Main Manuscript for:

Unlocking new mechanisms for future ALS therapies: early interventions with cholinergic antagonists reduce neuromuscular decline.

Reynaldo Popoli, Tyler L. Wells, *Turgay Akay

Atlantic Mobility Action Project, Brain Repair Center, Dept. of Medical Neuroscience,

Dalhousie University, Halifax, NS, CANADA, B3H 0A8

***Email**: Turgay.Akay@Dal.ca

Author Contributions: T.A. and R.P. designed research; R.P and T.L.W. performed research; R.P. analyzed data; R.P. wrote the paper.

Number of pages: 50

Number of figures: 7

Number of videos: 1

Competing Interest Statement: No competing interests.

Acknowledgements: We thank Brenda Ross and Janette Nason for their expert technical assistance throughout this project. We would like to thank the Biological Mass Spectrometry (BMS) CORE facility at Dalhousie University for providing mass spectrometry services used in this study. We thank Dr. James Fawcett for his guidance in the area of pharmacology. **Running title: Cholinergic antagonists for early intervention in ALS.**

New and Noteworthy: This study shows that blocking M2 receptors with methoctramine improves motor function and muscle innervation in an ALS mouse model, particularly in the absence of exercise. Further evaluation suggests that some of these beneficial effects are related to a peripheral mechanism. These findings highlight a potential new therapeutic approach for ALS that targets cholinergic signalling.

ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative condition characterized by motor neuron loss, leading to muscle paralysis and death. C-boutons have been shown to be part of the compensatory mechanism behind delayed symptom onset, and are most active during vigorous exercises, like swimming. When mutant mice with silenced C-boutons perform this exercise, disease progression and behavioral performance drastically improve. Genetic manipulation of C-boutons in human patients remains limited, therefore, we sought to manipulate these synapses using cholinergic antagonists in the presence and absence of exercise in a mouse model of ALS. We demonstrate that atropine and methoctramine administration yield significant improvements in human endpoints, weight maintenance, treadmill performance, and grip strength. Most remarkably, muscle innervation was greatly enhanced at humane endpoints compared to controls, suggesting these drugs provide a protective effect against loss of motor control. We found that methoctramine provided greater benefits in the absence of exercise, hinting at the presence of novel cholinergic mechanisms that can be manipulated in order to preserve motor function. Moreover, we provide evidence that these results are independent of C-boutons, and that methoctramine does not appear to cross the blood-brain barrier. Our results reveal pharmacological mechanisms by which muscle denervation can be reduced, thereby decreasing the rate of disease progression. We have uncovered a promising avenue for improving ALS symptoms by pharmacologically manipulating cholinergic transmission. This mechanism presents as a possible therapy translatable to the clinical setting, which has the potential to prevent the loss of motor control in patients with ALS.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that affects the nervous system, leading to the loss of motor neurons in the spinal cord and cerebral cortex. The clinical manifestation of ALS varies, with some cases showing progressive degeneration in upper motor neuron or lower motor neuron, or both (Ravits and La Spada, 2009). The rate at which motor neurons are lost varies across individuals, with a mean survival of approximately three years from symptom onset (Gubbay et al., 1985). Despite extensive research and efforts spanning decades, the etiology of ALS remains unclear. Currently, there is no cure for ALS and patients suffer from loss of muscle control and movement, affecting their quality of life. Only a few drugs have been approved that show, to a limited degree, a modest improvement in life expectancy: Riluzole, Edaravone, and Tofersen (Li and Bedlack, 2024).

Surviving motor units undergo a substantial increase in force output, effectively compensating for muscle denervation during earlier stages of the disease (McComas et al., 1971). The etiology of these mechanisms has been an important area of research over the last two decades (Schoenfeld et al., 2005). Our previous work has highlighted the critical role of C-boutons as an indispensable part of the motor compensation present in transgenic model mice that carry the mutant form of superoxide dismutase 1 ($mSOD1^{G93A}$) (Landoni et al., 2021). C-boutons are large cholinergic terminals that synapse with motor neurons in the spinal cord, and are involved in amplitude modulation in certain tasks, such as swimming (Zagoraiou et al., 2009). Moreover, our findings

demonstrate that exercise that activates C-boutons exacerbates disease progression in *mSOD1*^{G93A} mice (Wells et al., 2021). Intriguingly, when C-boutons are silenced, and mice are exercised in the form of swimming (an activity which would normally activate C-boutons), an improvement is seen in disease progression and behavioural performance. This suggests that in the absence of functionally active C-boutons, swimming recruits another system or mechanism, and it provides beneficial effects to the *mSOD1*^{G93A} mouse (Wells et al., 2021). C-boutons derive from V0c interneurons. These synapses release acetylcholine (ACh), which then binds to M2 receptors in motor neurons of the spinal cord (Hellström et al., 2003). The silencing of C-boutons in Wells et al., 2021 is highly specific to V0c interneurons, and it therefore inactivates only motor neuron synapses in the spinal cord (Zagoraiou et al., 2009). Other key motor neuron sites, such as the neuromuscular junction, are not genetically modified in this model (See methods). However, since genetic silencing of C-boutons is not feasible in humans, this study aimed to explore the therapeutic potential of blocking M2 receptors using cholinergic antagonists as a novel approach for treating ALS. Atropine is a non-selective muscarinic antagonist with high affinity for all muscarinic receptors, with Ki (in nM) of 0.28, 0.76, 0.19, 0.13, and 0.24, for the M1, M2, M3, M4, and M5 receptors, respectively (Moriya et al., 1999). This antagonist was selected due to its common use in hospital settings to treat pesticide poisoning and organophosphorus chemical warfare (“Atropine Sulfate Injection,” 2015). Methoctramine, a cholinergic antagonist exhibiting a strong affinity for M2 receptors, was selected based on its specificity and potency (Melchiorre et al., 1987). Methoctramine’s affinities (Ki in nM) for muscarinic receptors have been shown to be 49.8, 14.3, 277, 38, and 313 for M1, M2, M3, M4, and M5,

103 respectively (Melchiorre et al., 2001). We hypothesized that combining exercise with
104 either of these drugs would improve behavioural performance and muscle innervation.
105
106 In the present study, we investigate the effect of atropine and methoctramine,
107 compared to saline controls of *mSOD1*^{G93A} mice. Each group was further subdivided
108 into behavioural training (swimming condition) and resting conditions (absence of
109 exercise). We report that both drugs improve behavioural performance, independent of
110 exercise. Furthermore, both cholinergic antagonists prolong humane endpoints
111 exclusively under resting conditions. Additionally, the administration of methoctramine
112 improves muscle innervation at humane endpoints in both resting and exercise
113 conditions. Methoctramine experiments were repeated in the resting condition with drug
114 administration beginning at postnatal day 90 (P90). Notably, methoctramine
115 administration beginning at symptomatic stages appears detrimental, indicating a limited
116 window for intervention efficacy. Muscarinic M2 receptors are located in throughout the
117 body. In order to show that the effects of methoctramine are due to the post-synaptic
118 receptor of the V0c interneurons, experiments were repeated in mice with genetically
119 silenced C-boutons.
120
121 We show that the impact of methoctramine on disease progression is likely C-
122 bouton independent, suggesting peripheral effects. We conclude by providing
123 electromyogram data on the effects of methoctramine and mass spectrometry data from
124 blood samples and cerebrospinal fluid of mice injected with methoctramine.
125

METHODS

All experiments were performed in accordance with the Canadian Council on Animal Care guidelines and were approved by the Dalhousie University Committee on Laboratory Animals. All mice were housed on a 12-hour light/dark cycle (light period from 07:00 AM to 07:00 PM) with access to laboratory chow and water *ad libitum*.

ANIMALS

The following three lines of mice were used in this study: wild-type (WT) *C57BL/6* mice, *mSOD1*^{G93A} mice (The Jackson Laboratory), and *mSOD1*^{G93A}*; Dbx1::cre*; *ChAT*$^{fl/fl}$ (*mSOD1*^{G93A}*/C*^{off}) mice, in which the C-boutons of *mSOD1*^{G93A} mice are genetically silenced, as previously shown in Zagoraiou et al., 2009. Briefly, the silencing of C-boutons is accomplished by crossing *ChAT*$^{fl/fl}$ mice, in which LoxP sites are inserted in the ChAT gene, with mice in which the Cre recombinase gene is expressed under the control of the Dbx1 promoter, a transcription factor present in V0c interneurons. Only male mice were used during this research. We used three criteria to determine the humane end point of every animal: inability to walk on the treadmill at speeds faster than 0.05 m/s, inability to hold onto the grip strength apparatus, and weight loss of 15% of maximum weight over the course of their life. Mice were followed from postnatal day 30 (P30) until their humane endpoints. Quantitative PCR (qPCR) was used to determine the copy numbers in the *mSOD1*^{G93A} mice in at least one animal of every litter. No differences in gene expression between all groups were found.

BEHAVIOURAL EXPERIMENTS

In our first experiment, mice were randomly assigned to each drug group (atropine, methoctramine, and saline). Within the three groups, mice were further subdivided into swimming and resting groups for a total of 6 groups. Starting at P30, mice began to swim for thirty minutes, three times a week (Mondays, Wednesdays, and Fridays) and continued until an age when mice could no longer swim, despite significant intervention. Mice were injected with their respective drug twenty minutes prior to commencing the swimming exercise (see: 2.3 Drugs). The protocol for swimming has been previously described(Wells et al., 2021). The mice swam freely in a swimming pool with the following dimensions: 30 x 3 x 7 inches. The initial water temperature was 38°C and the end temperature after 30 minutes was 31°C. Lightly poking the mice in the back of their head with mouse-holding forceps (catalogue # NC1239918, Thermo Fisher Scientific) was done whenever they stopped swimming to ensure they swam as vigorously as possible. Mice swam for four minutes and immediately rested in a heated paper towel for one. This cycle was repeated six consecutive times for a total of thirty minutes. Immediately after swimming, mice were placed back in their cage, which was placed on a heating pad for 15 minutes. Behavioural and weight recordings were performed once a week, on a day when the mice did not swim and were not injected.

The maximum speed on a treadmill was recorded using a 25 x 6 x 16 cm^3 treadmill (model 802, Zoological Institute, University of Cologne, Cologne, Germany). The protocol for the treadmill has been previously described in Wells, et al., 2021. The speed on the treadmill was set to 0.1 m/s prior to commencing the recordings. Whenever a mouse could not walk at this speed, the speed was changed to 0.05 m/s. If the mouse

was still unable to walk at this speed, the mouse was considered to be at their humane endpoint. If the mouse was able to walk at 0.1 m/s, the speed of the treadmill was steadily increased until the mouse was unable to continue and approached the back wall of the treadmill. After stopping the treadmill and recording the maximum speed reached, the treadmill was adjusted to a new speed by rounding down to the nearest 0.05 m/s interval. Prior to commencing the following trial, mice were given a short break of 60 seconds after each trial. A total of three trials were performed, and the maximum speed reached was used for analysis.

The muscular strength of each mouse was assessed through the use of a grip strength apparatus (model GT3, BIOSEB) with a T-shape bar for mice accessory (BIO-GRIPBS). The test consisted of allowing the mouse to hold onto the bar and carefully pulling them from the apparatus until they could no longer hold on. Each trial consisted of allowing the animal to hold on to the bar and release a total of eight times, and the maximum force was recorded. A total of five trials were performed, and the average maximum force of said trials was recorded for statistical analyses.

