptoms peaked, MHV-3-infected female mice (Fig. 5d) but not male mice (Fig. S4b) showed a significant decrease in the total distance traveled compared to non-infected controls, indicating locomotor impairment in females. At 4 days post-infection, female infected mice displayed anxiety-like behavior, as indicated by a significant decrease in the percentage of time spent in the open arms of the elevated plus maze (EPM) (Fig. 5e), which was not observed in male mice (Fig. S4c). Both female and male infected mice displayed an anhedonic-like behavior in the Marble Burying test (MBT) at 4 days post-infection, as indicated by a significant reduction in the number of marbles buried (Fig. 5f and S4d). In addition, infected male mice had an increase in immobility time in the Forced Swimming Test (FST) (Fig. S4e), but no change in the frequency of immobility (Fig. S4f). Infected females, on the other hand, did not present significant differences in the time or frequency of immobility in the FST when compared to their control group (Fig. 5g–h). When performing the sucrose preference test (SPT), females infected with MHV-3 showed an anhedonic behavior on the third and fifth dpi. In summary, these results suggest that MHV-3 infection induces acute behavioral changes in mice, especially in females, including locomotor impairment, anxiety-like and anhedonic-like behavior.

Discussion

COVID-19 has been associated with a wide range of clinical manifestations, including neurological and psychiatric alterations. In this study, we investigated whether MHV-3 can directly affect the CNS and serve as a suitable platform to study COVID-19-related neurobiological mechanisms and identify novel therapeutic targets. The main findings of this study are: (i) MHV-3 delivered intranasally is capable of infecting and replicating in the CNS, causing mild histopathological alterations; (ii) MHV-3 infection results in increased glutamate release, intracellular calcium levels and pro-inflammatory mediators in the cortex; (iii) MHV-3 infection decreases the production of neuroprotective mediators such as BDNF and CX3CL1 in the brain; and (iv)

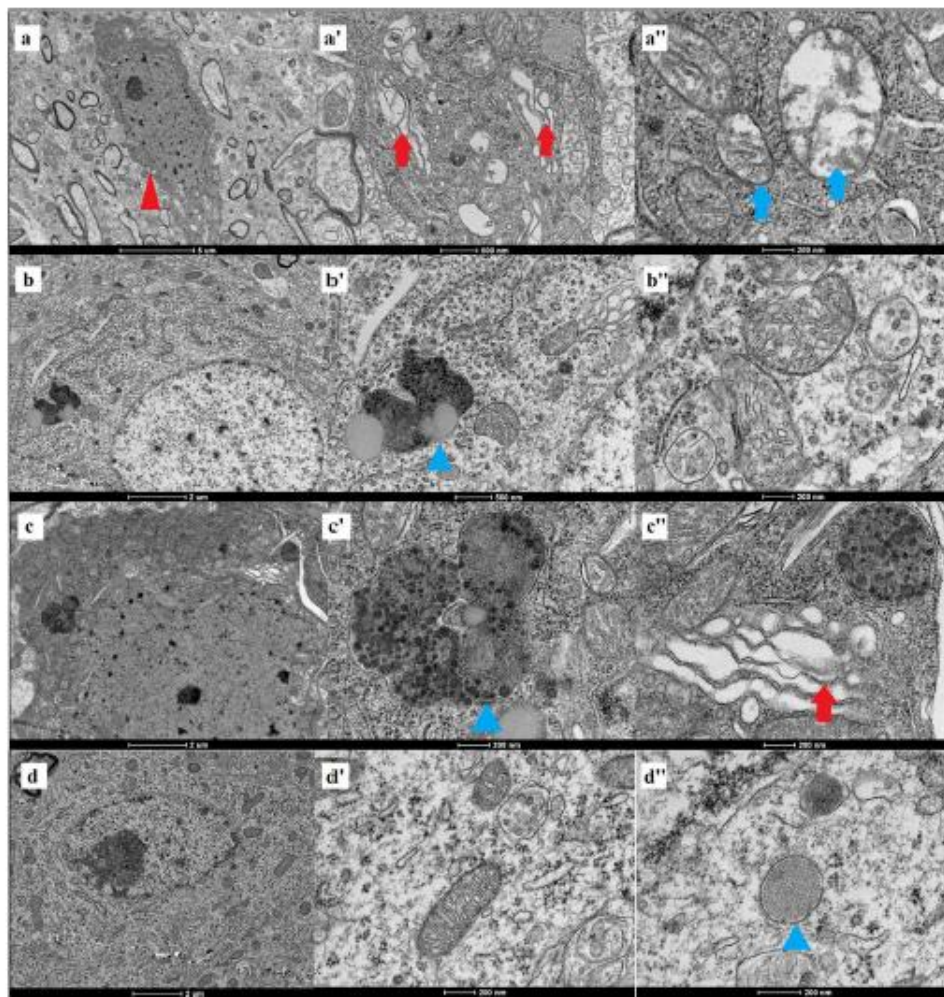


Fig. 2 Cerebral cortex neurons from MHV-3 infected mice show signs of degeneration at the ultrastructure level. **a–a''** Female C57BL/6 mice were infected intranasally with 3×10^3 PFU/mL of MHV-3. A dark degenerating neuron profile from cerebral cortex of MHV-3 infected mouse (red arrowhead) with enlarged Golgi cisternae (a' red arrows) and mitochondria with disrupted cristae (a''

blue arrows). **b–b''** and **c–c''** cerebral cortex neurons from two distinct MHV-3 infected mice presenting complex lipofuscin granules (b' and c' blue arrowheads) and enlarged Golgi cisternae (red arrow c''). **d–d''** A neuron profile from a mock mouse cerebral cortex with regular features

Animals infected with MHV-3 showed signs of anxiety-like and anhedonic-like behaviors and motor dysfunction, especially female mice.

