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Sympathomimetics Regulate Neuromuscular Junction Transmission through TRPV1, P/Q- and N-type Ca^{2+} Channels

Anna Zaia Carolina Rodrigues^{1,2,#}, Zhong-Min Wang^{1,#}, María Laura Messi¹, and Osvaldo Delbono^{1,2,§}

¹Department of Internal Medicine, Section on Gerontology and Geriatric Medicine, Wake Forest School of Medicine, Winston-Salem, North Carolina

²Neuroscience Program, Wake Forest School of Medicine, Winston-Salem, North Carolina

Abstract

Increasing evidence indicates that, first, the sympathetic nervous system interacts extensively with both vasculature and skeletal muscle fibers near neuromuscular junctions (NMJs) and, second, its neurotransmitter, noradrenaline, influences myofiber molecular composition and function and motor innervation. Since sympathomimetic agents have been reported to improve NMJ transmission, we examined whether two in clinical use, salbutamol and clenbuterol, affect the motor axon terminal via extracellular Ca^{2+} and molecular targets, such as TRPV1 and P/Q- and N-type voltage-activated Ca^{2+} channels. Electrophysiological recordings in ex-vivo preparations of peroneal nerves and lumbricalis muscles from young adult mice focused on spontaneous miniature end-plate potentials and singly and repetitively evoked end-plate potentials. Adding one dose of salbutamol or clenbuterol to the nerve/muscle preparation or repeatedly administering salbutamol to a mouse for 4 weeks increased spontaneous and evoked synaptic vesicle release but induced a steep decline in EPP amplitude in response to repetitive nerve stimulation. These effects were mediated primarily by ω -agatoxin IVA-sensitive P/Q-type and secondarily by ω -conotoxin GVIA-sensitive N-type Ca^{2+} channels. Presynaptic arvanil-sensitive TRPV1 channels seem to regulate Ca^{2+} at the motor neuron terminal at rest, while putative presynaptic β -adrenergic receptors may mediate sympathomimetic and catecholamine effects on presynaptic Ca^{2+} channels during NMJ activation.

Keywords

neuromuscular transmission; sympathetic nervous system; neuromuscular junction; skeletal muscle; calcium channels; sympathomimetics

[§]Correspondence to: Osvaldo Delbono, MD, PhD, Wake Forest School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157, Phone: 336-716-9802, odelbono@wakehealth.edu.

[#]These authors contributed equally to this work

Declaration of interest

The authors declare that they have no conflicts of interest

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Introduction

Neuromuscular junction (NMJ) transmission is mediated by nerve terminal (preterminal or presynapsis) release of acetylcholine (ACh) quanta that bind to the ACh receptor (AChR) on the surface of muscle cells (postterminal or postsynapsis), activating voltage-gated sodium channels to trigger an action potential.(Slater, 2008) This physiological mechanism is profoundly altered in such diseases as congenital myasthenic syndromes (CMS) and anti-MuSK myasthenia gravis. The recognized clinical improvements to NMJ transmission in response to treatment with selective β 2-adrenergic receptor (β 2-AR) agonists like salbutamol (a.k.a. albuterol)(Clausen et al., 2018; McMacken et al., 2018; Rodríguez Cruz et al., 2018) have been attributed to postsynaptic modulation,(Lynch and Ryall, 2008) but a potential presynaptic action has not been fully explored. This study was designed to elucidate how β 2-AR agonists regulate synaptic vesicle release at the nerve terminal and how sympathetic neurotransmitters regulate motor axon function within the complex structure of the distal spinal nerve.

Increasing evidence supports the concept that skeletal muscle sympathetic innervation plays a role in NMJ stability and motor innervation. Innervation of the skeletal muscle by both motor and sympathetic axons has been established, (Boeke, 1909a, b; Boeke, 1913) sparking interest in determining how the sympathetic nervous system (SNS) and its neurotransmitters (noradrenaline, ATP, and neuropeptide Y) affect skeletal muscle physiology. Research on the association between progressive muscle atrophy and sympathetic paralysis of voluntary muscles identified “functional synapses” between preganglionic sympathetic axons and skeletal muscle. (Ackerknecht, 1974; Hunter, 1925; Langley and Anderson, 1904; Wigston and Sanes, 1985) Sympathetic axons reach the muscle fiber (Khan et al., 2016) via Remak fibers traveling through the peripheral nervous system (Griffin and Thompson, 2008) and surrounding blood vessels.(Barker and Saito, 1981; Eichmann and Brunet, 2014) This finding prompted our interest in examining the influence of the SNS on neuromuscular transmission. Recently, we demonstrated that the SNS regulates the organization and function of NMJ transmission by maintaining optimal G-protein α_{i2} expression and preventing any increase in *Hdac4*, *myogenin*, *MuRF1*, and miR-206, while SNS ablation leads to upregulation of *MuRF1*, muscle atrophy, and downregulation of postsynaptic AChR. (Rodrigues et al., 2018) These results indicate crosstalk between sympathetic and motor axons at the spinal nerve.

If sympathetic nerves innervate the skeletal muscle, SM stimulate it by activating β 2-AR. (Lynch and Ryall, 2008) Noradrenaline (NA), the main neurotransmitter of ganglionic sympathetic neurons, modulates neuromuscular transmission,(Kuba, 1970) but its targets remain ill-defined. We and others are investigating how postganglionic sympathetic axons and their neurotransmitter(s) affect myofiber composition and function. SM were found to reestablish the compound muscle action potential disrupted by SNS ablation.(Khan et al., 2014) We demonstrated that regional surgical removal or systemic chemical ablation of mouse hindlimb SNS, which causes dramatic decrease in muscle NA,(Silveira et al., 2014) reduced release of synaptic vesicles.(Rodrigues et al., 2018) These findings support a novel presynaptic mechanism based on crosstalk between the motor and sympathetic nervous systems at the NMJ. Since these studies support the concept that the SNS neurotransmitter

NA modulates motor axon terminal ACh quantum release, here, we examined whether and how β 2-AR agonists modulate NMJ presynaptic vesicle release in response to acute ex-vivo or chronic repetitive in vivo administration. This study focused on the role of extracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]$), TRPV1, and P/Q- and N-type voltage-activated Ca^{2+} channels in synaptic vesicle release evoked by β 2-AR agonists at the neuromuscular junction in young adult mice as a means to examine how the SNS influences NMJ transmission.