For our second experiment, new groups of *mSOD1*^{G93A} mice were categorized into either a control group, where saline was injected, or an experimental group, where methoctramine was injected. These two groups consisted of non-exercise conditions (drug or vehicle administration without swimming). Behavioural experiments were performed from P30 until humane endpoints as previously mentioned. The delivery of the drug (or vehicle) commenced at symptomatic stages (postnatal day 90, P90).

Our third experiment consisted of groups of *mSOD1*^{G93A}*/C*^{off} mice

(*mSOD1*^{G93A} with genetically silenced C-boutons) which were categorized into either a control group or a methoctramine group, beginning at drug administration at P30, and in the absence of exercise.

Electromyogram (EMG) experiments were conducted in wild-type mice (WT) at least two weeks after electrodes were implanted (See 2.10 EMG IMPLANT SURGERIES). Mice were anesthetized with isoflurane in order to attach the headpiece of the EMG array. Once mice had woken up, they were placed in the mouse treadmill previously mentioned, and the connector was attached to an amplifier (model 102, Zoological Institute, University of Cologne, Cologne, Germany). The animals were given 5 minutes to recover from the anesthesia prior to commencing the experiments. Immediately after, mice were made to walk at speeds of 0.150 m/s for about ten seconds while EMG activity was recorded with the Spike 2 software (Version 10.19, Cambridge Electronic Design Ltd.). This was considered their baseline recording. Five minutes after the activity had stopped, mice were injected with either saline or methoctramine (see 2.3 DRUGS). Five minutes after the injection, mice were made to walk again, and their EMG activity was recorded. Recordings were done every five minutes for 45 minutes. Once experiments were finished, the connector was disconnected, and the mice were returned to their cages.

DRUGS

Drug administration was based on published studies. Protocols in mice were as follows. Atropine sulphate (Sigma) was prepared weekly by dissolving it in isotonic (NaCl 0.9%) saline solution and was stored at 4°C. Atropine was injected subcutaneously (s.c.) with a dose of 10 µg/kg, based on previous literature findings in which the dose did not affect the locomotor activity of mice(Ghelardini et al., n.d.). Methoctramine (Sigma) was prepared by dissolving it in isotonic (NaCl 0.9%) saline solution and separated into vials with a final concentration of 0.05mg/mL. Vials were frozen at -20°C until they had to be used. After thawing, vials were kept at 4°C for a maximum of seven days before discarding. For experiments beginning at P30 and P90, and for EMG experiments, methoctramine was injected intraperitoneally (i.p.) in saline with a dose of 200 µg/kg. For the P30 experiments, saline (NaCl 0.9%) was used as a control with a dose of 10 mL/kg injected. For P90 and EMG experiments, saline (NaCl 0.9%) was injected intraperitoneally (i.p.) with a dose of 10 mL/kg.

TISSUE PREPARATION

Mice were anesthetized by injecting them with Euthanyl (100 µL sodium pentobarbital, 100 mg/kg, i.p.; Bimeda-MTC). Transcardial perfusion was subsequently performed by injecting saline (NaCl, 0.9%) in the left ventricle, followed by 4% paraformaldehyde (PFA) injection in the same location. Both legs were submerged in phosphate-buffered saline (PBS) and the vertebral columns were submerged in PFA overnight prior to performing dissections. The gastrocnemius (GS) muscles, tibialis anterior (TA) muscles, and soleus (Sol.) muscles were dissected, and their weights were

recorded using an analytical balance (Sartorius, QUINTIX224-1S). For every type of muscle, the weights of the right and left leg were averaged and then normalized to the final weight of the mice. All muscles, as well as the spinal cord, were then placed in 30% sucrose solution overnight to cryoprotect. The spinal cords were partitioned into L2, L3-L4, and L5 segments. Muscles and spinal cord segments were frozen in Optimal Cutting Temperature compound (Tissue-Tek, Sakura FineTek) and stored at -80°C. Muscle segments and spinal cord segments were sectioned at 40 μm and 30 μm, respectively, using a cryostat (model CM 3050S, Leica).

MUSCLE IMMUNOFLUORESCENCE

Red fluorescent α-bungarotoxin conjugated to tetramethylrhodamine (BTX; 1:200, 5 μg/mL; catalogue #T1175, Thermo Fisher Scientific) was used to label the nicotinic acetylcholine receptors of the neuromuscular junction (NMJ). Goat anti-VAChT primary (1:1000; catalogue # ABN100, Millipore Sigma) and donkey anti-goat secondary (1:1000; catalogue # A32814, Thermo Fisher Scientific) were used to label presynaptic terminals of the NMJ. NMJs were manually assessed for muscle innervation using a microscope (model DM LB2, Leica) for muscle innervation. The number of NMJs counted was between 97 and 267 for each mouse. NMJs labelled with BTX that had more than 80% co-labelled with $VAChT^{+}$ axon terminals were considered *Fully innervated*, while anything under 80% overlap was considered *Partially innervated*. NMJs that had no co-labelling of $VAChT^{+}$ terminals were considered *Denervated*. The percentage of *Fully innervated, Partially innervated,* and *Denervated* NMJs related to the total number counted for each mouse were used for statistical analysis.

SPINAL CORD IMMUNOFLUORESCENCE

In order to confirm the genetic silencing of C-boutons in *mSOD1*^{G93A}/*C*^{off} mice compared to *mSOD1*^{G93A} mice, the following antibodies were used: goat anti-VAChT primary 1:1000; catalogue # ABN100, Millipore Sigma), rabbit anti-ChAT primary (1:250, catalogue # PA5-29653, Thermo Fisher), donkey anti-goat secondary (1:1000; catalogue # AB32814, Thermo Fisher), and donkey anti-rabbit secondary (1:1000; catalogue # A32794; Thermo Fisher). C-boutons were confirmed to be genetically silenced by the absence of co-labelling of ChAT+ and VAChT+ in motor neurons of the spinal cord. This was only seen in *mSOD1*^{G93A}/*C*^{off} mice.

MICROSAMPLING OF BLOOD

Adult wild-type mice of at least 50 days of age were used. Both legs were shaved to expose the saphenous vein. Mice were weighed and injected with methoctramine (i.p.) with a dose of 200 µg/kg and were placed back in their cages until the determined amount of time had passed. Once 10, 20, 30, or 40 minutes had passed, mice were placed in a 50 mL Falcon tube (Fisher Scientific) with holes previously drilled. One of the hind legs was immobilized by applying gentle pressure above the knee joint, therefore allowing clear visualization of the saphenous vein. Using a 23 G needle, the vein was lightly punctured, and blood was collected using 70 µL microhematocrit heparinized capillary tubes (Fisher Scientific, Catalogue # 22-362566). Once sufficient blood was collected, bleeding was stopped by applying light force on the puncture site with a 2” x 2” gauze. Mice were returned to their cages only after confirming the puncture site was no longer bleeding. Blood was transferred to 1.5 mL Eppendorf tubes and placed

immediately on ice. This procedure was repeated on different days to collect samples at 10, 20, 30, and 40 minutes after the administration of methoctramine. Blood samples were centrifuged at 2,000 RCF at 4°C for 15 minutes to separate plasma from whole blood (Eppendorf, Centrifuge 5418 R, Catalogue # 5401000137). Plasma samples were then transferred to new tubes and immediately frozen at -20°C until they were ready for mass spectrometry.

CEREBROSPINAL FLUID COLLECTION

Adult wild-type mice of at least 50 days of age and *mSOD1*^{G93A} mice of 128 and 129 days of age were used. Mice were anesthetized with 5% isoflurane at a rate of one litre per minute. Once deep anesthesia was achieved, isoflurane was reduced to 2-3% and maintained for the duration of the surgery. Buprenorphine slow release (0.1 mg/kg) and meloxicam (0.5 mg/kg) were administered subcutaneously. Methoctramine was injected with a dose of 200 µg/kg and the time was noted. Ophthalmic eye ointment was applied to the eyes. Mice were placed in a stereotaxic frame (Stereotaxic for mouse, SGLM, Model # 68018, RWD Life Science Inc.) and their temperature was maintained through a heating pad (ThermoStar Homeothermic Temperature Controller, Model # 69024, RWD Life Science Inc.). The skin on the neck and above was shaved, and a three-part skin scrub was applied using hibitane (4% chlorhexidine gluconate), alcohol, and povidone-iodine. The skin on the back of the neck was tugged with blunt forceps and cut with scissors to expose a 1.5 cm length area under the skin. An incision was made on the superficial connective tissue and the first layer of muscle was separated carefully by using blunt forceps. A micro-dissecting retractor was used to

maintain the connective tissue and the first layer of muscle separated (Stoelting Co., Item # 52124P). Using blunt forceps, the muscles at the midline were separated by pulling them sideways until part of the cisterna magna (CM) was exposed. A borosilicate glass tube previously pulled (capillary tube settings: Heat = 700, Fil = 4, Vel = 60, Del = 145, Pul = 175), was fixed to a 90° electrode holder (David Kopf Instruments, Model 1769). The capillary arm was advanced toward the mouse until it reached the CM. The capillary arm continued to be advanced until the membrane was punctured. Immediately after puncturing the membrane, the arm was retracted slightly to allow cerebrospinal fluid (CSF) to flow into the tube. Once a sufficient amount was collected (~10 µL), the tube was retracted from the CM and the mouse was euthanized. CSF was transferred to a 0.2 mL tube and immediately placed on ice. For WT mice, CSF was collected between 8- and 26- minutes post-injection. For *mSOD1*^{G93A} mice, CSF was collected between 11- and 17- minutes post-injection.

CSF was centrifuged at 4°C for 15 minutes at 2,000 RCF. After centrifugation, CSF samples were inspected for any blood at the bottom of the tube (seen as a visible red dot). Any samples that contained any amount of visible blood were discarded. The supernatant was transferred into a new tube and stored at -20°C until they were ready for mass spectrometry analysis. In the original sampling tub, 6 µL of ultra-pure water was added to the pellet. Absorptions of 145, 455, and 540 nm wavelengths were determined using a spectrophotometer (NanoDrop 2000, Thermo Fisher, Catalogue # ND-2000). Samples with a wavelength absorption of 0.02 AU or higher were considered contaminated and were discarded.

MASS SPECTROMETRY

Sample processing protocol

For extraction, the initial sample material was diluted to 100µL with water. Extraction was performed using Oasis HLB 96-well µElution plates (2mg of sorbent, Waters) on a vacuum manifold. Prior to sample loading, HLB wells were rinsed with 100% methanol followed by 0.1% (v/v) trifluoroacetic acid (TFA) in water. After sample loading, wells were rinsed with 0.1% (v/v) formic acid in water followed by 100% methanol for elution. Eluted sample material was concentrated in a SpeedVac centrifuge and reconstituted in water containing 12.5uM of isotopically labeled threonine (Cambridge Isotopes) prior to mass spectrometry (MS) analysis. Mass spectrometry analysis protocol Targeted mass spectrometry analysis was carried out on a QTRAP5500 instrument interfaced to an Agilent 1290 Infinity II liquid chromatography (LC) system. For analysis, samples were directly separated by an analytical column (2.1mm x 50mm, Kinetix F5 1.7µm, Phenomenex) at a flow rate of 0.4mL/min. For analyte elution he gradient held at an initial starting condition of 2% mobile phase B (99.9% acetonitrile, 0.1% formic acid) for 2-minutes before ramping to 60% B over 0.9 minutes, holding at 60% B for 3.1- minutes, ramping to 90% B over 0.5-minutes and holding for 1-minute, ramping to 2% B in 0.25-minutes, with a final equilibration at 2% B for 2.25-minutes (total run time of 10- minutes, mobile phase A = 0.1% formic acid in water). Data acquisition on the QTRAP5500 utilized a targeted selected reaction monitoring (SRM) acquisition scheme with transitions for methoctramine (Q1 mass = 292.0, Q3 mass = 121.1) and the labeled threonine (Q1 = 125.1, Q3 = 60.1).