Models that assess behavioral changes in the context of betacoronavirus infections in the CNS are still scarce. We have demonstrated the applicability of a murine betacoronavirus model as a platform to recapitulate the acute neurological and psychiatric symptoms observed in patients with COVID-19. All analyses were performed up to the 5th

day post infection, i.e., at the peak of systemic disease [13]. First, the neurotropism of MHV-3 was confirmed by recovering viable virus particles in the brain of mice. In addition, histopathological analysis of the brain of MHV-3-infected mice revealed mild changes, such as dilated blood vessels in the meninges, an inflammatory infiltrate, and hyperemic vessels in the cerebral cortex. These findings support human studies showing that SARS-CoV-2 was detected in the post-mortem brain of half of COVID-19 patients along with mild

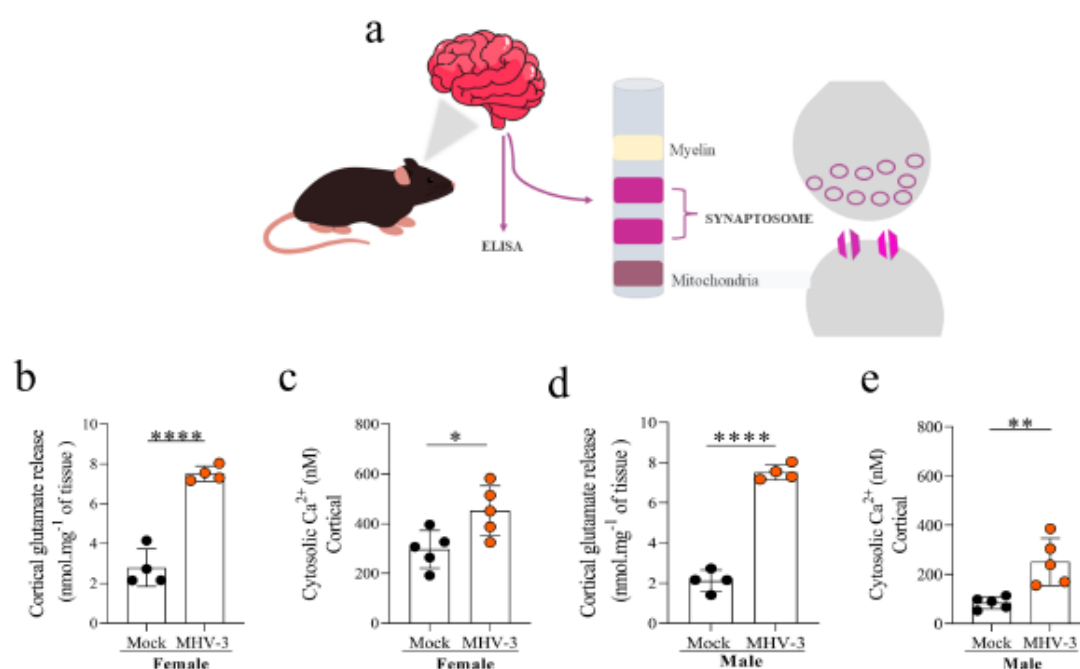


Fig. 3 MHV-3 infection induces increased cortical glutamate levels and intrasynaptosomal calcium levels in mice female and male C57BL/6 mice infected i.n. with 3×10^3 PFU/mL of MHV-3. **a** Schematic figure of cortical synaptosome preparation. **b** Cortical glutamate levels and **(c)** cortical intrasynaptosomal calcium levels were measured in female mice infected with MHV-3 and control

group. **d** and **e** Cortical glutamate levels and cortical intrasynaptosomal calcium levels were also measured in male mice infected with MHV-3 and mock group, respectively. Statistical analysis was performed using unpaired Student *t*-test (mean $\pm$ SD) or Mann-Whitney test (median with 95% CI); $n=4$ to 5/group. * $p<0.05$; ** $p<0.01$, **** $p<0.001$

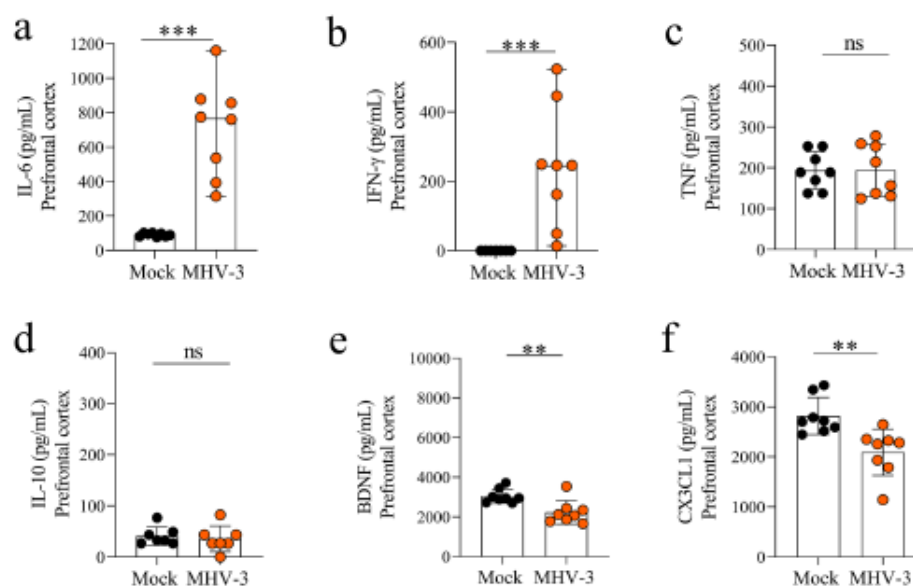
neuropathological lesions [3]. Although many studies have not found SARS-CoV-2 in the brain, it is already known that the immune response triggered by the virus can culminate in a “cytokine storm”, which reaches and compromises the homeostasis of the CNS, as well as other tissues [8].

In addition, we conducted a quantitative analysis of neurons (NeuN), microglia/macrophage (IBA1), and astrocytes (S100B), as well as evaluated apoptosis (cleaved caspase 3). None of these markers were found to be altered in the cortex of infected animals when compared to mock. However, this finding does not necessarily indicate that glia and neurons were not affected. In fact, brain tissues from individuals who died with COVID-19 showed that microglia abundantly expressed the lysosomal marker CD68, which is a marker of phagocytic activity, especially in the olfactory bulb and cerebellum [52]. Boroujeni et al. [53] also demonstrated that the inflammatory response in COVID-19 caused neuronal death in the cerebral cortex (post-mortem) of critically ill patients [53]. NeuN quantification revealed a similar number of marker-positive neurons when we compared the infected and control groups. Nonetheless, the analysis of

the ultrastructure of cerebral cortex samples from animals infected with MHV-3 revealed the presence of darkened neurons, known as Dark Neurons (DNs), indicating ongoing degeneration. Golgi complexes and dilated endoplasmic reticulum and mitochondria with ruptured cristae were also observed in these cases. Although the NeuN quantification showed no change, about 90–99% of DN recover with time, while a small number of neurons die [54]. Electron microscopy studies have shown that these DN have low functional activity. However, the origin and mechanism of the emergence of these dark neurons remain undefined [54].