The World Anti-Doping Agency prohibits athletes from consuming the two β 2-AR agonists used in this study, salbutamol and clenbuterol, either during competition or at all times due to their reported benefits to endurance.(Decorte et al., 2013) However, SM seem to have a place in treating neuromuscular transmission defects.(Rodríguez Cruz et al., 2018) Despite these reported potential benefits, conflicting effects of salbutamol on maximal inspiratory mouth pressure have been recorded in human respiratory muscles.(Javaheri et al., 1988), (Martineau et al., 1992) Ex-vivo recordings showed that salbutamol enhances force contraction in rat diaphragm after systemic in-vivo administration or ex-vivo application to diaphragm strips;(Van der Heijden et al., 1996) this effect might be mediated by membrane potential hyperpolarization.(Clausen and Flatman, 1980; Whittaker and Cardwell, 1981)

Elucidating the molecular pathway by which β 2-AR agonists regulate synaptic vesicle release at the motor axon terminal will transform our understanding of neuromuscular synaptic transmission and may lead to better, safer SM to treat related debilitating diseases.

Materials and Methods

Mice and nerve-muscle preparation

Young (2-3-month-old) male and female C57BL/6 mice were obtained from the National Institute on Aging (NIA) and housed in the pathogen-free Animal Research Program of the Wake Forest School of Medicine (WFSM) at 20-23°C with a 12:12-hour dark-light cycle. They were all fed chow ad libitum and had continuous access to drinking water. All experimental procedures were conducted in compliance with National Institutes of Health laboratory animal care guidelines. We made every effort to minimize suffering. Protocol A15-219 was approved by the WFSM Institutional Animal Care and Use Committee for this study. After euthanasia by cervical dislocation, the lumbricalis muscle was dissected with its plantar nerve attached, and fat, blood vessels, and gross connective tissue were removed using fine surgical tools under a stereomicroscope.

Mouse treatment

Five days per week for 4 consecutive weeks, 7 mice were injected subcutaneously with PBS, and 8 mice with 20 μ g/kg salbutamol diluted in saline. This treatment schedule was used to induce potentiation of rat diaphragm muscle contractility in a previous study.(Van der Heijden et al., 1996) Mice from both treatment groups were sacrificed within 24 hours of the last injection for ex-vivo recordings. No mouse exhibited gross pathology or changes in motor activity or behavior with treatment.

Electrophysiological recordings

We conducted functional recordings in a plantar nerve/lumbricalis muscle preparation as reported.(Rodrigues et al., 2018) Several advantages make it suitable for prolonged electrophysiological recordings: ~1.5cm segments of the plantar nerve can be recognized and dissected free from surrounding tissues; the lumbricalis muscle consists of a few myofiber layers arranged in parallel; and the NMJs are organized on a band aligned half-way between tendons. The preparations were incubated for 30 minutes in μ -conotoxin GIIIB (Alomone Labs, Jerusalem, Israel) to a final concentration of 1 μ M to prevent muscle contraction (see below). The NMJ transmission was then recorded intracellularly in oxygenated normal mammalian Ringer's solution (in mM, 136.8 NaCl, 5 KCl, 1 MgCl₂, 15 NaHCO₃, 1 Na₂HPO₄, 11 D-glucose, 2.0 CaCl₂, pH 7.4) or low Ca²⁺/high Mg²⁺ solution (in mM, 136.8 NaCl, 5 KCl, 4 MgSO₄, 15 NaHCO₃, 1 Na₂HPO₄, 11 D-glucose, 0.5 CaCl₂, pH 7.4) using a TEV-200A amplifier (Dagan, Minneapolis, MN) and DigiData 1322A and pClamp10.5 software (Molecular Devices, San José, CA). The intracellular electrodes (~40m Ω) were filled with 2M K-citrate and 10 mM K-chloride and mounted on the stage of a MP-285 micromanipulator (Sutter, Novato, CA). To visualize preparation, we used an upright, fixed-stage Axioscope FS microscope (Zeiss GmbH, Jena, Germany) with a 10 or 20X, water-immersion FLUAR objective mounted on a microscope translator, which enabled us to measure spontaneous miniature end-plate potential (MEPP) amplitude and the distance between the microelectrode and the NMJ.

End-plate potentials (EPPs) were elicited by electrical stimulation at increasing frequencies from 2 to 150 Hz, while MEPPs were baseline corrected, and their amplitude, frequency, time-to-peak, half-decay time, and duration were retrieved by setting basic selection criteria to conduct peak selection. We measured the ratio between the last and first EPP amplitudes of the run to calculate EPP facilitation or depression. EPP quantal content was calculated by dividing its mean amplitude by the mean amplitude of the MEPPs as described.(Sugiura et al., 2011) For data analyses, we used pClamp10.5 software and macros recorded in Excel.

Drugs and reagents

Salbutamol (α -[(*tert*-butylamino)methyl]-4-hydroxy-*m*-xylene- α,α' -diol or albuterol; Sigma, cat. S8260) was used for *in-vivo* and *ex-vivo* experiments. For *ex-vivo* experiments, it was dissolved in Ringer solution to a final concentration of 0.06 μ M or 20 μ g/l.

Clenbuterol (4-amino- α -(*t*-butylaminomethyl)-3,5-dichlorobenzyl alcohol hydrochloride; Sigma, cat. C5423) was diluted in Ringer solution and used exclusively in *ex-vivo* experiments at a final concentration of 1 μ M. Salbutamol and clenbuterol concentrations used in this study are within those reported in the literature for the treatment of asthmatic patients. (Jacobson et al., 2003; Yamamoto et al., 1985) **ω -conotoxin GIIIB** (a.k.a. myotoxin II, geographutoxin II, GTx-II; Alomone, cat. C270), a selective blocker of skeletal muscle Nav1.4 channels, was used at a concentration of 1 μ M. **ω -conotoxin GVIA** (a.k.a. SNX-124; Alomone, cat. C-300), which blocks N-type Ca²⁺ channels, was used at a final concentration of 3 μ M. **ω -agatoxin IVA** (a.k.a. ω -agatoxin-Aa4a, ω -AGTX-Aa4a, ω -aga-IVA, ω -agatoxin-4a; Alomone, cat. STA-500), which blocks P/Q-type Ca_v channels, was used at a concentration of 0.2 μ M. **Arvanil** (N-arachidonoyl-vanillyl-amine; Sigma, cat. SML0520), which activates TRPV1, was used at a final concentration of 0.5 μ M.