Specifically, the QTRAP was globally set to use a positive ion spray voltage of 5500, collision gas of 5, curtain gas of 25, temperature of 450, gas 1 of 45, and a gas 2 setting of 50. The declustering potential was globally set to 60, entrance potential to 10, and cell exit potential to 10. Collision energy values were set for each SRM transition (methoctramine = 21V, threonine = 21V). All SRM transitions were set to a scan duration setting of 100msec, yielding a total cycle time of 0.21 seconds. A mixture of 3.125µg/mL methoctramine and 12.5uM labeled threonine was used as a system suitability standard to monitor system performance throughout the analysis.

Data analysis

The resulting data were analyzed using Skyline software (version 24.1.0.199) with additional downstream processing in R.

EMG IMPLANT SURGERIES

Animals of at least 60 days of age were anesthetized with 5% isoflurane at a rate of 1L/min until deep anesthesia was achieved. Isoflurane was then adjusted to 1-3% for the remainder of the surgery. Meloxicam (0.5mg/kg) and buprenorphine (slow release, 0.1mg/kg) were injected subcutaneously, and ophthalmic eye ointment was applied to the eyes. The neck and hind limbs were shaved and a three-part skin scrub of hibitane (4% chlorhexidine gluconate), alcohol, and povidone-iodine was applied to the sites of incision for sterilization. Bipolar EMG electrodes were passed under the skin from the neck incision to the leg incisions. The electrodes were implanted into the gastrocnemius and tibialis anterior muscles on both legs. The headpiece was sutured to the skin around

the site of the neck incision. After the incisions were closed and anesthesia had been discontinued, animals were placed in a heated cage for a minimum of three days prior to returning them to their regular mouse rack. Food mash and hydrogel were placed in their cage daily until full recovery had been achieved.

EMG RECORDINGS

On day 1, mice were anesthetized with 5% isoflurane until deep anesthesia was achieved. A custom-made connector was attached to the EMG array headpiece that was previously implanted. Once connected, anesthesia was discontinued and the animal was placed on a treadmill (a 25 x 6 x 16 cm^3 treadmill (model 802, Zoological Institute, University of Cologne, Cologne, Germany). The connector was attached to an amplifier (model 102, Zoological Institute, University of Cologne, Cologne, Germany), and mice were given a minimum of five minutes to recover from anesthesia. Mice were made to walk for a minimum of 10 seconds at speeds between 0.2 and 0.3 m/s, and EMG activity was recorded with Spike2 (Version 10.19, Cambridge Electronic Design Ltd.). Five minutes after the baseline trial, mice were injected with either saline or methoctramine (200 μg/kg) and were given five additional minutes before subsequent trials. From this point on, trials were repeated every five minutes, for a total of eight trials post-injection. On day 2, the same procedure was repeated with saline or methoctramine, whichever had not been administered the previous day. EMG amplitudes were normalized to the baseline for its corresponding day and comparisons were made between saline and methoctramine at every timepoint using paired t-test (expressed as datapoints connected by lines, e.g., **Fig. 6**A).

EXPERIMENTAL DESIGN AND STATISTICAL ANALYSES

Behavioural data was collected weekly and averaged according to the disease stage as follows: presymptomatic (P30 to P51), muscle denervation (P58 to P79), symptom onset (P86 to P107), early symptomatic (P114 to P135), and late symptomatic (P142 to 163). In our first longitudinal experiments, 31 male mice were randomly assigned to one of three groups: saline injections, atropine injections, or methoctramine injections. Every group was subdivided into a swimming or a resting condition (n = 4 to 6 per group). Statistical analyses were performed using a two-way ANOVA to reveal main effects (drug effects without swimming subdivisions) and interactions between subdivisions on Weight, Maximum Speed on a Treadmill, and Grip Strength. A one-way ANOVA was conducted to determine differences in the Humane endpoint. Differences in muscle innervation were determined by conducting a chi-square test of homogeneity. In our second longitudinal experiment, where methoctramine was administered beginning at P90, mice were randomly assigned to a control group where saline was injected (n = 4) or a methoctramine group (n = 6). Statistical analyses were performed using a one- way ANOVA. Power analyses were performed on all statistical tests to ensure a minimum power of 0.8.

In our third experiment $mSOD^{G93A}/C^{off}$ mice were injected with saline or methoctramine (n = 3 per group), and independent-samples t-tests were conducted. For EMG recordings (n = 3), paired t-tests were conducted to compare GS amplitude between saline and methoctramine administration. SPSS Statistics 26.0 (IBM) was used for statistical analyses. Excel 2016 (Microsoft) was used for data presentation.

RESULTS

Cholinergic antagonists improve behavioural performance in *mSOD1^{G93A}* mice.

We have previously shown that in the absence of functional C-boutons, exercise in the form of swimming, a task that typically activates these cholinergic synapses, can positively influence disease progression in *mSOD1^{G93A}* mice(Wells et al., 2021). In order to assess whether these results can be reproduced with a pharmacological approach, we injected either atropine, a non-selective cholinergic antagonist, methoctramine, an M2-selective cholinergic antagonist, or saline as control, in both a swimming condition and a resting condition. Injections were administered three times a week from P30 until humane endpoint. In the swimming groups, mice were injected 20 minutes prior to the commencement of the exercise. Behavioural capabilities as measured by maximum speed on a treadmill and grip strength were recorded once weekly on days when mice were not injected and were averaged in four-week periods, based on disease stages (**Fig. 1**). A two-way ANOVA revealed no interactions between drug administered and exercise condition on maximum speed on a treadmill. Therefore, analyses of the main effects for drug and exercise were performed. Main effect for drug administered was statistically significant at the muscle denervation stage ($F(1,25) = 9.832$, $p < .001$), symptom onset stage ($F(1,24) = 8.816$, $p = .001$), early symptomatic stage ($F(2,25) = 7.451$, $p = .003$), and late symptomatic ($F(2,24) = 7.605$, $p = .003$), with a large effect 408 size for each of these stages ($\eta^2 = 0.440$, $\eta^2 = 0.424$, $\eta^2 = 0.373$, and $\eta^2 = 0.388$, respectively). Pairwise comparisons show that mice in the atropine condition were found to run faster at the muscle denervation stage (P58-P79), with a

statistically significant mean difference of 0.106 ± 0.036 m/s, p = .007 (**Fig. 1***A*). At this stage, mice in the methoctramine condition, independent of exercise, also ran faster, with a maximum speed of 0.541 ± 0.028 m/s, while mice in the saline group ran at a maximum speed of 0.377 ± 0.025 m/s, a statistically significant mean difference of 0.164 ± 0.038 m/s, p = .001. Mice in the atropine condition continued to run faster than their saline counterparts, but only in the early symptomatic stage and in the late symptomatic stage, with mean differences of 0.090 ± 0.033 m/s (p = .033) and 0.054 ± 0.021 m/s (p = .045), respectively. Mice in the methoctramine condition also continued to run faster than their saline counterparts in the symptom onset stage, early symptomatic stage, and late symptomatic stage, with a statistically significant mean difference of 0.159 ± 0.038 m/s (p = .001), 0.128 ± 0.035 m/s (p = .003), and 0.085 ± 0.022 m/s (p = .003), respectively. These results suggest that both atropine and methoctramine improve maximum speed on a treadmill at various stages of the disease independent of exercise, and these results are more pronounced in the methoctramine group. Notably, mice in the saline, resting condition have a difficult time maintaining a treadmill speed of 0.1 m/s at age P140 (Supplemental Video S1, *A*), while mice in the methoctramine, resting condition appear to walk effortlessly at double this speed at a similar age (Supplemental Video S1, *B*). Qualitatively, the performance of mice in the saline, resting condition at age P140 appears to be similar to the performance of mice in the methoctramine, resting condition at age P170 (Supplemental Video S1, *C*).

A two-way ANOVA revealed no interaction between drug administered and

exercise condition on grip strength in the presymptomatic stage (P30-P51). There was a statistically significant main effect for drug administered at this stage ($F(2,25) = 15.227$, $p < .001$, $\eta^2 = 0.559$) (**Fig. 1***B*). Pairwise comparisons show that mice in the atropine condition, independent of exercise, had a better grip strength performance compared to mice injected with saline, a statistically significant mean difference of 8.625 ± 2.615 g ($p = .009$). Mice in the methoctramine condition, independent of exercise, also had a better grip strength performance compared to mice injected with saline (methoctramine mice, 107.929 ± 1.982 g; saline mice, 93.393 ± 1.789 g, $p < .001$). A two-way ANOVA revealed an interaction between drug administered and exercise condition on Grip Strength in the muscle denervation stage ($F(2,25) = 10.218$, $p < .001$), symptom onset stage ($F(2,25) = 81.955$, $p < .001$), and early symptomatic stage ($F(2,25) = 16.408$, $p < .001$) with a large effect size for each stage ($\eta^2 = 0.450$, $\eta^2 = 0.603$, and $\eta^2 = 0.568$, respectively). In the muscle denervation stage, mice in the atropine, no swimming condition performed better than mice in the saline, no swimming condition with a statistically significant mean difference of 11.44 ± 4.06 g, $p = .009$. In this same stage, mice in the methoctramine and no swimming condition also had a better grip strength performance (123.11 ± 3.51 g) compared to both mice injected with atropine, no swimming (102.52 ± 2.87 g) and mice injected with saline, no swimming (91.09 ± 2.87 g), both a statistically significant mean difference ($p < .001$). These interactions were maintained in the symptom onset stage and the early symptomatic stage, with mice in the atropine, no swimming condition performing better than mice in the saline, no swimming condition, with statistically significant mean differences of 12.53 ± 3.82 g ($p = .003$) and 9.63 ± 3.73 g ($p = .016$), respectively. In these stages, mice in the

methoctramine, no swimming condition also performed better than mice in the saline, no swimming condition, with statistically significant mean differences of 32.17 ± 4.27 g (p < .001) and 20.26 ± 4.18 g (p < .001), respectively. Independent-samples t-tests were conducted between saline, no swimming and saline, swimming groups to assess differences in grip strength due to exercise. In the muscle denervation stage, grip strength was higher in the saline, swimming condition (97.75 ± 3.520 g) compared to the saline, no swimming condition (91.086 ± 3.343 g), a statistically significant difference of 6.665 g (95% CI, 1.976 to 11.353), $t(9) = 3.216$, $p = .011$. In the symptom onset stage, grip strength was higher in the saline, swimming condition (89.587 ± 4.081 g) compared to the saline, no swimming condition (77.283 ± 6.664 g), a statistically significant difference of 12.349 g (95% CI, 4.591 to 20.107), $t(9) = 3.601$, $p = .006$. In the early symptomatic stage, grip strength was higher in the saline, swimming condition (79.859 ± 5.672 g) compared to the saline, no swimming condition (67.248 ± 10.937 g), a statistically significant difference of 12.612 g (95% CI, 0.302 to 24.921), $t(9) = 2.318$, $p = .046$.