In the present study, we also measured the levels of the excitatory neurotransmitter glutamate and calcium (Ca^{2+}) released at presynaptic terminals in the cerebral cortex [55–59]. Our results showed that MHV-3 infection led to massive glutamate release and increased intracellular Ca^{2+} levels. Other viruses such as HIV, ZIKV, and H1N1 have been reported to impair glutamatergic transmission, which can result in excitotoxicity and impaired cell signaling [18, 32, 60, 61]. Additionally, HCoV-OC43, a human coronavirus, can infect human CNS neuronal and glial cells and

Female



Male

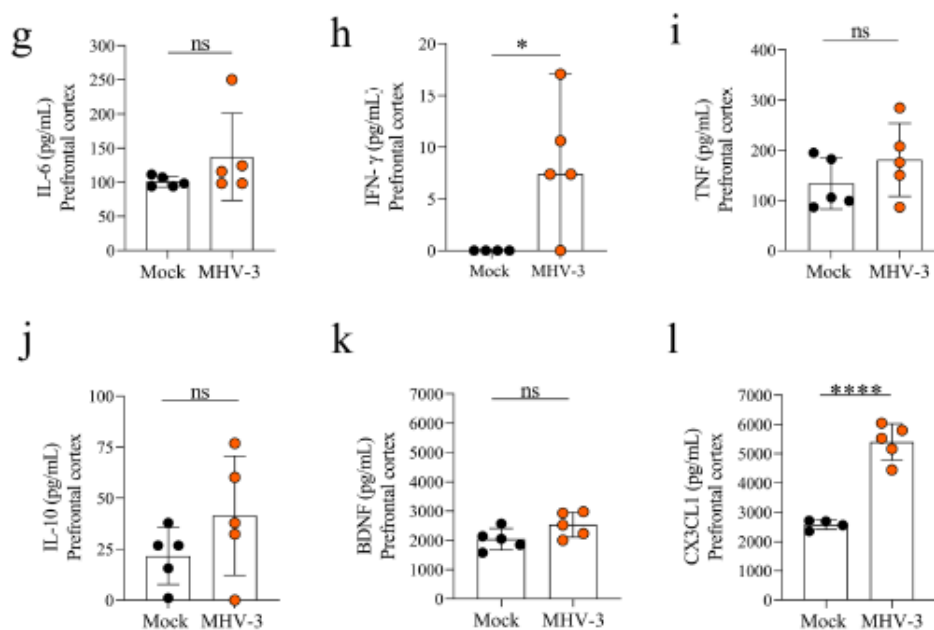


Fig. 4 Inflammatory mediator levels in the cortex of female and male mice upon MHV-3 infection. Female and male C57BL/6 mice were infected i.n. with 3×10^5 PFU/mL of MHV-3. Levels of IL-6, IFN- γ , TNF, IL-10, BDNF and CX3CL1 (fractalkine) (a–f) were quantified by enzyme-limited immunosorbent assay (ELISA) in the prefrontal cortex of control and MHV-3-infected mice. Statistical analysis was performed using unpaired Student *t*-test (mean $\pm$ SD) or Mann–Whitney test (median with 95% CI); *n* = 7 to 8/group (female) and *n* = 4 to 5/group (male). *ns* not significant (*p* > 0.05); **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001

activate neuroinflammatory mechanisms [33]. Infection of mice with HCoV-OC43 has been shown to induce neuronal dysfunction and decrease the expression of the glutamate transporter GLT-1 in astrocytes, potentially leading to increased central glutamate levels [34]. We also observed an increase in IL-6 levels in the PFC of animals after MHV-3 infection. IL-6 can be produced within the CNS by various cell types and infiltrating inflammatory cells under neuroinflammatory conditions [35]. It has been reported that membrane depolarization is one of the primary mechanisms for the upregulation of IL-6 in neurons, which is dependent on glutamatergic activation of N-methyl-D-aspartate receptors (NMDA-R), an increase in Ca^{2+} levels, and activation of Ca^{2+} -dependent kinases, such as calmodulin [36]. In a ZIKV infection model, we demonstrated that neuronal cell death could be prevented when infected animals were treated with an NMDA-R non-competitive inhibitor. Neurodegeneration and microgliosis induced by the infection were also inhibited *in vitro* and *in vivo* [8, 61].

It is interesting to note that increased levels of IL-6 have been associated with anxiety and depressive symptoms [37, 38], and play a role in neurogenesis [39]. Additionally, mounting evidence suggests that inflammation and alterations in glutamate neurotransmission may contribute to the pathophysiology of mood disorders [40]. There is evidence indicating that fractalkine has protective effects against glutamate-mediated excitotoxicity, as fractalkine increases BDNF levels and TrkB phosphorylation [41]. In our study, we observed a decrease in cortical levels of fractalkine and BDNF, which leads us to speculate that neuroprotection mechanisms are reduced, contributing to depressive-like behaviors in mice [62]. While IFN- γ contributes to virus clearance [42], we observed a decrease in IFN- γ levels after MHV-3 infection. Interestingly, at low physiological glutamate concentrations, glutamate can directly activate naïve T cells via AMPA-R. However, when glutamate concentration markedly increases, this neurotransmitter usually inhibits T cell function [43].

To evaluate the behavioral consequences of MHV-3 infection, we conducted several specific tests. In the elevated plus maze test, we observed anxiety-like behavior in the infected animals. This finding is consistent with a previous study on DENV-3 encephalitis, which showed that infected mice

also displayed anxiety-like behavior and increased mRNA expression of pro-inflammatory cytokines in the hippocampus associated with neuronal loss [55]. BDNF has been shown to have an antidepressant function in the PFC [45] and hippocampus [46]. In addition, another study demonstrated that administration of BDNF into the hippocampus of rats reduced anxiety-like behavior in the elevated plus maze test [47], suggesting that the reduction in BDNF levels may be associated with the development of anxiety disorders. Moreover, impaired neuron-microglia communication, specifically the CX3CL1/CX3CR1 pathways, has been increasingly linked to the development of psychiatric conditions [48].

Studies performed in transgenic mice (K18-hACE2) that overexpress human ACE-2 and are infected with SARS-CoV-2 strongly support the acute CNS effects observed in our MHV-3 model. Kumari and colleagues [58] reported that intranasal inoculation of SARS-CoV-2 in K18-hACE2 mice was associated with neuroinvasion and encephalitis. These findings were corroborated by Oladunni et al. [62], who demonstrated that intranasal infection of K18-hACE2 mice resulted in viable viral titers in various organs, including the nostril, lungs, and brain. Moreover, the infection was linked to local and systemic chemokine storms, with increased levels of CXCL-2, CXCL-10, and CCL-3 in the brain of SARS-CoV-2-infected mice. Finally, another recent study showed that primary neurons isolated from K18-hACE2 mice are susceptible to SARS-CoV-2 infection, and that the infection upregulates the expression of genes involved in innate immunity and inflammation, including IFN- α , ISG-15, CXCL-10, CCL-2, IL-6, and TNF, in the neurons [63].