Statistical Analysis

For statistical analysis we used Sigma Plot, version 12.5 (Systat Software, Inc., San José, CA) and Microsoft Excel software. All data were expressed as mean $\pm$ S.E.M. Student's t-test was used to compare two groups, and analysis of variance (ANOVA) followed by Bonferroni's *post hoc* analysis to compare three or more.

Results

Acute and chronic salbutamol administration increases MEPP frequency and EPP quantal content

First, we tested whether MEPP and EPP properties differed between peroneal nerve/lumbricalis muscle preparations acutely exposed to the β_2 -AR agonist salbutamol (0.06 μ M) or Ringer solution alone (henceforth, *control*) for 2-4 hours. The statistical analysis shows that, compared to control (125 fibers from 21 mice), salbutamol (25 fibers from 7 mice) significantly increased MEPP frequency, time to peak, half-decay time, and duration. Examining EPPs evoked by electrical stimulation of the peroneal nerve, we found that salbutamol increased the amplitude and quantal content but decreased the interval between the stimulation pulse and EPP generation (Table 1).

To determine whether repeated systemic administration of salbutamol 5 days per week for 4 weeks would have effects similar to those recorded in acute and *ex-vivo* experiments, we sacrificed the mouse within 24 hours of the last injection and measured spontaneous and evoked synaptic potentials. Figure 1 shows a 6-second fragment of a continuous 5-minute recording of spontaneous ACh release from a mouse injected with saline (*chronic control*) (**a**) and a mouse treated with salbutamol (*chronic salbutamol*) (**b**), both in Ringer solution. MEPP frequency, but not amplitude, increased, and differences in MEPP frequency were greater (~80%) than those recorded in the *ex-vivo* preparation acutely exposed to salbutamol (~30%). The effect on MEPP kinetics, time to peak, and half-decay time were less obvious (Table 1). Chronic salbutamol administration significantly increased EPP amplitude, time to peak, half-decay time, and quantal content but decreased latency compared to the chronic control. Figure 1c shows that EPP amplitude was greater in salbutamol than control in both conditions, followed the stimulation pulses at frequencies ranging from 2 to 150Hz. Changes in EPP properties evoked by acute or chronic salbutamol administration did not differ significantly (Table 1). These data indicate that both acute and chronic salbutamol administration facilitate NMJ transmission predominantly at the axon terminal.

Like salbutamol, clenbuterol increases MEPP frequency and EPP quantal content

To define whether the changes in MEPP and EPP properties reported above could be induced only by salbutamol or by other β_2 -AR agonists with different chemical structures, we exposed the *ex-vivo* peroneal nerve/lumbricalis muscle preparation to 1 μ M clenbuterol. Table 2 summarizes MEPP and EPP properties in the treated conditions (n = 27 fibers, 4 mice). Like salbutamol, clenbuterol increased MEPP frequency but decreased half-decay time compared to control. It also increased EPP amplitude and quantal content but reduced their time to peak and half-decay time. Note that, compared to the control recordings (2Hz: -2.35 ± 0.43 , 10 Hz: -13.17 ± 2.25 , 25 Hz: -17.86 ± 2.85 , 30 Hz: -20.55 ± 2.76 , 50 Hz:

–25.14±3.12, 100Hz: –34.31± 4.77, and 150 Hz: –37.34±5.63), the last peak/first peak EPP amplitude ratio shows a more pronounced decline (Table 2) and is statistically significant in the 30-150 Hz range.

Reducing extracellular Ca²⁺ prevents clenbuterol-mediated enhancement of neuromuscular transmission

To examine the role of extracellular Ca²⁺ in the increased synaptic transmission seen with clenbuterol, we used a modified Ringer solution in which Ca²⁺ concentration was reduced to 0.5 mM, and Mg²⁺ concentration increased to 4 mM, as described in *Methods*. Figure 2 shows a continuous 6-second MEPP recording in this low Ca²⁺/high Mg²⁺ solution (a) and with the addition of 1μM clenbuterol (b). Compared to control, MEPP amplitude, frequency, and time to peak were significantly lower in the low Ca²⁺/high Mg²⁺ solution (n = 4 fibers, 1 mouse) (Table 3). Strikingly, reducing external Ca²⁺ prevented clenbuterol (n = 7 fibers, 1 mouse) from affecting MEPPs and EPPs (Fig. 2, Table 3). Figure 2c shows the stimulation artefacts, occasionally followed by EPPs of varying amplitude, in low Ca²⁺/high Mg²⁺ and Ca²⁺/high Mg²⁺ plus clenbuterol solutions. The absence of EPPs or their varying amplitude prevented us from analyzing the last peak/first peak ratio reliably. Clearly, clenbuterol had no effect on NMJ transmission, and statistical differences in MEPP and EPP properties recorded in the two treatments were not significant. These results indicate that β2-AR agonists' action on NMJ transmission requires a physiological [Ca²⁺] range.

P/Q-type Ca²⁺ channel blockade prevents clenbuterol-mediated enhancement of neuromuscular transmission

Incubating the ex-vivo preparation in 0.2 μM ω-agatoxin IVA, a P/Q-type Ca_v channel blocker, for 15-20 min (n = 9 fibers per group, 2 mice) did not significantly modify MEPP properties (Fig. 3a-b, Table 4). EPP amplitude was much lower; half-decay time was slower; and their quantal content was strongly reduced to approximately one third of that recorded under control conditions (Table 1). Like clenbuterol alone (Table 2), clenbuterol with ω-agatoxin IVA (n = 8 fibers, 2 mice) increased MEPP frequency and half-decay time and duration but had no significant effect on EPP amplitude or quantal content (Fig. 3c, Table 4). These results indicate that ω-agatoxin IVA does not affect spontaneous synaptic potentials but significantly ablates clenbuterol's effects on evoked synaptic vesicle release at the motor axon terminal since EPP amplitude in the presence of clenbuterol plus ω-agatoxin IVA was less than 50 percent of that recorded in clenbuterol alone, and the toxin suppressed increases in clenbuterol-induced EPP quantal content.

Compared to control, ω-agatoxin IVA evoked a significant decline in EPP amplitude in response to repetitive nerve stimulation at 2-50Hz, which persisted after adding ω-agatoxin IVA plus clenbuterol. These data, together with the strong reduction in EPPs amplitude evoked by the toxin, lack of improvement in the LP/FP EPPs ratio, and non-significant increased EPP amplitude in the presence of the toxin plus clenbuterol suggest that β2-AR agonists use the P/Q-type Ca_v channel to enhance neuromuscular transmission.