Taken together, in the absence of swimming, mice injected with atropine or methoctramine have a stronger grip strength compared to saline controls. These findings suggest that cholinergic antagonists in *mSOD1G93A* mice provide beneficial effects on behavioural performance throughout disease progression, and these effects are more pronounced with the M2-selective antagonist methoctramine and in the absence of swimming.

Methoctramine improves weight, humane endpoint, muscle weights, and muscle innervation at humane endpoint in *mSOD1^{G93A}* mice.

The weights of mice were assessed once weekly and were averaged in four-week periods according to disease stage for mice in saline, atropine, and methoctramine conditions, in both swimming and no swimming groups. A two-way ANOVA revealed no interactions at any stage between drug administered and exercise conditions. Analyses of the main effects for drug administered were statistically significant at the muscle denervation stage ($F(2,24) = 3.562$, $p = .044$), symptom onset stage ($F(2,25) = 6.383$, $p = .006$), early symptomatic stage ($F(2,25) = 7.552$, $p = .003$), and late symptomatic stage ($F(2,25) = 8.727$, $p = .001$), with a large effect size for each of these stages ($\eta^2 = 0.229$, $\eta^2 = 0.338$, $\eta^2 = 0.377$, and 0.411, respectively). For the muscle denervation stage, the ANOVA observed power was 0.605. Pairwise comparisons show that mice in the methoctramine conditions, independent of exercise, weighted 23.847 ± 0.486 g during the muscle denervation stage (P58-P79), while mice in the saline conditions weighted 22.049 ± 0.468 g, a statistically significant mean difference of 1.798 ± 0.674 g ($p = .041$; **Fig 2***A*). Mice in the methoctramine conditions continued to show a greater weight than their saline counterparts in the symptom onset stage, early symptomatic stage, and late symptomatic stage, with a statistically significant mean difference of 2.384 ± 0.681 g ($p = .003$), 2.655 ± 0.685 g ($p = .002$), and 2.815 ± 0.675 g ($p < .001$). This suggests that mice injected with methoctramine, independent of their exercise activity, can maintain a heavier body weight compared to controls throughout disease progression, and these differences are more notable as the disease progresses.

A one-way ANOVA was conducted to determine differences in humane endpoint between mice injected with atropine and mice injected with saline, in both the swimming and no swimming conditions (**Fig. 2***B*, top panel). Humane endpoint was statistically significantly different across groups ($F(3,18) = 4.995$, $p = .011$, $\eta^2 = 0.454$), indicating at least one group differed from the others. Humane endpoints were as follows: mice in the saline, no swimming group had 151.7 ± .5.61 days, mice in the saline, swimming group had 152.4 ± 4.56 days, mice in the atropine, no swimming group had 164.0 ± 8.34 days, and mice in the atropine, swimming group had 160.4 ± 6.42 days. Tukey post hoc analysis revealed that the increase from saline, no swimming to atropine, no swimming (12.33, 95% CI (1.774 to 22.892)) was statistically significant ($p = .019$). The increase from saline, swimming to atropine, no swimming (11.60, 95% CI (0.525 to 22.675)) was also statistically significant ($p = .038$). A one-way ANOVA was conducted to determine differences in humane endpoint between mice injected with methoctramine and mice injected with saline, in both the swimming and no swimming conditions (**Fig. 2***B*, lower panel). Humane endpoint was statistically significantly across all groups ($F(3,16) = 3.329$, $p = .046$, $\eta^2 = 0.384$), suggesting that at least one group differed from the others. Mice in the methoctramine, no swimming condition had a humane endpoint of 163.5 ± 7.51 days, while mice in the methoctramine, swimming condition had a humane endpoint of 154.2 ± 7.40 days. Tukey post hoc analysis revealed that the increase in humane endpoint from saline, no swimming to methoctramine, no swimming (11.833, 95% CI (0.260 to 23.406) was statistically significant ($p = .044$). Together, these findings suggest that the administration of either atropine or methoctramine can increase humane endpoint in *mSOD1*^{G93A} mice compared to saline controls, but only in the

559 absence of exercise in the form of swimming.

560

561 After reaching the humane endpoint, mice weights were recorded and then
562 euthanized. The gastrocnemius (GS), tibialis anterior (TA), and soleus (Sol.) muscles
563 were dissected and weighed. Muscle weights were then normalized to the final weight
564 of the animal. A two-way ANOVA was performed on normalized GS weights, TA weights,
565 and Sol weights (**Fig. 2***C*). The analysis revealed an interaction between drug
566 administered and exercise condition in the GS weight with a large effect size ($F(2,25) =$
567 7.843, $p < .01$, $\eta^2 = 0.386$). Pairwise comparisons show that mice in the atropine,
568 swimming condition had a lower normalized GS weight (0.263 ± 0.022%) compared to
569 mice in the saline, swimming condition (0.376 ± 0.022%), a statistically significant
570 difference ($p = .004$). Mice in the methoctramine, no swimming condition had a higher
571 normalized GS weight (0.352 ± 0.025%) compared to mice in the saline, no swimming
572 condition (0.270 ± 0.020%), a statistically significant difference (**Fig. 2***C,* top panel**;** $p =$
573 $.048$). An interaction was also found in the normalized TA weight ($F(2,25) = 5.879$, $p =$
574 $.008$, $\eta^2 = 0.320$). Mice in the atropine, swimming condition had a lower normalized TA
575 weight (0.084 ± 0.012%) compared to mice in the saline, swimming condition (0.110 ±
576 0.006%), a statistically significant difference (**Fig. 2***C*, middle panel*;* $p = .009$). Mice in
577 the methoctramine, no swimming condition had a higher normalized TA weight (0.113 ±
578 0.016%) compared to mice in the saline, no swimming condition (0.093 ± 0.007%), a
579 statistically significant difference ($p = .046$). No interactions or main effects were found
580 in the normalized Sol. weight. These findings show that both GS and TA weights are
581 heavier in the methoctramine group compared to saline controls, but only in the absence

582 of exercise. The weights of both GS and TA are lower in the atropine condition
583 compared to saline controls, but only in the presence of exercise. An independent-
584 samples t-test also shows that GS weight was higher in the saline, swimming condition
585 (0.376 ± 0.047) than in the saline, no swimming condition (0.270 ± 0.048), a statistically
586 significant difference of 0.101 (95% CI, 0.0415 to 0.172), $t(9) = 3.702$, $p = .005$.
587 Similarly, TA weight was higher in the saline, swimming condition (0.110 ± 0.006) than
588 in the saline, no swimming condition (0.093 ± 0.007), a statistically significant difference
589 of 0.018 (95% CI, 0.009 to 0.026), $t(9) = 4.588$, $p = .001$.
590
591 We examined the muscle innervation of GS, TA, and Sol. muscles at humane
592 endpoint in the saline, atropine, and methoctramine conditions, in both swimming and no
593 swimming groups (**Fig. 2***D*). 5044 neuromuscular junctions (NMJ) were analyzed (n =
594 689-830 per group) and a chi-square test of homogeneity was conducted (**Fig. 2***E*). A
595 total of 255 NMJ (30.72%) were fully innervated in the methoctramine, no swimming
596 group compared to 188 (18.36%) in the saline, no swimming group and 186 (18.29%) in
597 the atropine, no swimming group, a statistically significant difference in proportions, $p <$
598 .001. A total of 234 NMJ (32.5%) were fully innervated in the methoctramine, swimming
599 group compared to 86 (12.48%) in the saline, swimming group and 108 (14.14%) in the
600 atropine, swimming group, a statistically significant difference in proportions. Post hoc
601 analysis involved pairwise comparisons using the z-test of proportions with a Bonferroni
602 correction. These findings reveal that the administration of methoctramine in mice,
603 independent of exercise, improves muscle innervation at Humane Endpoint compared to
604 the administration of saline or atropine.

Methoctramine is detrimental to *mSOD1*^{G93A} mice when administration begins at symptomatic stages.

Our findings reveal that the administration of methoctramine beginning at P30 can have beneficial effects on behavioural performance, humane endpoint, and muscle innervation in *mSOD1*^{G93A} mice. We sought to determine if these beneficial effects could be seen when methoctramine is administered after symptom onset. Weight, maximum speed on a treadmill, and grip strength were recorded weekly beginning at P30. Administration of methoctramine began at P90 and continued until Humane Endpoint. An independent-sample t-test was run to determine if there were differences in weight, maximum speed on a treadmill, grip strength, and humane endpoint between mice injected with methoctramine (n = 6) and saline (n = 4). No statistically significant differences were found in Weight or Maximum Speed on a Treadmill at any stages of the disease (**Fig. 3***A, B*). In the early symptomatic stage, grip strength was lower in mice injected with methoctramine (68.349 ± 3.898 g) compared to mice injected with saline (88.704 ± 13.023 g), a statistically significant difference of 20.355 (95% CI, 6.04 to 34.66), $t(7) = 3.364$, $p = .012$ (**Fig. 3***C*). In the late symptomatic stage, mice injected with methoctramine also had lower grip strength (43.851 ± 3.561 g) compared with mice injected with saline (59.649 ± 4.902 g), a statistically significant difference of 15.797 (95% CI, 8.38 to 23.21), $t(6) = 5.214$, $p = .002$. No statistically significant differences were observed in the Humane Endpoint (**Fig. 3***D*). These results suggest that the beneficial effects of methoctramine are limited to the administration beginning at earlier stages, and administration beginning at symptomatic stages can be detrimental to grip strength.

Methoctramine improves humane endpoint in *mSOD1^{G93A}* mice with non-functional C-boutons.

Our findings show that the administration of methoctramine, an M2-selective cholinergic antagonist, beginning at P30 can provide beneficial effects to *mSOD1^{G93A}* mice. Given that M2 muscarinic receptors are located throughout the body, including post-synaptic to V0c interneurons, we wanted to investigate whether the beneficial effects of methoctramine are dependent on C-boutons. To do this, we repeated our longitudinal experiment where methoctramine administration began at P30, but in mice with genetically silenced C-boutons (*mSOD1^{G93A}/C^{off}*). Similar to our previous experiments, *mSOD1^{G93A}/C^{off}* mice were randomly assigned to either a saline condition or a methoctramine condition (n = 3 per group). Weight, Maximum Speed, and Grip strength were assessed weekly on days when injections were not taking place. Histological assessment demonstrates prevalent C-boutons in *mSOD1^{G93A}* mice (**Fig. 4***A*), and complete absence of ChAT at the sites of C-boutons in the lumbar spine of *mSOD1^{G93A}/C^{off}* mice (**Fig. 4***B*).