According to Andrade et al. [13], there are no sex differences in the lung disease caused by MHV-3 in mice. This finding is supported by Oladunni et al. (2020) who found no significant difference in chemokine levels in the lung between male and female K18-hACE2 transgenic-infected mice, except for CXCL-10 at early time points of infection, which was significantly higher in female K18-hACE2 mice. However, our results indicate significant sex differences in the central nervous system (CNS) of MHV-3 infected mice, particularly in terms of the inflammatory response. Female mice infected with MHV-3 had increased levels of IL-6 in the analyzed brain regions (PFC, hippocampus, and striatum), while this cytokine did not change in male mice. On the other hand, infected male mice showed an increase in CX3CL1 in the PFC and BDNF in the hippocampus compared to the control group, while females infected had reduced levels of these mediators in the PFC. Previous studies have also reported the role of BDNF in the pathogenesis of depression, and its action is directly related to the brain region [44].

The Brazilian COVID Registry Project, a multicenter study that collected data from 39 Brazilian hospitals in 17

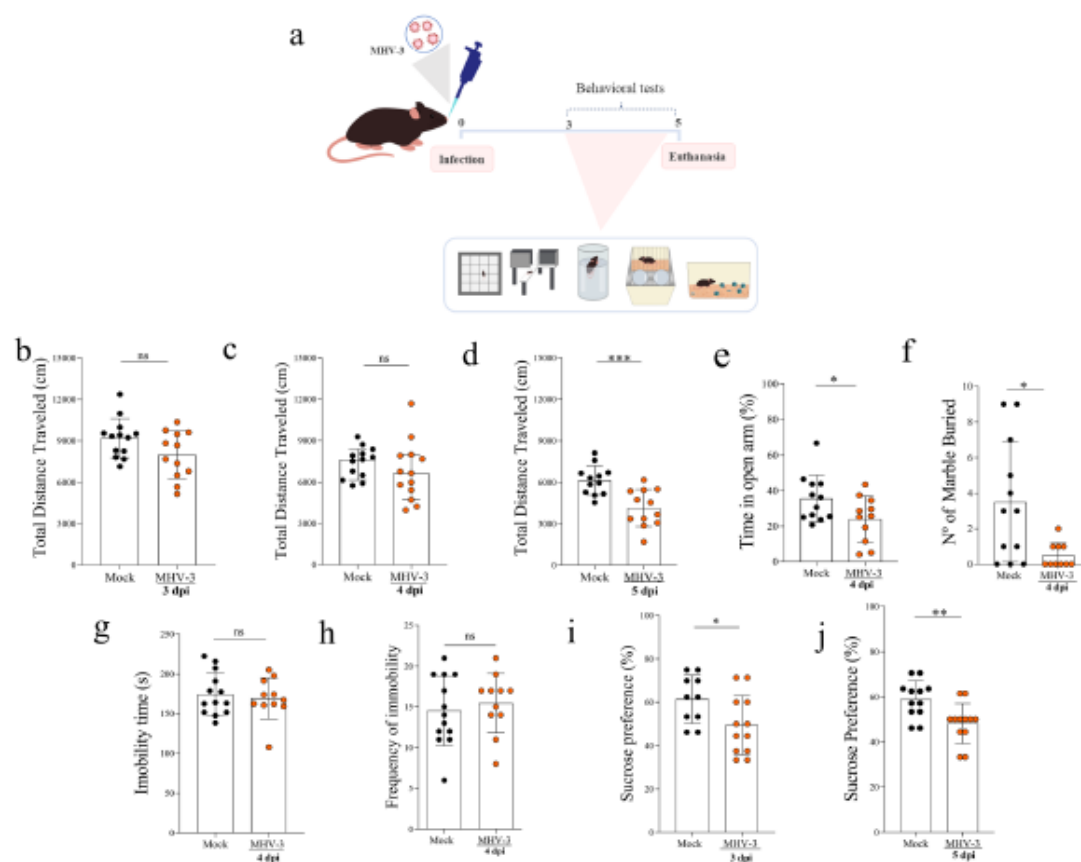


Fig. 5 MHV-3 infection induces behavioral alterations in female C57BL/6 mice. **a** Experimental design. **b–d** Locomotor activity was assessed using the open field test. **e** Anxiety-like behavior was assessed by calculating the percentage of time the animals remained in the open arms of the elevated plus maze. **f** Anhedonic-like behavior

was assessed by the Marble Burying test. **(g, h)** Forced Swimming test and **(i, j)** Sucrose preference test. Statistical analysis was performed using unpaired Student *t*-test (mean $\pm$ SD) or Mann-Whitney test (median with 95% CI); *n* = 12 to 13/group. *ns* not significant (*p* > 0.05); **p* < 0.05; ***p* < 0.01; ****p* < 0.001

cities, observed that patients admitted with COVID-19 and clinically diagnosed with neurological syndromes had a higher incidence of septic shock, ICU admission and death compared to controls [49]. Furthermore, the study analyzed the incidence of neurological manifestations at hospital admission by gender and age group and found that women were more likely to experience headaches and less likely to have seizures [50]. In MHV-3 infection in females, there was a reduction of BDNF and CX3CL1, which may result in behavioral changes and loss of protection mechanisms. On the other hand, infected males show an increase in CX3CL1 and maintain homeostatic BDNF levels, which may help to mitigate possible behavioral repercussions.

The MHV-3 model, like other models used to study the neurological effects of COVID-19, has several limitations.

First, it is not suitable for investigating the viral entry step, as SARS-CoV-2 uses a different receptor, ACE-2, while MHV-3 enters the cell through the CEACAM-1 adhesion molecule. This limits the usefulness of the model in studying drugs that target viral entry. Additionally, the MHV-3 model is very acute and lethal, with mice succumbing to the infection within a short period of time (around 6–7 dpi). As a result, it may be challenging to detect tissue damage in refractory organs such as the brain and heart [64] using regular techniques. In addition, future studies should be performed on the third and fourth day after infection to better understand the early inflammatory and biochemical mechanisms of infection related to neuropsychiatric symptoms. Another limitation of this work is the evaluation of a single protein (cleaved caspase 3)

involved in the apoptotic pathway, so there is a need to explore other caspases/pathways related to programmed cell death. We found some differences between males and females in some experiments, while the sex differences are intriguing and warrant further investigation, such an exploration would require dedicated research and analysis and are beyond the scope of this study.

In fact, fatigue is a very common symptom in cases of COVID and, above all, post-COVID. The frequency of immobility can be considered as an indication of motor activity in the forced swimming test [66, 67]. In the present study, no significant differences were found in the frequency of immobility when comparing mice infected with MHV-3 and controls, regardless of sex. These results, along with the open field data, suggest that the observed depressive-like behavior in males may not be solely attributed to fatigue. However, we acknowledge that further comprehensive investigations are warranted to systematically explore the role of fatigue during MHV-3 infection. Nevertheless, despite these limitations, the MHV-3 model can still be valuable in mimicking a severe betacoronavirus infection and may serve as a useful platform for further studies on acute neuropsychiatric changes caused by COVID-19, as well as testing potential novel therapeutic strategies against severe disease.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00011-023-01798-w>.