N-type Ca_v channel blockade prevents clenbuterol-evoked decline in EPP amplitude in response to repetitive nerve stimulation

To determine whether β_2 -AR agonists influence neuromuscular transmission through another channel than P/Q-type Ca^{2+} , we studied the effects of clenbuterol in the presence of the N-type Ca_v channel blocker ω -conotoxin GIVA. Figure 4 shows MEPP recordings in the ex-vivo preparation incubated for 15-20 min in 3 μM ω -conotoxin GIVA ($n = 10$ -12 fibers per group, 2 mice) (**a**) or 3 μM ω -conotoxin GIVA plus 1 μM clenbuterol (**b**). The toxin significantly decreased MEPP frequency and prolonged half-decay time compared to the control or clenbuterol, while ω -conotoxin GIVA plus 1 μM clenbuterol lowered MEPP frequency compared to clenbuterol alone but not ω -conotoxin GIVA alone (Table 5). EPP half-decay time was slower in ω -conotoxin GIVA compared to control, and time to peak and half-decay time were slower in the presence of the toxin compared to clenbuterol (Table 2). Compared to ω -conotoxin GIVA alone, the addition of clenbuterol increased EPP amplitude and quantal content. EPPs recorded in 3 μM ω -conotoxin GIVA or 3 μM ω -conotoxin GIVA plus 1 μM clenbuterol followed the stimulation frequency from 2 to 150 Hz without fail, and no major differences in amplitude or kinetics were observed (Fig. 4c). Surprisingly, ω -conotoxin GIVA prevented decreases in LP/FP EPP amplitude in response to repetitive nerve stimulation at frequencies higher than 2 Hz, and adding clenbuterol to the bath solution did not significantly modify these effects (Table 5). The data indicate that β_2 -AR agonists do not use N-type Ca^{2+} channels to enhance neuromuscular transmission.

TRPV1 channel mediates Ca^{2+} conductance at the motor axon terminal to modulate synaptic vesicle release

NMJ transmission in the presence of arvanil (N-arachidonoyl-vanillyl-amine), a vanilloid TRPV1 (VR1) agonist, (Di Marzo et al., 2002) or arvanil plus clenbuterol were recorded in both muscle fiber sets and sequentially in the same fiber. Compared to control, arvanil increased MEPP frequency almost 85% and modestly decreased its half-decay time but did not change quantal content or EPP amplitude measured in two separate sets of fibers (arvanil: $n = 18$ fibers from 3 mice; $n = 19$ fibers from 3 mice). However, it induced a significant decline in EPP amplitude in response to repetitive stimulation at all frequencies. Notably, arvanil prevented clenbuterol-evoked increases in MEPP frequency, EPP amplitude, and quantal content (Table 2) recorded in fiber sets or the same fiber ($n = 9$ fibers from 3 mice) (Table 6). We propose that the TRPV channel mediates Ca^{2+} conductance at the motor axon terminal to modulate synaptic vesicle release at rest.

Putative presynaptic β -ARs regulate Ca^{2+} influx at rest and during neuron depolarization

Next, we examined the role of the β_1/β_2 -AR blocker propranolol in NMJ transmission. Data included in Table 7 show, that compared to control, 1 μM propranolol induced a slower, more prolonged MEPP half-decay time, consistent with postsynaptic activity ($n = 26$ fibers from 4 mice). Also, clenbuterol exerted no presynaptic or postsynaptic action in the presence of propranolol ($n = 27$ fibers from 4 mice). Compared to control, propranolol or propranolol plus clenbuterol had no effect on the generation of repetitive EPPs or the magnitude of their amplitude decline over time. These data show that propranolol acts on the pre- and

postterminal NMJ, precluding any effects of clenbuterol on putative presynaptic and reported postsynaptic β -adrenoreceptors. (Khan et al., 2016; Lynch and Ryall, 2008)

Discussion

Sympathomimetic agents enhance neuromuscular transmission

Studies of the effects of SM drugs on neuromuscular transmission have mainly focused on the postsynaptic membrane β 2-AR, neglecting their interaction with presynaptic molecular targets. The complexity of the SNS terminal trajectory, the number of neurotransmitters it releases, and the number of potential targets may explain this oversight. Note that β 1- and β 2-AR antagonists, such as propranolol, cause muscle weakness, (Howard, 1990) while β 2-AR agonists enhance muscle strength in diseases characterized by congenital impaired neurotransmission. (Lorenzoni et al., 2013) β 2-AR expression predominates in skeletal muscle, (Lynch and Ryall, 2008) mediates SNS action on the myofiber, and its agonists correct the size of AChR clusters at the postterminal and the amplitude of the compound muscle action potential after sympathetic denervation. (Khan et al., 2016) However, chronic administration of SM has serious side effects that preclude their use as a therapy for muscle-wasting conditions. (Baker et al., 2006) Diseases characterized by altered neuromuscular transmission benefit from their therapeutic use, (Ghazanfari et al., 2014; Sadeh et al., 2011) but their mechanism of action remains unclear. We sought to clarify the role of SM in NMJ pre- and postsynapsis in the hindlimb lumbricalis muscle of adult mice.

NA, the main neurotransmitter of ganglionic sympathetic neurons, and adrenaline, the catecholamine secreted by the adrenal medulla, modulate neuromuscular transmission, but their effects and sites of action differ. NA acts at the nerve ending, increasing transmitter release, while adrenaline has both presynaptic and postsynaptic actions. (Kuba, 1970) SM drugs produce similar effects by acting on different targets at the NMJ. Our data show that, like NA, salbutamol and clenbuterol increase MEPP frequency but not amplitude and increase EPP amplitude and half-decay time, indicating that SNS neurotransmitter and SM agents share a mechanism of action at the NMJ pre- and postterminal. We have no information about NA's effects on EPP quantal content in mammalian species. A report in an amphibian shows that NA facilitates synaptic transmission by making quanta release more synchronous. (Bukcharaeva et al., 1999) Here, we examined the molecular targets of SM agents at the terminal axons. The fact that clenbuterol exacerbated the decline of the LP/FP EPP amplitude ratio recorded in control conditions is consistent with its role in the reported decrease in evoked Ca^{2+} transient amplitude, Ca^{2+} spark amplitude and frequency, and sarcoplasmic reticulum Ca^{2+} load in EDL muscle. (Sirvent et al., 2014).