An independent-samples t-test was conducted to determine differences in Weight, Maximum Speed, Grip Strength, and Humane Endpoint between both conditions. There was no statistically significant difference in Weight or Grip Strength between the saline and methoctramine conditions (**Fig.** 4*C*, *D*). Mice in the methoctramine condition had a greater Maximum Speed compared to their saline counterparts in the presymptomatic stage, muscle denervation stage, symptom onset

stage, and early symptomatic stage, with a mean increase of 0.119 ± 0.075, 0.122 ± 0.130, 0.049 ± 0.152, and 0.109 ± 0.126, respectively, however, these differences did not reach statistical significance (**Fig 4***E*). Humane endpoint was higher in *mSOD1*^{G93A}*/C*^{off} mice injected with methoctramine (157.33 ± 2.517 days) compared to their saline counterparts (144.33 ± 1.155 days), a statistically significant difference of 13 days (95% CI, 8.56 to 17.44), $t(4) = 8.132$, $p = .001$ (**Fig. 4***F*). These results indicate that the beneficial effects of methoctramine are, at least partly, independent of C-boutons, suggesting that the positive effects seen in our first experiment are likely due to a mechanism on the peripheral nervous system.

Methoctramine reaches peak plasma concentration within ten minutes and does not appear to cross the blood-brain barrier.

Our findings demonstrate that the effects of methoctramine are independent of C-boutons, raising questions about whether this pharmacological agent is crossing the blood-brain barrier. Therefore, we sought to determine if methoctramine could be detected in blood plasma and CSF. To do this,10 wild-type mice were injected with methoctramine on four different days and blood was collected from the saphenous vein as previously described. Plasma was separated by centrifugation and mass spectrometry was performed on all samples. Our findings demonstrate that methoctramine reaches its highest plasma concentration within ten minutes and decreases, on average, by approximately 58% 40 minutes after administration (**Fig. 5***A*). Additionally, 5 wild-type mice and 3 *mSOD1*^{G93A} mice had CSF collected from the cisterna magna after administration of methoctramine as previously described. CSF was

673 collected at different timepoints, centrifuged, and quality control was performed to
674 ensure absence of hemoglobin. WT and mSOD1 CSF samples were compared to water
675 samples, CSF samples where no methoctramine had been previously injected, and CSF
676 samples in which methoctramine was manually added to them (Supplemental Figure
677 **S1**). CSF samples show no differences in peak area compared to water samples or CSF
678 samples where methoctramine had not been previously injected (**Fig. 5***B*). Although
679 methoctramine is readily found in plasma through mass spectrometry, the absence of
680 this drug in CSF suggests that it is not able to cross the blood-brain barrier, at least in
681 quantities within our detection limits. Therefore, our findings suggest that the beneficial
682 effects of methoctramine are likely due to peripheral effects.
683
684 **Methoctramine increases GS amplitude in WT as measured by EMG.**
685 Our findings demonstrate that the effects of methoctramine are C-bouton
686 independent and methoctramine is likely to not cross the blood-brain barrier (BBB),
687 suggesting a peripheral effect. The muscarinic receptor M2 has previously been shown
688 to modulate ACh release in the neuromuscular junction(Minic et al., 2002; Slutsky et al.,
689 2004). Therefore, we sought to determine the effects of methoctramine on EMG
690 amplitude in WT mice at different timepoints. To do this, mice were implanted with
691 electrodes and EMG activity was measured 5 minutes prior to administration of saline or
692 methoctramine (baseline) and every 5 minutes post-injection. GS amplitude was
693 normalized to the baseline of each mouse on a given day. Recordings for saline and
694 methoctramine were done on separate days, and the order of injections was alternated
695 to mitigate practice effects.

A paired-samples t-test was used to determine differences in EMG amplitude between saline and methoctramine conditions. Compared to mice injected with saline, mice injected with methoctramine showed a higher normalized GS amplitude at 10 minutes (1.143 ± 0.075), 20 minutes (1.285 ± 0.231), 30 minutes (1.280 ± 0.126), and 40 minutes post-injection (1.289 ± 0.174), a statistically significant increase of 0.298 (95% CI, 0.256 to 0.337), $t(2) = 33.185$, $p < .001$, 0.445 (95% CI, 0.114 to 0.776), $t(2) = 5.777$, $p = .029$, 0.460 (95% CI, 0.063 to 0.857), $t(2) = 4.990$, $p = .038$, and 0.421 (95% CI, 0.037 to 0.804), $t(2) = 4.713$, $p = .042$, respectively. (**Fig 6***A*). In addition, a consistent increase was also observed in the 15-, 25-, and 25-minutes post-injection timepoints, although these did not reach statistical significance.

These results indicate that methoctramine moderately increases GS EMG amplitude during slow walking, and the improvements seen in our previous *mSOD1*^{G93A} experiments are likely related to peripheral effects of methoctramine.

DISCUSSION

We have shown that systemic injection of cholinergic receptor antagonists, atropine that antagonizes a wide range of metabotropic receptors and methoctramine that is selective to M2 receptors, significantly improves motor behavior in ALS mouse model, the *mSOD1*^{G93A}, independent of swimming. Both drugs improved the humane endpoint only if they were not paired with swimming. This improvement was contingent

upon early start of the drug delivery at pre-symptomatic ages as the late start at symptomatic ages had rather detrimental effect on some behavioural performances and no effect on humane endpoint. Methoctramine, but not atropine, improved muscle innervation at humane endpoint. Lastly, we show evidence that the beneficial effects of methoctramine are C-bouton independent, as improvements are seen in *mSOD1*^{G93A}*/C*^{off} mice, and we were unable to detect methoctramine in cerebrospinal fluid through mass spectrometry. Overall, our study suggests that the administration of cholinergic antagonists, especially methoctramine, provides beneficial effects for disease progression in the *mSOD1*^{G93A} mouse model when administered at earlier stages of the disease, and these are likely due to peripheral effects. The mechanisms behind these improvements present a crucial opportunity for the development of therapies for neurological disorders that destroy motor neurons by which disease progression can be significantly slowed down. Importantly, atropine appeared to be less effective compared to methoctramine, likely due to the wide array of muscarinic receptors that it targets (M1-M5), as well as its ability to cross the BBB. Future research must be aimed at delineating the contribution of each muscarinic receptors in the pathophysiology of ALS.

Peripheral M2 receptor antagonism improves disease progression in ALS mouse model.

We have previously shown that when *mSOD1*^{G93A} mice with genetically silenced C- boutons engage in swimming, an activity that would typically upregulate these cholinergic synapses, their behavioural performance and muscle innervation

improve (Wells et al., 2021). We hypothesized that the administration of cholinergic antagonists in combination with swimming would have similar effects. We found this to not be the case, and our results suggest these cholinergic antagonists provide greater beneficial effects in the absence of swimming. Methoctramine has previously been shown to delay in loss of strength in ALS mutant mice after 15 days of *in vivo* administration during presymptomatic stages, followed by accelerated denervation, loss of strength, and death after discontinuation (Saxena et al., 2013). In our longitudinal experiments, we determined that long-term administration of methoctramine in *mSOD1*^{G93A} provides beneficial effects in the absence of functional C-boutons (**Fig. 4**). However, the fact that weight, maximum speed, and grip strength did not significantly improve in the methoctramine condition suggests that silencing C-boutons present some limitations to how much improvement methoctramine can bring about. Although the exact limitations remain to be investigated. Nevertheless, the improvements seen in humane endpoint, in combination with our data suggesting that methoctramine does not cross the blood-brain-barrier into the cerebrospinal fluid, provides evidence that the positive effects of methoctramine are likely due to M2 receptors in the periphery and not in the spinal cord postsynaptic to the C-boutons. Two possible candidates to carry out these effects are the Schwann cells (SC) and the M2 autoreceptor at the motor neurons presynaptic endings at the neuromuscular junction.

SC are glial cells in the peripheral nervous system that are essential for the homeostasis and repair of nervous tissue during disease and after injury (Jessen and Mirsky, 2016). Previous research has shown that SC in rats express several types of

765 muscarinic receptors, with M2 being the prevalent subtype (Loreti et al., 2006). This
766 suggests that the beneficial effects of methoctramine could be due to its action in this
767 cell population. For example, a specific type of SC called terminal Schwann cells (tSC)
768 has been found to be influenced by methoctramine. When injected subcutaneously
769 twice daily for 7 days, tSC are activated, and terminal sprouting is induced in
770 neuromuscular junctions of adult mice (Wright et al., 2009). This effect could be
771 responsible for the improved NMJ innervation we have noted in our study. There are
772 other effects that could be occurring as a result of methoctramine administration. For
773 example, the transcription factor c-Jun is involved in SC injury response, and c-Jun c-
774 KO mice exhibit impaired axonal repair after injury (Arthur-Farraj et al., 2012). In
775 agreement with this idea, arecaidine, an M2-selective agonist, has been shown to
776 reduce the expression of c-Jun. Therefore, it is possible that methoctramine influences
777 c-Jun expression and enhances the repair capabilities of these cells. Moreover,
778 methoctramine could be inducing the proliferation of SC, given that the use of
779 arecaidine in cultures of SC results in cell-cycle arrest and accumulation of cells in the
780 G1 phase (Loreti et al., 2008). In addition to proliferation, methoctramine could also
781 enhance migration in response to injury. Supporting this idea, arecaidine has been
782 shown to inhibit cell migration in a wound assay in a wound assay (Botticelli et al.,
783 2022). Therefore, methoctramine is likely inducing several actions in SC that contribute
784 to the effects we have seen in our work.
785
786 In spinal cord *in vitro* preparations, methoctramine has been shown to decrease
787 firing frequency in motor neurons and amplitude in ventral root bursts during fictive

788 locomotion (Nascimento et al., 2019). Concurrent with this idea, the activation of the
789 muscarinic receptor M2 is known to increase motor neuron excitability through the C-
790 boutons as seen by the reduction in afterhyperpolarization (Miles et al., 2007). However,
791 in our study, we found an increase in EMG amplitude in wild-type mice following
792 administration of methoctramine (**Fig. 6**). This increase is possibly due to the effects of
793 blocking the autoreceptor at the NMJ, which has been shown to inhibit synaptic
794 transmission when activated(Garcia et al., 2005). Therefore, the effects of
795 methoctramine could be enhancing the release of acetylcholine at the synapse, rather
796 than affecting the M2 receptors at C-boutons (Nascimento et al., 2019). This selective
797 peripheral effect could be explained by methoctramine's inability to cross the BBB in our
798 *in vivo* experiments. Despite our findings regarding the absence of methoctramine in
799 CSF through the use of mass spectrometry (**Fig. 5**), it is important to note that it is
800 possible the drug is crossing the BBB but in such small quantities that it is beyond the
801 detection limit in our assay. However, even if methoctramine is crossing the BBB *in* vivo
802 in amounts under our detection limit, it is likely that these quantities are not of clinical
803 significance. In order to determine the effects of methoctramine in the CNS, such as
804 blocking the M2 receptors postsynaptic to C-boutons, future research could be
805 conducted where methoctramine is injected intrathecally. This would eliminate the
806 challenge posed by methoctramine's inability to cross the BBB, therefore allowing direct
807 assessment of its central effects.
808
809 **Some of the effects of methoctramine rely on its early administration and**
810 **avoidance of exercise.**

The effects of exercise on disease progression in ALS has long been an area of research that has produced mixed results. High intensity exercise has been shown to hasten the onset of motor performance deficits in *mSOD1*^{G93A} mice, while moderate exercise can delay motor deficits slightly (Carreras et al., 2009). Other studies have shown that high-intensity endurance exercise can hasten motor performance deterioration and death in male *mSOD1*^{G93A} mice (Mahoney et al., 2004). In our current study, we found that high-intensity exercise in the form of swimming abolishes the beneficial effects of methoctramine on grip strength and humane endpoint (**Fig. 1** and **Fig. 2**, respectively). As previously mentioned, it is possible that methoctramine increases the release of acetylcholine at the NMJ by blocking the presynaptic autoreceptor, and in combination with the additional demand of swimming, no positive effects are seen. In this regard, methoctramine could be mimicking the effects of low-intensity exercise, a strategy that has been shown to provide beneficial effects on disease progression in *mSOD1*^{G93A} mice (Kirkinezos et al., 2003).