Author contributions J.C. Pimenta: Conceptualization, Methodology, Investigation, Writing – Original Draft, Writing – Review & Editing, Project administration. B. D. S. Oliveira: Investigation, Writing – Original Draft; A.L.D. Lima: Investigation, Methodology. C.A. Machado: Investigation. L. Rossi: Investigation, Writing – Original Draft- Review & Editing. L.D.S.B. Lacerda: Investigation. C.M. Queiroz-Junior: Investigation, Writing – Original Draft- Review & Editing. A.C. D. S. P. Andrade: S.R. Oliveira: Conceptualization, Methodology. M.R. Gonçalves: Investigation. B. Mota: Investigator. F. M. Marim: Investigator- Review & Editing. R. Santana: Review & Editing. P. Pires: Resources. A. L. Teixeira: Writing -Review & Editing. L.B. Vieira: Writing - Review & Editing, Resources. C. Guatimosim: Writing – Review & Editing, Resources. M.M. Teixeira: Writing – Review & Editing, Resources, Project administration, Funding acquisition. A.S.D. Miranda: Conceptualization, Methodology, Resources, Writing – Review & Editing, Project administration. V.V. Costa: Conceptualization, Methodology, Resources, Writing – Review & Editing, Supervision, Project administration, Funding acquisition.

Funding This study was funded by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, 88881.507175/2020-01, Fundação de Amparo à Pesquisa do Estado de Minas Gerais, APQ 02281-18, APQ 02281-18, Conselho Nacional de Desenvolvimento Científico e Tecnológico, 465425/2014-3

Data availability The data that support the findings of this study are included within the article and available if required.

Declarations

Competing interests The authors declare no competing interests.

References

- Bougakov D, Podell K, Goldberg E. Multiple neuroinvasive pathways in COVID-19. *Mol Neurobiol*. 2021;58(2):564–75. <https://doi.org/10.1007/s12035-020-02152-5>.
- Harapan BN, Yoo HJ. Neurological symptoms, manifestations, and complications associated with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and coronavirus disease 19 (COVID-19). *J Neurol*. 2021;268(9):3059–71. <https://doi.org/10.1007/s00415-021-10406-y>.
- Matschke J, Lütgehetmann M, Hagel C, Sperhake JP, Schröder AS, Edler C, Mushumba H, Fitzek A, Allweiss L, Dandri M, Dottermusch M, Heinemann A, Pfefferle S, Schwabenland M, Sumner Magruder D, Bonn S, Prinz M, Gerloff C, Püschel K, Glatzel M. Neuropathology of patients with COVID-19 in Germany: a post-mortem case series. *Lancet Neurol*. 2020;19(11):919–29. [https://doi.org/10.1016/S1473-4422\(20\)30308-2](https://doi.org/10.1016/S1473-4422(20)30308-2).
- Meinhardt J, Radke J, Dittmayer C, Franz J, Thomas C, Mothes R, Laue M, Schneider J, Brünink S, Greuel S, Lehmann M, Hassan O, Aschman T, Schumann E, Chua RL, Conrad C, Eils R, Stenzel W, Windgassen M, Heppner FL. Olfactory transnasal SARS-CoV-2 invasion as a port of central nervous system entry in individuals with COVID-19. *Nat Neurosci*. 2021;24(2):168–75. <https://doi.org/10.1038/s41593-020-00758-5>.
- Puelles VG, Lütgehetmann M, Lindenmeyer MT, Sperhake JP, Wong MN, Allweiss L, Chilla S, Heinemann A, Wanner N, Liu S, Braun F, Lu S, Pfefferle S, Schröder AS, Edler C, Gross O, Glatzel M, Wichmann D, Wietz T, Huber TB. Multiorgan and renal tropism of SARS-CoV-2. *New Engl J Med*. 2020;383(6):590–2. <https://doi.org/10.1056/NEJMc2011400>.
- Song E, Zhang C, Israelow B, Lu-Culligan A, Prado AV, Skriabine S, Lu P, Weizman O-E, Liu F, Dai Y, Szigeti-Buck K, Yasumoto Y, Wang G, Castaldi C, Heltke J, Ng E, Wheeler J, Alfajaro MM, Levavasseur E, Iwasaki A. Neuroinvasion of SARS-CoV-2 in human and mouse brain. *J Experim Med*. 2021. <https://doi.org/10.1084/jem.20202135>.
- Krasemann S, Haferkamp U, Pfefferle S, Woo MS, Heinrich F, Schweizer M, Appelt-Menzel A, Cubukova A, Barenberg J, Leu J, Hartmann K, Thies E, Littau JL, Sepulveda-Falla D, Zhang L, Ton K, Liang Y, Matschke J, Ricklefs F, Pless O. The blood-brain barrier is dysregulated in COVID-19 and serves as a CNS entry route for SARS-CoV-2. *Stem Cell Rep*. 2022;17(2):307–20. <https://doi.org/10.1016/j.stemcr.2021.12.011>.
- Boldrini M, Canoll PD, Klein RS. How COVID-19 affects the brain. *JAMA Psychiat*. 2021;78(6):682. <https://doi.org/10.1001/jamapsychiatry.2021.0500>.
- Burks SM, Rosas-Hernandez H, Alejandro Ramirez-Lee M, Cuevas E, Talpos JC. Can SARS-CoV-2 infect the central nervous system via the olfactory bulb or the blood-brain barrier? *Brain Behav Immun*. 2021;95:7–14. <https://doi.org/10.1016/j.bbi.2020.12.031>.
- Perlman S, Evans G, Afifi A. Effect of olfactory bulb ablation on spread of a neurotropic coronavirus into the mouse brain. *J Exp Med*. 1990;172(4):1127–32. <https://doi.org/10.1084/jem.172.4.1127>.
- Cowley TJ, Weiss SR. Murine coronavirus neuropathogenesis: determinants of virulence. *J Neurovirol*. 2010;16(6):427–34. <https://doi.org/10.1007/BF03210848>.
- Cheng Q, Yang Y, Gao J. Infectivity of human coronavirus in the brain. *EBioMedicine*. 2020;56:102799. <https://doi.org/10.1016/j.ebiom.2020.102799>.
- Andrade AC, dos SP, Campolina-Silva GH, Queiroz-Junior CM, de Oliveira LC, Lacerda L de SB, Gaggino JCP, de Souza FRO, de Meira Chaves I, Passos IB, Teixeira DC, Bittencourt-Silva PG, Valadão PAC, Rossi-Oliveira L, Antunes MM, Figueiredo