In contrast to short treatments, long-term use of SM agents results in loss of specificity by acting on cells other than those involved in NMJ transmission (e.g. cardiac myocytes), and muscle hypertrophy, a PKA- and Akt-dependent process evoked by direct effect of catecholamines on muscle β 2-AR. Whether SM-evoked phosphorylation promotes β 1- and β 2- adrenoreceptors desensitization has been reported, (Hausdorff et al., 1990; Lefkowitz et al., 1990) but it did not seem to have a significant effect during the period of drug administration in our experiments. Our study concludes that both acute and chronic

salbutamol administration facilitate NMJ transmission predominantly at the motor and sympathetic axons.

Complex interaction of SM with motor and sympathetic axons. Reducing extracellular Ca^{2+} concentration suppresses SM-evoked synaptic vesicle release

Our data show that drastically reducing extracellular Ca^{2+} significantly prevents clenbuterol from enhancing NMJ transmission. Although the dependence of synaptic vesicle release on external Ca^{2+} is well established in mammalian species, (Losavio and Muchnik, 1997) whether Ca^{2+} influx at the nerve terminal mediates sympathomimetic action on NMJ transmission is unknown. That low Ca^{2+} prevents clenbuterol-evoked increases in MEPP frequency and EPP quantal content implies that Ca^{2+} influx takes place at the motor axon terminal in resting conditions. Whether adrenoreceptors are present in *terminal* Schwann cells in vivo is unknown, but they have been reported in adult rat and guinea pig Schwann cells in vitro. (Fink et al., 1999; Yasuda et al., 1988) Precisely locating adrenoreceptors will help to define how Schwann cells affect Ca^{2+} signaling at the NMJ. (Arbour et al., 2017) Although a fine equilibrium between motor axon and perisynaptic Schwann cells is required for Ca^{2+} homeostasis, (Castonguay and Robitaille, 2001; Ko and Robitaille, 2015) the mechanism of Ca^{2+} conductance at rest is poorly understood.

Wong et al. reported that a transient receptor potential channel of the vanilloid subtype (TRPV), *Inactive* (*Iav*), regulates presynaptic resting Ca^{2+} concentration. (Wong et al., 2014) If it mediates Ca^{2+} conductance at the motor axon terminal in mammalian species and participates in the regulation of synaptic vesicle release, it could explain how extracellular Ca^{2+} regulates intracellular Ca^{2+} in resting conditions. We propose that the TRPV1 channel mediates Ca^{2+} conductance at the motor axon terminal to modulate synaptic vesicle release. This hypothesis would explain how extracellular Ca^{2+} regulates intracellular Ca^{2+} and how, at rest, low extracellular Ca^{2+} prevents clenbuterol-evoked increases in MEPP frequency and EPP quantal content. These experiments indicate that, at rest, the TRPV1 channel mediates Ca^{2+} conductance and clenbuterol-evoked synaptic vesicle release at the motor axon, consistent with a prior report showing that TRPV1 activation elicits a Ca^{2+} signal in brain tumor cells. (Stock et al., 2012) Sympathomimetic effects on the presynapse are prevented because the TRPV1 channels are fully activated. By increasing resting $[\text{Ca}^{2+}]$, arvanil may prevent SM-mediated increase in EPP amplitude and quantal content.

Voltage-gated Ca^{2+} channels mediate SM-evoked potentiation of neuromuscular transmission in response to nerve stimulation

As expected, ω -agatoxin IVA had no effect on MEPP properties since the activation of P/Q-type Ca^{2+} channels requires axolemma depolarization; in contrast, clenbuterol increased MEPP frequency in the presence of the toxin. These results support the concept that P/Q-type Ca^{2+} channels do not play a role in spontaneous vesicle release. The observed increase in MEPP halfdecay time and duration in the presence of salbutamol or ω -agatoxin IVA plus clenbuterol and decrease in half-decay time in the presence of clenbuterol alone indicates that the toxin does not have a postsynaptic effect, and clenbuterol and salbutamol modify AChR gating properties differentially, probably by modifying the time the channel remains in the open configuration.

P/Q-type Ca^{2+} channels are involved in ACh release from adult mammalian motor nerve terminals, and the N-type is responsible for transmitter release from peripheral autonomic nerve terminals.(Katz et al., 1996; Katz et al., 2015; Molina-Campos et al., 2015) Treating the peroneal nerve/lumbricalis muscle preparation with ω -agatoxin IVA, a P/Q-type Ca^{2+} channel blocker, sharply reduced the LP/FP EPP amplitude ratio at various nerve stimulation frequencies (2-50Hz). This effect is similar to that recorded in Lambert-Eaton Myasthenic Syndrome (LEMS),(Lambert and Elmqvist, 1971) which involves antibodies against P/Q-type Ca^{2+} channels.(Pinto et al., 1998) However, although decremental EPPs have been reported, their amplitude increased at frequencies equal to, or higher than, 10Hz, indicating that LEMS antibodies and ω -agatoxin IVA act on different P/Q-type Ca^{2+} channel residues, ω -agatoxin IVA evoked progressive decline in EPP amplitude in response to nerve stimulation and prevented any further effect of clenbuterol, indicating that sustained EPP generation requires Ca^{2+} influx through P/Q-type Ca_v channels at the nerve terminal.

Compared to control, blocking N-type Ca^{2+} channels with ω -conotoxin GIVA decreased spontaneous acetylcholine release ~50 percent and precluded clenbuterol-induced potentiation of MEPP frequency at rest. This toxin did not modify EPP amplitude or quantal content, consistent with previous reports in the adult mouse.(Giovannini et al., 2002; Katz et al., 1996) Note that clenbuterol increased EPP amplitude and quantal content in the presence of ω -conotoxin GIVA, which indicates that it does not require Ca^{2+} permeation through N-type Ca^{2+} channels to enhance postsynaptic potentials. Surprisingly, compared to control, ω -conotoxin GIVA prevented EPP amplitudes from declining in response to repetitive nerve stimulation, which seems paradoxical. A previous study shows that N-type Ca^{2+} channels are located at terminals that are immunoreactive to substance P,(Westenbroek et al., 1998) indicating that they are expressed in sympathetic axons, and consistent with reports that they are responsible for transmitter release from peripheral autonomic nerve terminals.(Katz et al., 1996; Katz et al., 2015; Molina-Campos et al., 2015) Since N-type Ca^{2+} channels play an essential role in evoked release of NA from sympathetic neurons,(Hirning et al., 1988) synaptic vesicle recycling depends on Ca^{2+} , (Neher and Marty, 1982; Schneggenburger et al., 2012; Yamashita et al., 2010) and sympathetic axons run along motor axons in spinal nerves, we propose that ω -conotoxin GIVA blockade of the neurotransmitter NA from sympathetic axons counters the time- and frequency-dependent decline in EPP amplitude.