The beneficial effects of methoctramine in the absence of swimming were limited by administration beginning at P30. Notably, when methoctramine administration was begun at P90, all positive effects disappeared, and grip strength performance was worsened (**Fig. 3**). This suggests there is a limited time window for methoctramine to be effective. Therefore, the administration of methoctramine can provide highly beneficial effects on disease progression in *mSOD1*^{G93A} mice, but only when its administration begins at earlier stages of the disease.

The longitudinal effects of long-term administration of cholinergic antagonists in ALS mouse models has not been previously shown. Our study provides clear evidence that the administration of said pharmacological agents provide remarkable benefits in the context of ALS, significantly decreasing loss of motor function over time and preserving muscle innervation, two hallmarks of motor neuron diseases that lead to muscle weakness and loss of function. Specifically, we conclude that these beneficial effects are due to the antagonism of the M2 receptors in the peripheral nervous system. These novel results propose two possible mechanisms by which disease progression can be slowed down: the antagonism of M2 receptors in SC, which are involved in axonal repair, cell migration, and proliferation, and the inhibition of the M2 autoreceptor in the NMJ that is likely to increase ACh release. Our study offers a critical opportunity for additional research to explore how these pathways can be further manipulated in the context of pre-clinical studies in order to uncover novel therapeutics that can minimize the detrimental effects seen in ALS.

SUPPLEMENTAL MATERIAL

Supplemental Figure S1 and Video S1 (https://dx.doi.org/10.21227/v313-a075)

GRANT

This research has been supported by the Canadian Institutes of Health Research (162357) and ALS Society of Canada (2017).

FIGURE LEGENDS

Fig. 1. Atropine and methoctramine improve behavioural performance at various disease stages. Two-way ANOVAs were conducted to examine the effects of Drug and Exercise on Maximum Speed on a Treadmill and on Grip Strength at every stage. *p*-values are Bonferroni-adjusted ***A***, Interaction effects between Drug and Exercise on Maximum Speed were not statistically significant at any stage. Analyses of the main effect of Drug on Maximum Speed were statistically significant. Mice injected with either atropine or methoctramine had greater Maximum Speed compared to mice injected with saline at various stages, independent of swimming. ***B***, There was a statistically significant interaction effect between Drug and Exercise conditions on Grip Strength at various stages. Mice injected with either atropine or methoctramine showed greater Grip Strength compared to mice injected with saline, only in the absence of swimming. In the Muscle Denervation and Symptom Onset stages, mice injected with methoctramine in the no swimming condition also performed better in Grip Strength compared to mice injected with atropine in the no swimming condition. Analysis of the main effect of Drug on Grip Strength was statistically significant in the Presymptomatic stage. At this stage, independent of swimming, mice injected with either atropine or methoctramine showed greater Grip Strength compared to mice injected with saline. Bars represent mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 2. Methoctramine improves weight, humane endpoint, normalized muscle weight, and muscle innervation at humane endpoint. ***A***, two-way ANOVA were conducted to examine the effects of Drug and Exercise on Weight at every stage. p-values are Bonferroni-adjusted. Interaction effects between Drug and Exercise on Average Weight were not statistically significant at any stage. Analyses of the main effect of Drug on Average Weight were statistically significant for methoctramine but not atropine at all stages except Presymptomatic stage. Mice injected with methoctramine had greater Weight compared to mice injected with saline at the Muscle Denervation stage, Symptom Onset stage, Early Symptomatic stage, and Late Symptomatic stage,

independent of swimming. ***B***, One-way ANOVAs were conducted to examine if Humane Endpoints were different with atropine or methoctramine, in the presence and absence of swimming. Humane Endpoint was statistically significantly different between groups. Tukey post hoc analysis revealed an increase in Humane Endpoint in mice that were injected with atropine in the no swimming condition compared to mice injected with saline in the swimming condition and in the no swimming condition. Mice injected with methoctramine in the no swimming condition had an increase in Humane Endpoint compared to mice injected with saline in the no swimming condition. ***C***, two-way ANOVA were conducted to examine the effects of Drug and Exercise conditions on Muscle Weight for GS, TA, and Sol. muscles. p-values are Bonferroni-adjusted. *Upper panel*: there was a statistically significant interaction effect between the Drug and Exercise conditions on GS Weight. Mice injected with atropine had a lower GS Weight compared to mice injected with saline, only in the swimming conditions. Mice injected with methoctramine had a larger GS Weight compared to mice injected with saline, only in the absence of swimming. *Middle panel*: There was a statistically significant interaction effect between the Drug and Exercise conditions on TA Weight. Mice injected with methoctramine had a higher TA Weight compared to mice injected with saline, only in the no swimming condition. Mice injected with atropine had a lower TA Weight compared to mice injected with saline, only in the swimming condition. *Lower panel*: No interactions or main effects were statistically significant in the Sol. Weights. ***D***, Muscle innervation at Humane Endpoint was calculated based on the number of BTX+ postsynaptic terminals that had complete overlapping VAChT+ presynaptic terminals, partially overlapping presynaptic VAChT+ terminals, and no overlapping terminals (→, ➤, and *, respectively). ***E***, A Chi-square test of homogeneity was conducted to determine if there was a difference in the proportion of NMJs that were fully innervated in the GS muscle in all groups. There was a statistically significant difference in proportions, $p < .001$. Post hoc analysis involved pairwise comparisons using the z-test of two proportions with a Bonferroni correction. The proportion of fully innervated NMJs was significantly higher in the methoctramine condition compared to both saline and atropine conditions, in both the swimming and no swimming groups. Bars represent mean ± SD. Boxplots display lower and upper extremes, lower and upper quartiles, and median. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Fig. 3. Methoctramine does not improve Weight or Maximum Speed, or Humane Endpoint, and it is detrimental to Grip Strength when injected at Symptomatic stages. Independent-sample t-tests were conducted to examine the effects of methoctramine when administration began at P90. ***A***, There were no statistically significant differences on Weight at any stages. ***B***, There were no statistically significant differences on Maximum Speed at any stage. ***C***, Mice injected with methoctramine beginning at P90 lower Grip Strength compared to mice that were injected with saline beginning at P90. ***D***, there were no statistically significant differences on Humane endpoint between mice that were injected with saline and methoctramine, beginning at P90. Bars represent mean ± SD. Boxplots display lower and upper extremes, lower and upper quartiles, and median. * $p < 0.05$, ** $p < 0.01$, $p < 0.001$.

Fig. 4. Methoctramine improves Humane endpoint but not Weight, Maximum Speed, or Grip Strength in mSOD1G93A/Coff mice. Independent sample t-test were run to determine if there were differences in Weight, Maximum Speed, Grip Strength, or Humane Endpoint between $mSOD1^{G93A}/C^{off}$ mice injected with methoctramine and $mSOD1^{G93A}/C^{off}$ injected with saline. ***A***, Intact C-Boutons in $mSOD1^{G93A}$ mice compared to silenced C-boutons in $mSOD1^{G93A}/C^{off}$. ***B***, as shown by absence of ChAT+ synapses to motor neurons. ***C***, There were no statistically significant differences on Weight at any stages. ***D***, There were no statistically significant differences on Grip Strength at any stages. ***E***, There were no statistically significant differences on Maximum Speed at any stage. ***F***, There was a statistically significant difference in humane endpoint. $mSOD1^{G93A}/C^{off}$ injected with methoctramine had a higher humane endpoint compared to $mSOD1^{G93A}/C^{off}$ mice injected with saline. Bars represent mean ± SD. Boxplots display lower and upper extremes, lower and upper quartiles, and median. * $p < 0.05$, ** $p < 0.01$, $p < 0.001$.

Fig. 5. Mass spectrometry of plasma and cerebrospinal fluid of mice injected with methoctramine. Mass spectrometry analyses were performed on plasma samples and CSF samples of WT and $mSOD1^{G93A}$ mice at different timepoints. ***A***, Plasma samples of WT mice injected with methoctramine (n = 10) collected at different timepoints show a peak signal at 10 minutes and a decrease in signal after 40 minutes. ***B***, CSF samples of WT mice (n = 5) injected with methoctramine collected between 8- and 26-minutes post-injection, blank solvent with no CSF or methoctramine, and CSF spiked with methoctramine. No differences are seen in the signal seen in blank samples or CSF of mice injected with methoctramine. ***C***, CSF samples of $mSOD1^{G93A}$ mice aged P127-128, blank solvent with no CSF or methoctramine, and CSF spiked with methoctramine. No differences were seen between the blank samples and the mSOD1 CSF samples.

Fig. 6. Methoctramine increases EMG amplitude during slow speed walking. A paired-samples t-test was used to determine whether there was a statistically significant mean difference between EMG amplitude when mice were injected with methoctramine compared to mice that were injected with saline at different timepoints. ***A***, Raw EMG waveforms were integrated to assess bursting amplitude in the GS muscle. ***B***, Integrated EMG waveforms demonstrating that mice injected with methoctramine achieved higher amplitude bursts after 10 and 20 minutes compared to mice injected with saline. ***C***, Mice injected with methoctramine achieved a higher normalized EMG amplitude at most timepoints relative to baseline. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

REFERENCES

1. Ravits JM, La Spada AR. ALS motor phenotype heterogeneity, focality, and spread: deconstructing motor neuron degeneration. Neurology. 2009;73:805–11.

2. Gubbay SS, Kahana E, Zilber N, Cooper G, Pintov S, Leibowitz Y. Amyotrophic lateral sclerosis. A study of its presentation and prognosis. Journal of Neurology. 1985;295–300.

3. Li X, Bedlack R. Evaluating emerging drugs in phase II & III for the treatment of amyotrophic lateral sclerosis. Expert Opinion on Emerging Drugs. 2024;93–102.

4. McComas AJ, Sica REP, Campbell MJ. Functional compensation in partially denervated muscles. Journal of Neurology, Neurosurgery & Psychiatry. 1971;453–60.

5. Schoenfeld MA, Tempelmann C, Gaul C, Kuhnel GR, Duzel E, Hopf JM, et al. Functional motor compensation in amyotrophic lateral sclerosis. Journal of Neurology. 2005;944–52.

6. Landoni LM, Myles JR, Wells TL, Mayer WP, Akay T. Cholinergic modulation of motor neurons through the C-boutons are necessary for the locomotor compensation for severe motor neuron loss during amyotrophic lateral sclerosis disease progression. Behavioural Brain Research. 2019 Sep;369:111914.

7. Zagoraiou L, Akay T, Martin JF, Brownstone RM, Jessel TM, Miles GB. A cluster of cholinergic pre-motor interneurons modulates mouse locomotor activity. Neuron. 2009;645–62.