- AFA, Wnuk NT, Temerozo JR, Ferreira AC, Cramer A, Costa VV (2021) A biosafety level 2 mouse model for studying betacoronavirus-induced acute lung damage and systemic manifestations. *J Virol* <https://doi.org/10.1128/JVI.01276-21>
14. Reza-Zaldivar EE, Hernández-Sapiéns MA, Minjarez B, Gómez-Pinedo U, Márquez-Aguirre AL, Mateos-Díaz JC, Matias-Guiu J, Canales-Aguirre AA. Infection mechanism of SARS-CoV-2 and its implication on the nervous system. *Front Immunol*. 2021. <https://doi.org/10.3389/fimmu.2020.621735>.
 15. Weiss SR, Leibowitz JL (2011) Coronavirus Pathogenesis (pp. 85–164). <https://doi.org/10.1016/B978-0-12-385885-6.00009-2>
 16. Garcia AB, de Moraes AP, Rodrigues DM, Gilioli R, de Oliveira-Filho EF, Durães-Carvalho R, Arns CW. Coding-complete genome sequence of murine hepatitis virus strain 3 from Brazil. *Microbiol Resour Announc*. 2021. <https://doi.org/10.1128/MRA.00248-21>.
 17. Amaral DH, Rachid MA, Vilela MC, Campos RD, Ferreira GP, Rodrigues DC, Lacerda-Queiroz N, Miranda AS, Costa VV, Campos MA, Kroon EG, Teixeira MM, Teixeira AL. Intracerebral infection with dengue-3 virus induces meningoencephalitis and behavioral changes that precede lethality in mice. *J Neuroinflamm*. 2011;8(1):23. <https://doi.org/10.1186/1742-2094-8-23>.
 18. Costa VV, Del Sarto JL, Rocha RF, Silva FR, Doria JG, Olmo IG, Marques RE, Queiroz-Junior CM, Foureaux G, Araújo JMS, Cramer A, Real ALCV, Ribeiro LS, Sardi SI, Ferreira AJ, Machado FS, de Oliveira AC, Teixeira AL, Nakaya HI, Teixeira MM. N-Methyl-D-Aspartate (NMDA) receptor blockade prevents neuronal death induced by Zika virus infection. *MBio*. 2017. <https://doi.org/10.1128/mBio.00350-17>.
 19. Dunkley PR, Heath JW, Harrison SM, Jarvie PE, Glenfield PJ, Rostas JAP. A rapid Percoll gradient procedure for isolation of synaptosomes directly from an S1 fraction: homogeneity and morphology of subcellular fractions. *Brain Res*. 1988;441(1–2):59–71. [https://doi.org/10.1016/0006-8993\(88\)91383-2](https://doi.org/10.1016/0006-8993(88)91383-2).
 20. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J Biol Chem*. 1985;260(6):3440–50.
 21. Nicholls DG, Sihra TS, Sanchez-Prieto J. Calcium-dependent and -independent release of glutamate from synaptosomes monitored by continuous fluorimetry. *J Neurochem*. 1987;49(1):50–7. <https://doi.org/10.1111/j.1471-4159.1987.tb03393.x>.
 22. Rodrigues HA, de Fonseca MC, Camargo WL, Lima PMA, Martinelli PM, Naves LA, Prado VF, Prado MAM, Guatimosim C. Reduced expression of the vesicular acetylcholine transporter and neurotransmitter content affects synaptic vesicle distribution and shape in mouse neuromuscular junction. *PLoS One*. 2013;8(11):e78342. <https://doi.org/10.1371/journal.pone.0078342>.
 23. Esposito Z, Belli L, Toniolo S, Sancesario G, Bianconi C, Martorana A. Amyloid β , glutamate, excitotoxicity in Alzheimer's Disease: are we on the right track? *CNS Neurosci Ther*. 2013;19(8):549–55. <https://doi.org/10.1111/cns.12095>.
 24. Mody I. NMDA receptor-dependent excitotoxicity: the role of intracellular Ca²⁺ release. *Trends Pharmacol Sci*. 1995;16(10):356–9. [https://doi.org/10.1016/S0165-6147\(00\)89070-7](https://doi.org/10.1016/S0165-6147(00)89070-7).
 25. Prediger RDS, Aguiar AS, Rojas-Mayorquin AE, Figueiredo CP, Matheus FC, Gineslet L, Chevarin C, Bel ED, Mongeau R, Hamon M, Lanfumey L, Raisman-Vozari R. Single intranasal administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in C57BL/6 mice models early preclinical phase of Parkinson's disease. *Neurotox Res*. 2010;17(2):114–29. <https://doi.org/10.1007/s12640-009-9087-0>.
 26. Oliveira TPD, Gonçalves BDC, Oliveira BS, de Oliveira ACP, Reis HJ, Ferreira CN, Aguiar DC, de Miranda AS, Ribeiro FM, Vieira EML, Palotás A, Vieira LB. Negative modulation of the metabotropic glutamate receptor type 5 as a potential therapeutic strategy in obesity and binge-like eating behavior. *Front Neurosci*. 2021. <https://doi.org/10.3389/fnins.2021.631311>.
 27. Camargos QM, Silva BC, Silva DG, Toscano ECB, Oliveira BS, Bellozi PMQ, Jardim BLO, Vieira ÉLM, de Oliveira ACP, Sousa LP, Teixeira AL, de Miranda AS, Rachid MA. Minocycline treatment prevents depression and anxiety-like behaviors and promotes neuroprotection after experimental ischemic stroke. *Brain Res Bull*. 2020;155:1–10. <https://doi.org/10.1016/j.brainresbull.2019.11.009>.
 28. de Miranda AS, Brant F, Vieira LB, Rocha NP, Vieira ÉLM, Rezende GHS, de Oliveira Pimentel PM, Moraes MFD, Ribeiro FM, Ransohoff RM, Teixeira MM, Machado FS, Rachid MA, Teixeira AL. A neuroprotective effect of the glutamate receptor antagonist MK801 on long-term cognitive and behavioral outcomes secondary to experimental cerebral malaria. *Mol Neurobiol*. 2017;54(9):7063–82. <https://doi.org/10.1007/s12035-016-0226-3>.
 29. Eltokhi A, Kurpiers B, Pitzer C. Baseline depression-like behaviors in wild-type adolescent mice are strain and age but not sex dependent. *Front Behav Neurosci*. 2021. <https://doi.org/10.3389/fnbeh.2021.759574>.
 30. Davis HE, Assaf GS, McCorkell L, Wei H, Low RJ, Re'em Y, Redfield S, Austin JP, Akrami A. Characterizing long COVID in an international cohort: 7 months of symptoms and their impact. *EClinicalMedicine*. 2021. <https://doi.org/10.1016/j.eclinm.2021.101019>.
 31. WHO Coronavirus (COVID-19) Dashboard. Available from: <https://covid19.who.int/>.
 32. Gorska AM, Eugenin EA. The glutamate system as a crucial regulator of CNS toxicity and survival of HIV reservoirs. *Front Cell Infect Microbiol*. 2020. <https://doi.org/10.3389/fcimb.2020.00261>.
 33. Brison E, Jacomy H, Desforges M, Talbot PJ. Glutamate excitotoxicity is involved in the induction of paralysis in mice after infection by a human coronavirus with a single point mutation in its spike protein. *J Virol*. 2011;85(23):12464–73. <https://doi.org/10.1128/JVI.05576-11>.
 34. Liu GJ, Nagarajah R, Banati RB, Bennett MR. Glutamate induces directed chemotaxis of microglia. *Eur J Neurosci*. 2009;29(6):1108–18. <https://doi.org/10.1111/j.1460-9568.2009.06659.x>.
 35. Sanchis P, Fernández-Gayol O, Comes G, Escrig A, Giral M, Palmiter RD, Hidalgo J. Interleukin-6 derived from the central nervous system may influence the pathogenesis of experimental autoimmune encephalomyelitis in a cell-dependent manner. *Cells*. 2020;9(2):330. <https://doi.org/10.3390/cells9020330>.
 36. Sallmann S, Jüttler E, Prinz S, Petersen N, Knopf U, Weiser T, Schwaninger M. Induction of interleukin-6 by depolarization of neurons. *J Neurosci*. 2000;20(23):8637–42. <https://doi.org/10.1523/JNEUROSCI.20-23-08637.2000>.
 37. Lotrich FE. Inflammatory cytokine-associated depression. *Brain Res*. 2015;1617:113–25. <https://doi.org/10.1016/j.brainres.2014.06.032>.
 38. Dowlati Y, Herrmann N, Swardfager W, Liu H, Sham L, Reim EK, Lancôt KL. A meta-analysis of cytokines in major depression. *Biol Psychiat*. 2010;67(5):446–57. <https://doi.org/10.1016/j.biopsych.2009.09.033>.
 39. Bauer S, Kerr BJ, Patterson PH. The neurotrophic cytokine family in development, plasticity, disease and injury. *Nat Rev Neurosci*. 2007;8(3):221–32. <https://doi.org/10.1038/nrn2054>.
 40. Haroon E, Miller AH, Sanacora G. Inflammation, glutamate, and glia: a trio of trouble in mood disorders. *Neuropsychopharmacology*. 2017;42(1):193–215. <https://doi.org/10.1038/npp.2016.199>.
 41. Lauro C, Catalano M, Di Paolo E, Chece G, de Costanzo I, Trettel F, Limatola C. Fractalkine/CX3CL1 engages different neuroprotective responses upon selective glutamate receptor overactivation.