In summary, blocking sympathetic neuron N-type Ca^{2+} channels decreases NA release, improves motor terminal Ca^{2+} handling,(Sirvent et al., 2014) and sustains EPP amplitudes at increasing frequencies by allowing Ca^{2+} permeation through P/Q-type Ca^{2+} channels. Figure 5 proposes a model to explain the relationship between adrenergic receptors and Ca^{2+} channels at the NMJ.

Two voltage-gated Ca^{2+} channels, R- and L-type, were not examined in this study. Transmission at the NMJ relies on P/Q-type channels, but when they are deleted, it must depend on both N- and R-type Ca^{2+} channels.(Urbano et al., 2003) Since most of our experiments were performed in the ex-vivo preparation, and R-type Ca^{2+} channels are mainly located at the motor neuron soma,(Plant et al., 1998) we did not expect them to participate in sympathomimetic effects on NMJ transmission. L-type Ca^{2+} channels are related to synaptic vesicle endocytosis,(Perissinotti et al., 2008) but they are difficult to

examine due to their partial inhibition by dihydropyridines, (Xu and Lipscombe, 2001) and their blockade does not significantly reduce the quantal content of Ca^{2+} -buffered NMJs. (Urbano and Uchitel, 1999)

Two important questions demand further analysis. First, how do these findings clarify the relationship between motor and sympathetic axons at the NMJ, and second, can sympathomimetic agents be used to treat diseases characterized by impaired neuromuscular transmission? The pharmacological manipulation of salbutamol and clenbuterol in this study shows that the SNS regulates NMJ function by enhancing spontaneous and evoked synaptic vesicle release via probably TRPV1 channels and P/Q-type Ca^{2+} channels, respectively. We propose that a fine-tuned equilibrium between P/Q- and N-type Ca^{2+} channel activation helps to sustain EPP amplitude, quantal content, and action potential generation. However, sympathomimetic effects on repetitive EPP generation raises questions about their efficacy in physiological and probably pathological conditions. (Crivelli et al., 2011)

Conclusions

This study addressed the concept that SM act on axon terminal molecular targets involved in Ca^{2+} homeostasis to regulate motor neuron/skeletal muscle synaptic transmission. Their effect is similar to that reported for NA, which targets the motor neuron terminal, but remains poorly understood. By elucidating the mechanism of action of clenbuterol and salbutamol, we now know that the SNS influences skeletal muscle physiology by acting, not only at the NMJ postterminal, but the motor neuron terminal, in P/Q- Ca^{2+} channels and TRPV1 channels, and the sympathetic axon, in N-type Ca^{2+} channels. In addition, prolonged NMJ activation depends on their effect on the balance between sympathetic and motor axon terminal activity.

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Abbreviations

(NMJ)	Neuromuscular junction
(AChR)	ACh receptor
(CMS)	congenital myasthenic syndrome
(β 2-AR)	β 2-adrenergic receptor
(MuSK)	muscle-specific kinase
(SNS)	sympathetic nervous system
(NA)	noradrenaline

(MEPP)	miniature end-plate potential
(EPP)	end-plate potential
(EDL)	extensor digitorum longus
(ANOVA)	analysis of variance
(TRPV1)	transient receptor potential channels of the vanilloid subtype-1
(LP/FP EPP)	last pulse/first pulse ratio
(AP)	action potential
(SM)	sympathomimetics
(G)	G-protein
(tSC)	terminal Schwann cell
(VGCC)	voltage-gated calcium channel

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Highlights

- Sympathomimetic (SM) agents regulate neuromuscular junction transmission.
- Clenbuterol and salbutamol enhance spontaneous and evoked synaptic vesicle release.
- $[Ca^{2+}]$, P/Q-, and N-type Ca^{2+} channels and TRPV1 mediate the action of SM agents.
- This study supports endogenous sympathetic regulation of the motor neuron terminal.

Figure 1. Salbutamol increases MEPP frequency and EPP quantal content

a-b. A continuous, 6-second trace of spontaneous ACh release in Ringer solution, **a.** Muscle fiber from a mouse injected with saline. **b.** Muscle fiber from a mouse injected with salbutamol. An increase in MEPP frequency but not amplitude is obvious. **c.** EPP recordings following stimulation pulses at frequencies ranging from 2 to 150 Hz. Compared to saline-, salbutamol-injected mice show increased EPP amplitude. The thin and thick arrows show the stimulation artefact and EPP, respectively.

Figure 2. Reduced extracellular Ca^{2+} prevents clenbuterol-enhanced neuromuscular transmission.

Continuous, 6-second MEPP recordings in low Ca^{2+} /high Mg^{2+} solution (a) and low Ca^{2+} /high Mg^{2+} solution plus 1 μM clenbuterol (b). Compared to control, MEPP amplitude, frequency, and time to peak were significantly reduced by the low Ca^{2+} /high Mg^{2+} solution and not significantly modified by adding clenbuterol (Table 3). c. The stimulation artefact was irregularly followed by EPPs of varying amplitude in low Ca^{2+} /high Mg^{2+} and Ca^{2+} /high Mg^{2+} plus clenbuterol solutions. Failures in EPP generation are obvious. The thin and thick arrows show the stimulation artefact and EPP, respectively.

Figure 3. Blocking P/Q-type Ca_v channels prevents clenbuterol-enhanced neuromuscular transmission.

Continuous MEPP recordings in 0.2 μ M ω -agatoxin IVA (a) and 0.2 μ M ω -agatoxin IVA plus clenbuterol (b). The amplitudes of EPPs recorded in ω -agatoxin IVA were sharply decreased compared to control. ω -agatoxin IVA prevented clenbuterol-evoked increases in EPP amplitude at all stimulation frequencies (c). The thin and thick arrows show the stimulation artefact and EPP, respectively.

Figure 4. Blocking N-type Ca_v channels with ω -conotoxin GIVA prevents clenbuterol-mediated decline in EPP amplitude in response to repetitive nerve stimulation.

MEPP recordings in 3 μM ω -conotoxin GIVA (a) or 3 μM ω -conotoxin GIVA plus 1 μM clenbuterol (b). EPPs recorded in 3 μM ω -conotoxin GIVA or 3 μM ω -conotoxin GIVA plus 1 μM clenbuterol followed the stimulation frequency, and no major differences in amplitude or kinetics were observed (c). The thin and thick arrows show the stimulation artefact and EPP, respectively.