8. Wells T, Miles JR, Akay T. C-Boutons and Their Influence on Amyotrophic Lateral Sclerosis Disease Progression. The Journal of Neuroscience. 2021;41:8088–101.

9. Hellström J, Oliveira ALR, Meister B, Cullheim S. Large cholinergic nerve terminals on subsets of motoneurons and their relation to muscarinic receptor type 2. The Journal of Comparative Neurology. 2003;460:476–86.

10. Moriya H, Takagi Y, Nakanishi T, Hayashi M, Tani T, Hirotsu I. Affinity profiles of various muscarinic antagonists for cloned human muscarinic acetylcholine receptor (mAChR) subtypes and mAChRs in rat heart and submandibular gland. Life Sciences. 1999;64:2351–8.

11. Food and Drug Administration [Internet]. 2015. Atropine Sulfate Injection. Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2015/021146s015lbl.pdf

12. Melchiorre C, Bolognesi ML, Minarini A, Rosini V M Tumiatti. Differential blockade of muscarinic receptor subtypes by polymethylene tretraamines. Novel class of selective antagonists of cardiac M-2 muscarinic receptors. Journal of Medicinal Chemistry. 1987;30:201–4.

13. Ghelardini C, Galeotti N, Gualtieri F, Marchese V, Bellucci C, Bartolini A. Antinociceptive and Antiamnesic Properties of the Presynaptic Cholinergic Amplifier PG-9. 1998; 284(3):806-16

14. Minic J, Molgo J, Karlsson E, Krejci E. Regulation of acetylcholine release by muscarinic receptors at the mouse neuromuscular junction depends on the activity of acetylcholinesterase. European Journal of Neuroscience. 2002;439–48.

15. Slutsky I, Parnas H, Parnas I. Presynaptic effects of muscarine on ACh release at the frog neuromuscular junction. The Journal of Physiology. 2004;769–82.

16. Saxena S, Roselli F, Singh K, Leptien K, Julien JP, Gros-Louis F, et al. Neuroprotection through excitability and mTOR required in ALS motoneoruons to delay disease and extend survival. Neuron. 2013;80–96.

17. Jessen KR, Mirsky R. The repair Schwann cell and its function in regenerating nerves. The Journal of Physiology. 2016;3521–31.

18. Loreti S, Vilaro MT, Visentin S, Rees H, I. LA, Tata AM. Rat Schwann cells express M1-M4 muscarinic receptor subtypes. Journal of Neuroscience Research. 2006;97–105.

19. Wright MC, Potluri S, Wang X, Dentcheva E, Gautam D, Tessler A, et al. Distinct Muscarinic Acetylcholine Receptor Subtypes Contribute to Stability and Growth, But Not Compensatory Plasticity, of Neuromuscular Synapses. Journal of Neuroscience. 2009;14942–55.

20. Arthur-Farraj PJ, Latouche M, Wilton DK, Quintes S, Chabrol E, Banerjee A, et al. c-Jun Reprograms Schwann Cells of Injured Nerves to Generate a Repair Cell Essential for Regeneration. Neuron. 2012;633–47.

21. Loreti S, Ricordy R, De Stefano ME, Augusti-Tocco G, Tata AM. Acetylcholine inhibits cell cycle progression in rat Schwann cells by activation of the M2 receptor subtype. Neuron Glia Biology. 2008;269–79.

22. Botticelli E, Salazar Intriago MS, Piovesana R, M. TA. Analysis of Signal Transduction Pathways Downstream M2 Receptor Activation: Effects on Schwann Cell Migration and Morphology. Life. 2022;

23. Nascimento F, Spindler LRB, Miles GB. Balanced cholinergic modulation of spinal locomotor circuits via M2 and M3 muscarinic receptors. Sci Rep. 2019 Oct 1;9(1):14051.

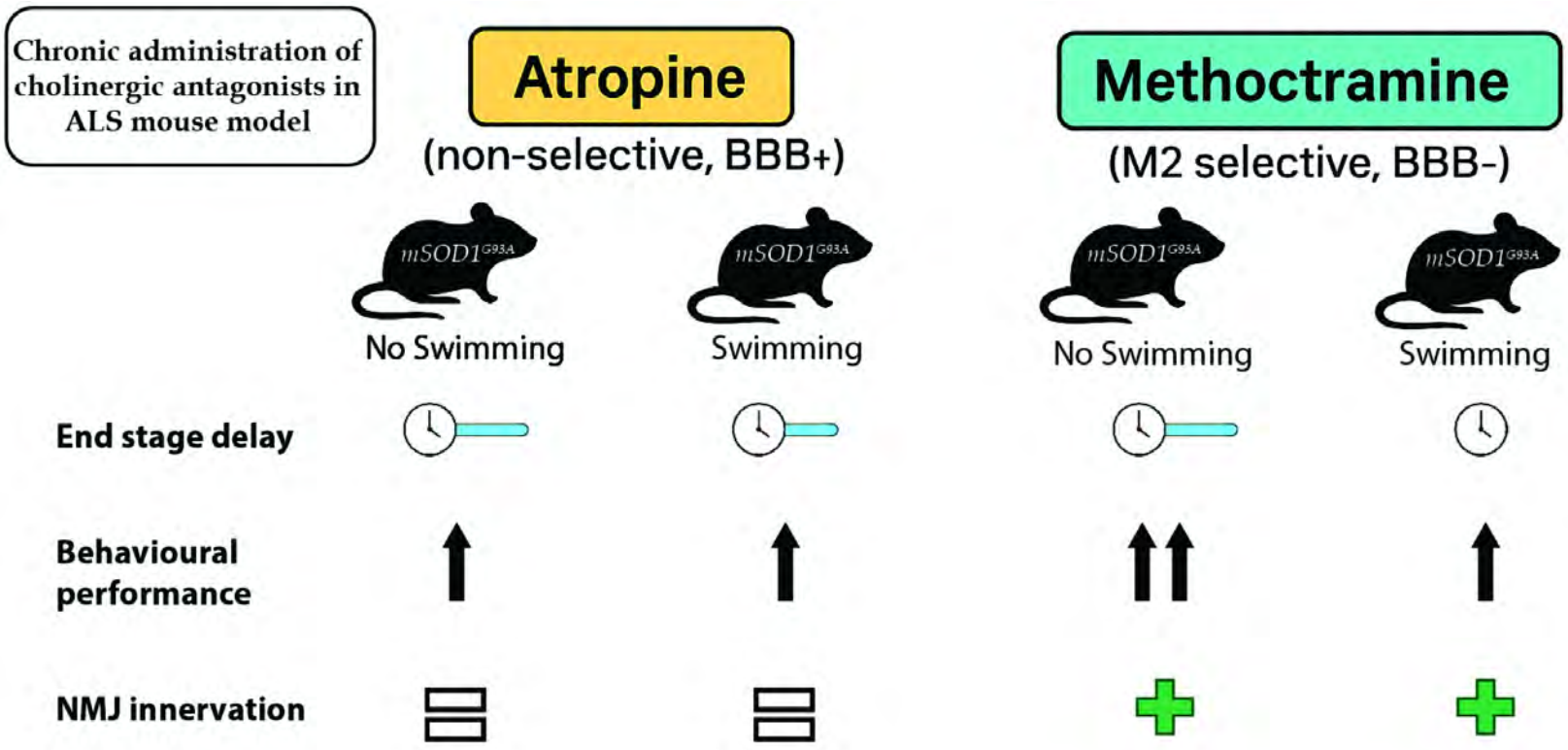
24. Miles GB, Hartley R, Todd AJ, Brownstone RM. Spinal cholinergic interneurons regulate the excitability of motoneurons during locomotion. Proc Natl Acad Sci USA. 2007 Feb 13;104(7):2448–53.

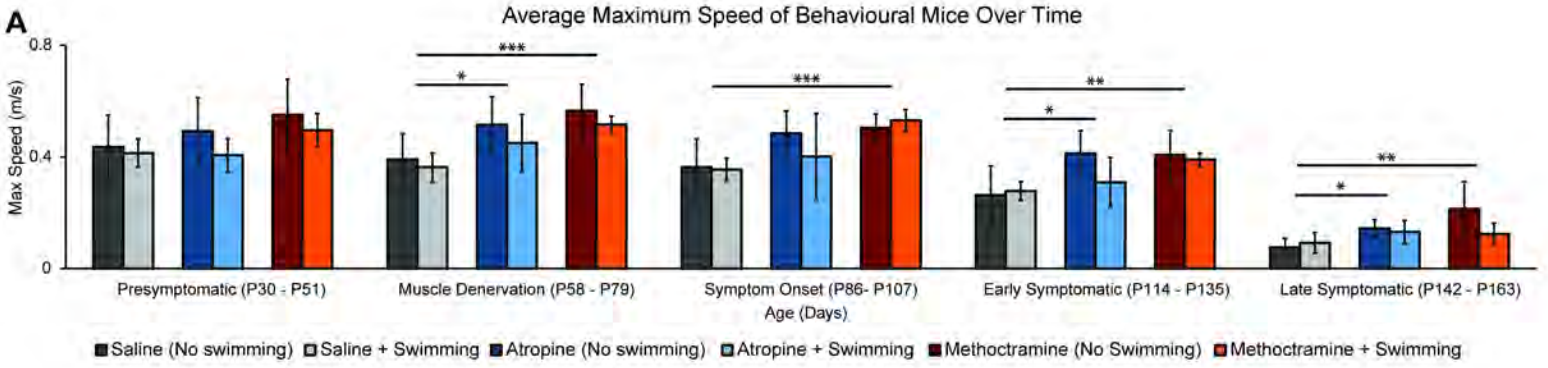
25. Garcia N, Santafe MM, Lanuza MA, Tomas. Expression of muscarinic acetylcholine receptors (M1-, M2-, M3- and M4-type) in the neuromuscular junction of the newborn and adult rat. Histology and Histopathology. 2005 Jul;(20):733–43.

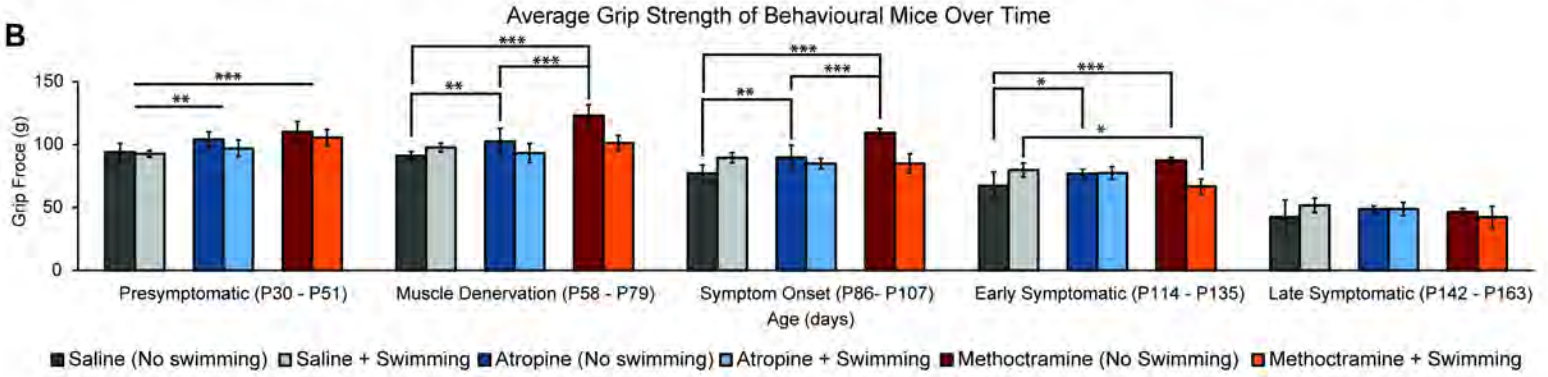
26. Carreras I, Yuruker S, Aytan N, Hossain L, Choi JK, Jenkins BG, et al. Moderate exercise delays the motor performance decline in a transgenic model of ALS. Brain Research. 2009;192–201.