- Front Cell Neurosci. 2015. <https://doi.org/10.3389/fncel.2014.00472>.
42. Binder GK, Griffin DE. Interferon- γ -mediated site-specific clearance of alphavirus from CNS neurons. *Science*. 2001;293(5528):303–6. <https://doi.org/10.1126/science.1059742>.
43. Ganor Y, Besser M, Ben-Zakay N, Unger T, Levite M. Human T cells express a functional ionotropic glutamate receptor GluR3, and glutamate by itself triggers integrin-mediated adhesion to laminin and fibronectin and chemotactic migration. *J Immunol*. 2003;170(8):4362–72. <https://doi.org/10.4049/jimmunol.170.8.4362>.
44. Miyazishi H, Nitta A. A role of BDNF in the depression pathogenesis and a potential target as antidepressant: The modulator of stress sensitivity “Shati/Nat8l-BDNF system” in the dorsal striatum. *Pharmacoeconomics*. 2021;14(9):889. <https://doi.org/10.3390/ph14090889>.
45. Li H, Wang T, Shi C, Yang Y, Li X, Wu Y, Xu Z-QD. Inhibition of GALR1 in PFC alleviates depressive-like behaviors in postpartum depression rat model by upregulating CREB-BDNF and 5-HT levels. *Front Psychiatry*. 2018. <https://doi.org/10.3389/fpsy.2018.00588>.
46. Li M, Li C, Yu H, Cai X, Shen X, Sun X, Wang J, Zhang Y, Wang C. Lentivirus-mediated interleukin-1 β (IL-1 β) knock-down in the hippocampus alleviates lipopolysaccharide (LPS)-induced memory deficits and anxiety- and depression-like behaviors in mice. *J Neuroinflammation*. 2017;14(1):190. <https://doi.org/10.1186/s12974-017-0964-9>.
47. Cirulli F, Berry A, Chiarotti F, Allea E. Intrahippocampal administration of BDNF in adult rats affects short-term behavioral plasticity in the Morris water maze and performance in the elevated plus-maze. *Hippocampus*. 2004;14(7):802–7. <https://doi.org/10.1002/hipo.10220>.
48. Chamera K, Trojan E, Szuster-Gluszcak M, Basta-Kaim A. The potential role of dysfunctions in neuron-microglia communication in the pathogenesis of brain disorders. *Curr Neuropharmacol*. 2020;18(5):408–30. <https://doi.org/10.2174/1570159X17666191113101629>.
49. Marcolino MS, Anschau F, Kopitke L, Pires MC, Barbosa IG, Pereira DN, Ramos LEF, Assunção LFI, Costa ASM, Nogueira MCA, Duani H, Martins KPMP, Moreira LB, Silva CTCA, Oliveira NR, de Ziegelmann PK, Guimarães-Júnior MH, Lima MOSS, Aguiar RLO, Teixeira AL. Frequency and burden of neurological manifestations upon hospital presentation in COVID-19 patients: Findings from a large Brazilian cohort. *J Neurol Sci*. 2022. <https://doi.org/10.1016/j.jns.2022.120485>.
50. Pereira DN, Bicalho MAC, Jorge AO, Gomes AGR, Schwarzbald AV, Araújo ALH, Cimini CCR, Ponce D, Rios DRA, Grizende GMS, Manenti ERF, Anschau F, Aranha FG, Bartolazzi F, Batista JL, Tupinambás JT, Ruschel KB, Ferreira MAP, Araújo PG, Marcolino MS. Neurological manifestations by sex and age group in COVID-19 inpatient patients. *ENeurologicalSci*. 2022;28:100419. <https://doi.org/10.1016/j.ensci.2022.100419>.
51. Chou SH-Y, Beghi E, Helbok R, Moro E, Sampson J, Altamirano V, Mainali S, Bassetti C, Suarez JJ, McNett M, Nolan L, Temro K, Cervantes-Arslanian AM, Anand P, Mukerji S, Alabasi H, Westover MB, Kavi T, John S, David K. Global incidence of neurological manifestations among patients hospitalized with COVID-19—a report for the GCS-NeuroCOVID Consortium and the ENERGY Consortium. *JAMA Network Open*. 2021;4(5):e2112131. <https://doi.org/10.1001/jamanetworkopen.2021.12131>.
52. Solomon IH, Normandin E, Bhattacharyya S, Mukerji SS, Keller K, Ali AS, Adams G, Hornick JL, Padera RF, Sabeti P. Neuropathological features of Covid-19. *N Engl J Med*. 2020;383(10):989–92. <https://doi.org/10.1056/NEJMc2019373>.
53. Boroujeni ME, Simani L, Bluysen HAR, Samadikhan HR, Zamanli Benisi S, Hassani S, Akbari Dilmaghani N, Fathi M, Vakili K, Mahmoudiasl G-R, Abbaszadeh HA, Hassani Moghaddam M, Abdollahifar M-A, Aliaghaei A. Inflammatory response leads to neuronal death in human post-mortem cerebral cortex in patients with COVID-19. *ACS Chem Neurosci*. 2021;12(12):2143–50. <https://doi.org/10.1021/acscchemneuro.1c00111>.
54. Csordás A, Mázló M, Gallyas F. Recovery versus death of “dark” (compacted) neurons in non-impaired parenchymal environment: light and electron microscopic observations. *Acta Neuropathol*. 2003;106(1):37–49. <https://doi.org/10.1007/s00401-003-0694-1>.
55. de Miranda AS, Rodrigues DH, Amaral DCG, de Lima Campos RD, Cisalpino D, Vilela MC, Lacerda-Queiroz N, de Souza KPR, Vago JP, Campos MA, Kroon EG. Dengue-3 encephalitis promotes anxiety-like behavior in mice. *Behav Brain Res*. 2012;230(1):237–42. <https://doi.org/10.1016/j.bbr.2012.02.020>.
56. Andrews MG, Mukhtar T, Eze UC, Simoneau CR, Ross J, Parikshak N, Wang S, Zhou L, Koontz M, Velmeshev D, Siebert C-V, Gemenes KM, Tabata T, Perez Y, Wang L, Mostajir-Radji MA, de Majo M, Donohue KC, Shin D, Kriegstein AR. Tropism of SARS-CoV-2 for human cortical astrocytes. *Proc Natl Acad Sci*. 2022. <https://doi.org/10.1073/pnas.2122236119>.
57. Winkler ES, Bailey AL, Kafai NM, Nair S, McCune BT, Yu J, Fox JM, Chen RE, Earnest JT, Keeler SP, Ritter JH, Kang L-I, Dort S, Robichaud A, Head R, Holtzman MJ, Diamond MS. SARS-CoV-2 infection of human ACE2-transgenic mice causes severe lung inflammation and impaired function. *Nat Immunol*. 2020;21(11):1327–35. <https://doi.org/10.1038/s41590-020-0778-2>.
58. Kumari P, Rothan HA, Natekar JP, Stone S, Pathak H, Strate PG, Arora K, Brinton MA, Kumar M. Neuroinvasion and encephalitis following intranasal inoculation of SARS-CoV-2 in K18-hACE2 mice. *Viruses*. 2021;13(1):132. <https://doi.org/10.3390/v13010132>.
59. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, Schiergens TS, Herrler G, Wu N-H, Nitsche A, Müller MA, Drosten C, Pohlmann S. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell*. 2020;181(2):271–280.e8. <https://doi.org/10.1016/j.cell.2020.02.052>.
60. Düttsedau HP, Steffen J, Figueiredo CA, Boehme JD, Schultz K, Erck C, Korte M, Faber-Zuschratter H, Smalla K-H, Dieterich D, Krüger A, Bruder D, Dunay IR. Influenza A Virus (H1N1) infection induces microglial activation and temporal dysbalance in glutamatergic synaptic transmission. *MBio*. 2021. <https://doi.org/10.1128/mBio.01776-21>.
61. Olmo IG, Carvalho TG, Costa VV, Alves-Silva J, Ferrari CZ, Izidoro-Toledo TC, da Silva JF, Teixeira AL, Souza DG, Marques JT, Teixeira MM, Vieira LB, Ribeiro FM. Zika virus promotes neuronal cell death in a non-cell autonomous manner by triggering the release of neurotoxic factors. *Front Immunol*. 2017. <https://doi.org/10.3389/fimmu.2017.01016>.
62. Oladunni FS, Park JG, Pino PA, Gonzalez O, Akhter A, Allu-Guardia A, Olmo-Fontánez A, Gautam S, Garcia-Vilanova A, Ye C, Chiem K, Headley C, Dwivedi V, Parodi LM, Alfson KJ, Staples HM, Schami A, Garcia JJ, Whigham A, Torrelles JB. Lethality of SARS-CoV-2 infection in K18 human angiotensin-converting enzyme 2 transgenic mice. *Nat Commun*. 2020;11(1):6122. <https://doi.org/10.1038/s41467-020-19891-7>.
63. Rothan H, Kumari P, Stone S, Natekar J, Arora K, Aurovi T, Kumar M. SARS-CoV-2 infects primary neurons from human ACE2 expressing mice and upregulates genes involved in the inflammatory and necroptotic pathways. *Pathogens*. 2022;11(2):257. <https://doi.org/10.3390/pathogens11020257>.