Figure 5. A model to explain the relationship between adrenergic receptors and Ca^{2+} channels at the NMJ

Depolarization of the sympathetic axon (AP: axon potential), leads to activation of the N-type Ca^{2+} channel, Ca^{2+} influx, and release of the neurotransmitter NA. NA effects on sympathetic, motor and muscle fiber are mimicked by clenbuterol and salbutamol (sympathomimetics, SM). SM act on sympathetic axon α_2 adrenergic receptor, which evokes Ca^{2+} channel inhibition mainly (G^*), and possibly exclusively, via $\text{G}(\text{o})/\text{G}(\text{i})$. Other rapid pertussis toxin (PTX)-insensitive G-protein pathways may involve NA/SM-independent, activating mechanisms ($\text{G}^\#$). (Mirotznik et al., 2000) The relative influence of these pathways would determine the amplitude and duration of increased NA concentration at the NMJ. The effects of NA/SM on the postterminal β_2 -AR has been extensively described. (Lynch and Ryall, 2008) Based on clenbuterol's lack of effect on MEPP frequency, EPP amplitude, and quantal content (QC) in the presence of propranolol (Table 7), we propose another potential target: the motor axon terminal. We also propose that Ca^{2+} influx through TRPV1 channels and P/Q-type Ca^{2+} channels ($\text{Ca}_v2.1$) determines the axoplasmic Ca^{2+} concentration at rest and during motor neuron depolarization, respectively. Since the channel agonist arvanil suppresses their effect (Table 6), SMs seem to modulate TRPV1 channels, while P/Q channel blockade ablates clenbuterol's effects on MEPP frequency and EPP quantal content (Table 4). Whether β_1 - and/or β_2 -AR are expressed presynaptically is uncertain. However, as proposed for the regulation of N-type Ca^{2+} channels, the balance between G-protein activation and inhibition may determine the levels

of P/Q-type Ca^{2+} channel activation and axoplasmic Ca^{2+} concentration, which ultimately affect ACh release and EPP quantal content. Whether ARs are present in *terminal* Schwann cells in vivo is unknown but they have been reported in adult rat and guinea pig Schwann cells in vitro.(Fink et al., 1999; Yasuda et al., 1988) Elucidating AR location is particularly important due to the role of these cells in Ca^{2+} signaling at the NMJ.(Arbour et al., 2017) Dashed lines indicate potential or untested pathways.

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Table 1

Acute and chronic salbutamol administration enhance neuromuscular transmission

MEPP	Control			Chronic Control			Acute Salbutamol			Chronic Salbutamol		
	Mean	SEM		Mean	SEM		Mean	SEM	p-value (*)	Mean	SEM	p-value (**)
Amplitude (mV)	1.403	± 0.022		1.482	± 0.089		1.561	± 0.045	0.178	1.421	± 0.028	0.511
Frequency (Hz)	0.812	± 0.036		0.794	± 0.069		1.082	± 0.056	<0.001	1.506	± 0.089	<0.001
Time to peak (ms)	0.807	± 0.029		0.885	± 0.103		1.046	± 0.059	<0.001	0.876	± 0.038	0.003
Half decay time (ms)	2.066	± 0.050		2.167	± 0.130		2.384	± 0.052	<0.001	2.025	± 0.043	<0.001
Duration (ms)	5.627	± 0.224		6.347	± 0.825		6.755	± 0.287	<0.001	5.938	± 0.196	0.111
EPP												
Amplitude (mV)	29.678	± 0.973		30.963	± 2.000		33.566	± 0.622	0.032	34.634	± 1.988	0.031
Latency (ms)	1.830	± 0.044		1.702	± 0.057		1.453	± 0.028	0.004	1.602	± 0.083	<0.001
Time to peak (ms)	2.466	± 0.066		2.175	± 0.079		2.428	± 0.043	0.765	2.446	± 0.072	<0.001
Half-decay time (ms)	4.935	± 0.215		4.960	± 0.224		5.576	± 0.112	0.371	5.432	± 1.146	0.039
Quantal content	21.055	± 0.573		20.866	± 0.947		24.295	± 0.558	<0.001	23.483	± 1.146	0.001

Compared to control (*), chronic control (**), or acute salbutamol (***). Statistically significant values are in bold.

Table 2**Clenbuterol Enhances Neuromuscular Junction Transmission**

MEPP	Clenbuterol		
	Mean	SEM	p-value (*)
Amplitude (mV)	1.410 ± 0.034		0.606
Frequency (Hz)	1.282 ± 0.176		0.005
Time to peak (ms)	0.818 ± 0.117		0.223
Half decay time (ms)	1.767 ± 0.092		0.010
Duration (ms)	4.929 ± 0.427		0.061
EPP			
	Mean	SEM	p-value (*)
Amplitude (mV)	32.547 ± 2.407		0.027
Latency (ms)	1.725 ± 0.138		0.946
Time to peak (ms)	2.054 ± 0.145		0.002
Half-decay time (ms)	3.306 ± 0.339		<0.001
Quantal content	23.037 ± 1.863		0.032
LP/FP - EPP 2 Hz (%)	-3.537 ± 1.399		0.621
LP/FP - EPP 10Hz (%)	-13.751 ± 1.461		0.918
LP/FP - EPP 25Hz (%)	-22.820 ± 3.101		0.069
LP/FP - EPP 30Hz (%)	-29.329 ± 3.297		0.004
LP/FP - EPP 50Hz (%)	-35.679 ± 2.409		0.002
LP/FP - EPP 100Hz (%)	-44.051 ± 3.344		0.024
LP/FP - EPP 150Hz (%)	-50.764 ± 3.634		0.024

LP/FP = Last peak/first peak EPP amplitude ratio.

Compared to control in Table 1 (*). Statistically significant values are in bold.