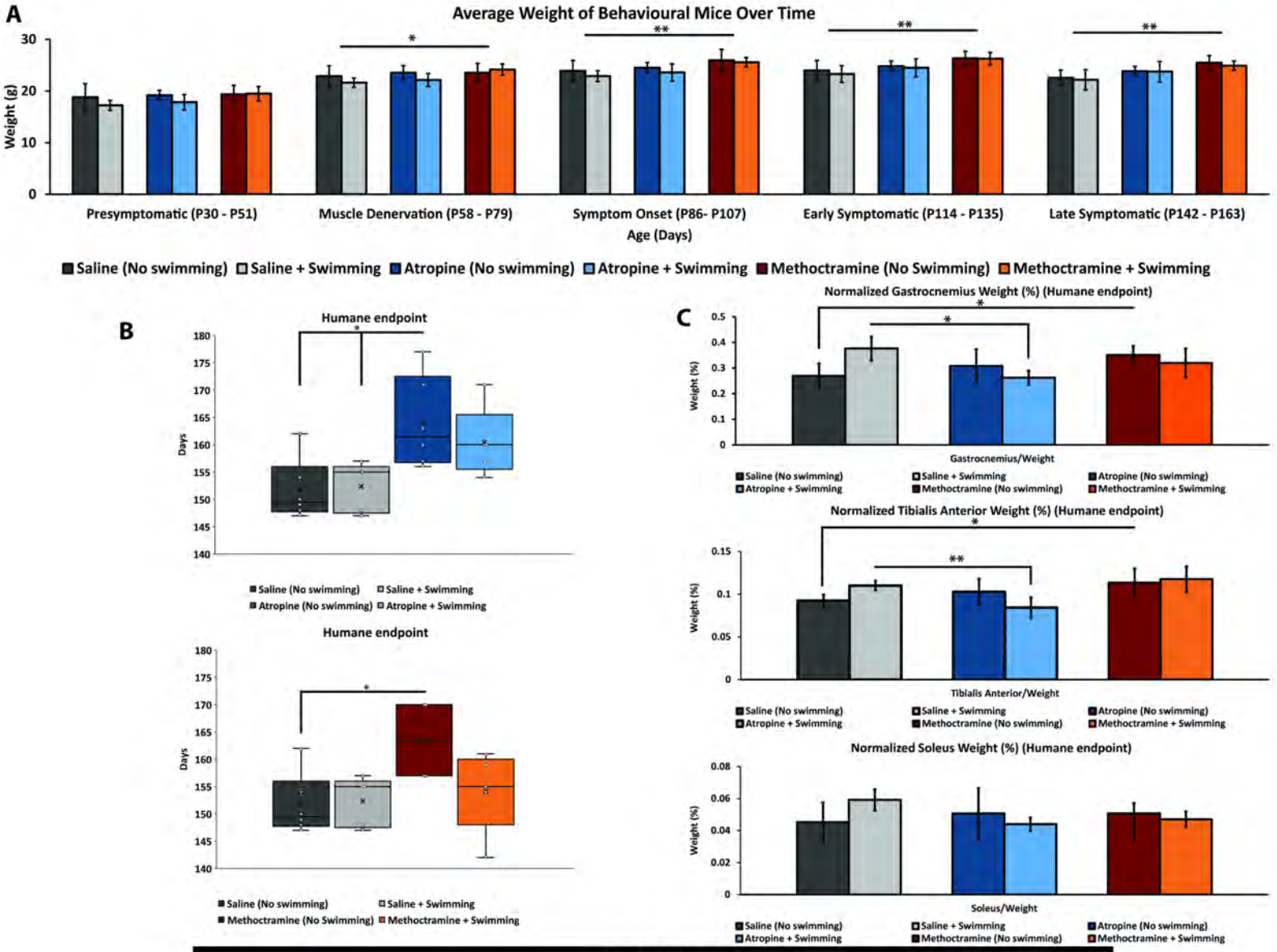
27. Mahoney DJ, Rodriguez C, Devries M, Yasuda N, Tarnopolsky MA. Effects of high-intensity endurance exercise training in the G93A mouse model of amyotrophic lateral sclerosis. Muscle & Nerve. 2004;656–62.

28. Kirkinezos IG, Hernandez D, Bradley WG, Moraes CT. Regular exercise is beneficial to a mouse model of amyotrophic lateral sclerosis. Annals of Neurology. 2003;804–7.

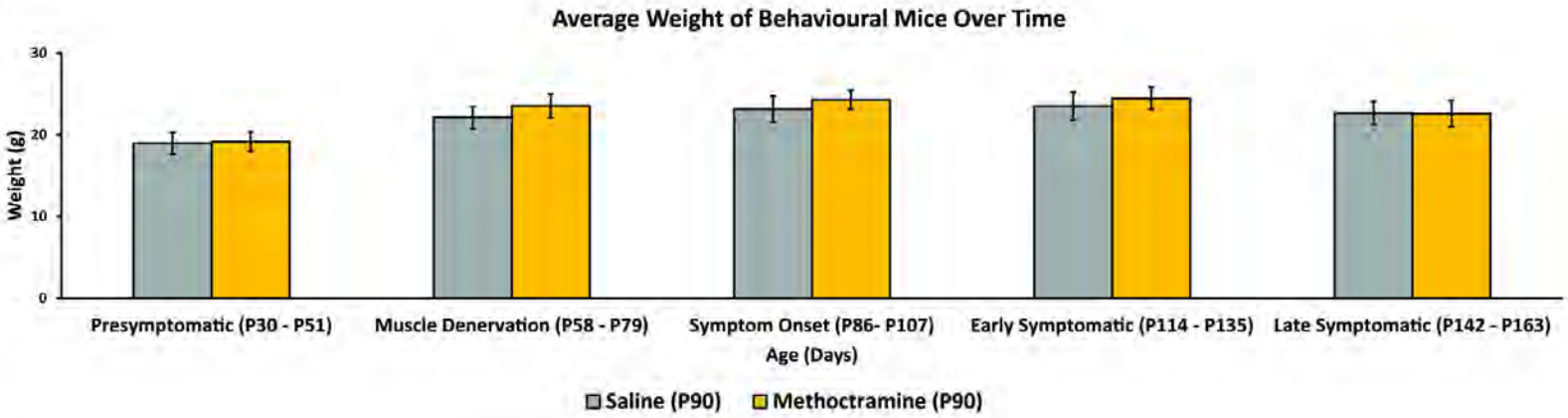




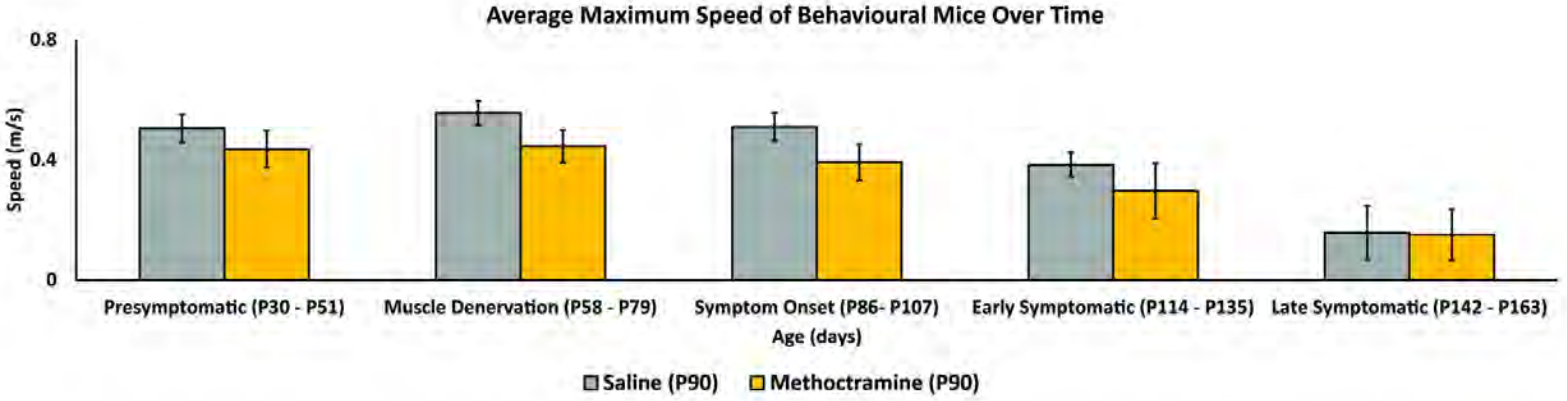




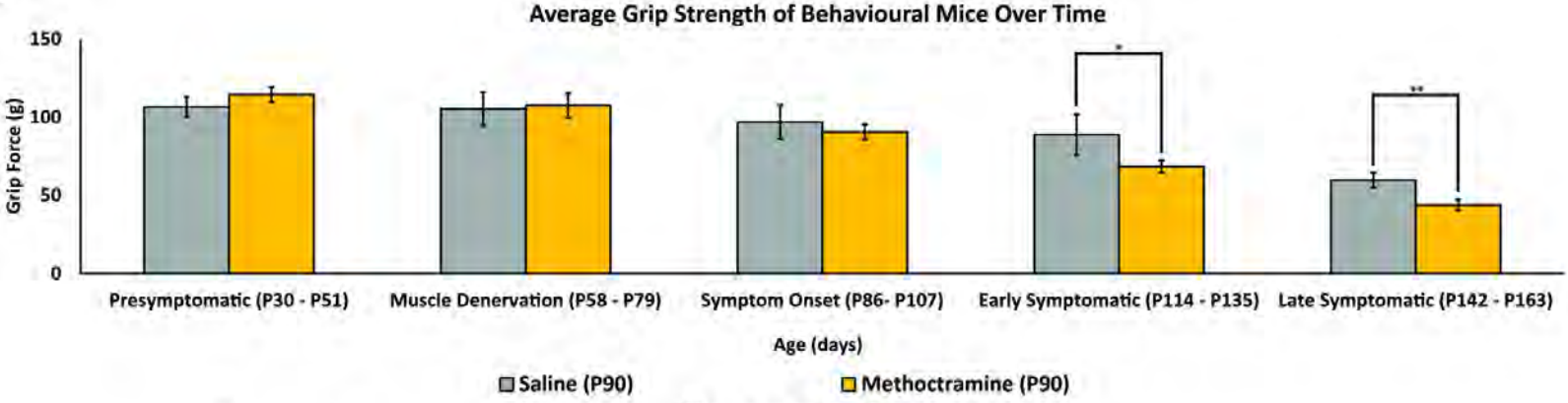
A



B



C



D