64. Vieira-Alves I, Alves ARP, Souza NMV, Melo TL, Coimbra Campos LMC, Lacerda LSB, Queiroz-Junior CM, Andrade ACSP, Barcelos LS, Teixeira MM, Costa VV, Cortes SF, Lemos VS. TNF/ α /NOS/NO pathway mediates host susceptibility to endothelial-dependent circulatory failure and death induced by betacoronavirus infection. *Clin Sci*. 2023;137(7):543–59. <https://doi.org/10.1042/CS20220663>.
65. Heap RE, Marín-Rubio JL, Peltier J, Heunis T, Dannoura A, Moore A, Trost M (2021) Proteomics characterisation of the L929 cell supernatant and its role in BMDM differentiation. *Life Sci Alliance* 4(6): e20200957. <https://doi.org/10.26508/lsa.20200957>
66. Costa AP, Vieira C, Bohner LO, Silva CF, Santos EC, De Lima TC, Lino-de-Oliveira C. A proposal for refining the forced swim test in Swiss mice. *Prog Neuropsychopharmacol Biol Psychiatry*. 2013;45:150–5. <https://doi.org/10.1016/j.pnpbp.2013.05.002>.
67. Suman P, Zerbinatti N, Theindl L, Domingues K, Lino de Oliveira C. Failure to detect the action of antidepressants in the forced swim test in Swiss mice. *Acta Neuropsychiatrica*. 2018;30(3):158–67. <https://doi.org/10.1017/neu.2017.33>.

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor (e.g. a society or other partner) holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.