Table 3 Low Ca^{2+} /High Mg prevents clenbuterol-enhanced neuromuscular transmission

MEPP	Low Ca^{2+} /High Mg^{2+}				Low Ca^{2+} /High Mg^{2+} Clenbuterol			
	Mean	SEM	p-value (*)	p-value (**)	Mean	SEM	p-value (*)	p-value (***)
Amplitude (mV)	1.145	± 0.026	0.013	0.007	1.202	± 0.034	0.023	0.142
Frequency (Hz)	0.082	± 0.019	0.001	0.002	0.277	± 0.139	0.002	0.230
Time to peak (ms)	0.335	± 0.046	0.001	0.005	1.579	± 1.198	0.041	0.927
Half decay time (ms)	1.908	± 0.137	0.689	0.286	1.825	± 0.336	0.145	0.788
Duration (ms)	4.518	± 0.415	0.419	0.576	7.852	± 2.661	0.633	0.527
EPP	Mean	SEM	p-value (*)	p-value (**)	Mean	SEM	p-value (*)	p-value (***)
	Mean	SEM	p-value (*)	p-value (**)	Mean	SEM	p-value (*)	p-value (***)
Amplitude (mV)	4.831	± 0.141	<0.001	0.008	6.093	± 1.141	0.027	0.073
Latency (ms)	0.225	± 0.066	<0.001	<0.001	0.410	± 0.176	0.064	0.230
Time to peak (ms)	0.400	± 0.057	<0.001	0.007	0.740	± 0.269	0.086	0.109
Half-decay time (ms)	0.488	± 0.065	<0.001	0.008	1.220	± 0.647	0.141	0.073
Quantal content	4.220	± 0.050	<0.001	0.009	5.249	± 1.025	0.027	0.073

Compared to control in Table 1 (*), clenbuterol (*), or low Ca^{2+} /High Mg^{2+} (*), or low Ca^{2+} /High Mg^{2+} (*). Statistically significant values are in bold.

Table 4

ω -agatoxin IVA prevents clenbuterol-enhanced neuromuscular transmission

MEPP	ω -agatoxin IVA				ω -agatoxin IVA + clenbuterol			
	Mean	SEM	p-value (*)		Mean	SEM	p-value (**)	
Amplitude (mV)	1.131	± 0.147	0.104		1.385	± 0.080	0.248	
Frequency (Hz)	1.147	± 0.078	0.080		1.283	± 0.088	0.031	
Time to peak (ms)	0.881	± 0.281	0.873		1.447	± 0.343	0.109	
Half decay time (ms)	1.476	± 0.286	0.061		2.214	± 0.306	0.049	
Duration (ms)	4.786	± 0.847	0.803		7.558	± 0.741	0.013	
EPP	Mean	SEM	p-value (*)		Mean	SEM	p-value (**)	
	Mean	SEM	p-value (*)		Mean	SEM	p-value (**)	
Amplitude (mV)	7.982	± 2.074	<0.001		14.991	± 2.959	0.053	
Latency (ms)	1.972	± 0.099	0.226		2.925	± 0.369	0.011	
Time to peak (ms)	2.756	± 0.113	0.081		2.938	± 0.300	0.39	
Half-decay time (ms)	6.039	± 0.378	0.021		6.769	± 0.776	0.27	
Quantal content	6.898	± 0.898	<0.001		10.779	± 1.982	0.12	
LP/FP - EPP 2 Hz (%)	-20.9	± 7.348	0.003		-18.26	± 6.671	0.123	
LP/FP - EPP 10Hz (%)	-32.6	± 6.640	0.004		-39.42	± 7.126	0.297	
LP/FP - EPP 25Hz (%)	-45.1	± 6.330	<0.001		-48.30	± 6.944	0.085	
LP/FP - EPP 30Hz (%)	-39.5	± 7.767	0.012		-32.81	± 3.472	0.460	
LP/FP - EPP 50Hz (%)	-41.8	± 5.039	0.001		-59.12	± 7.460	0.736	
LP/FP - EPP 100Hz (%)	-41.7	± 5.979	0.233		-53.25	± 5.141	0.596	
LP/FP - EPP 150Hz (%)	-39.7	± 3.521	0.542		-35.77	± 6.137	0.082	

LP/FP = Last peak (LP)/First peak (FP) EPP amplitude ratio. Compared to control (*) or ω -Agatoxin IVA (**). Statistically significant values are in bold.

Table 5

ω -conotoxin GVIA prevents clenbuterol-enhanced neuromuscular transmission

MEPP	ω -conotoxin GVIA				ω -conotoxin GVIA+ clenbuterol			
	Mean	SEM	p-value (*)	p-value (**)	Mean	SEM	p-value (**)	p-value (***)
Amplitude (mV)	1.375 $\pm$ 0.070		0.896	0.596	1.282 $\pm$ 0.060		0.086	0.164
Frequency (Hz)	0.417 $\pm$ 0.085		0.002	<0.001	0.415 $\pm$ 0.093		0.003	0.494
Time to peak (ms)	0.725 $\pm$ 0.130		0.746	0.878	0.675 $\pm$ 0.162		0.175	0.409
Half decay time (ms)	1.727 $\pm$ 0.226		0.044	0.423	1.777 $\pm$ 0.233		0.078	0.441
Duration (ms)	5.199 $\pm$ 0.524		0.824	0.330	5.231 $\pm$ 0.831		0.38	0.549
EPP	Mean	SEM	p-value (*)	p-value (**)	Mean	SEM	p-value (**)	p-value (***)
	Mean	SEM	p-value (*)	p-value (**)	Mean	SEM	p-value (**)	p-value (***)
Amplitude (mV)	26.805 $\pm$ 4.079		0.585	0.112	39.236 $\pm$ 4.226		0.076	0.03
Latency (ms)	1.685 $\pm$ 0.174		0.096	0.436	1.577 $\pm$ 0.162		0.757	0.32
Time to peak (ms)	2.925 $\pm$ 0.283		0.069	0.005	2.632 $\pm$ 0.160		0.073	1
Half-decay time (ms)	6.325 $\pm$ 0.475		0.008	<0.001	5.241 $\pm$ 0.305		0.073	0.226
Quantal content	18.968 $\pm$ 2.534		0.438	0.117	31.671 $\pm$ 4.212		0.003	<0.001
LP/FP - EPP 2 Hz (%)	-3.185 $\pm$ 0.820		0.359	0.278	-2.312 $\pm$ 0.939		0.433	0.266
LP/FP - EPP 10Hz (%)	-0.259 $\pm$ 1.482		<0.001	<0.001	-2.669 $\pm$ 1.031		<0.001	0.467
LP/FP - EPP 25Hz (%)	-2.385 $\pm$ 1.684		<0.001	<0.001	-1.968 $\pm$ 0.871		<0.001	0.071
LP/FP - EPP 30Hz (%)	-1.200 $\pm$ 0.994		<0.001	<0.001	-5.684 $\pm$ 2.843		<0.001	0.733
LP/FP - EPP 50Hz (%)	-0.579 $\pm$ 1-234		<0.001	<0.001	-1.116 $\pm$ 1.313		<0.001	0.232
LP/FP - EPP 100Hz (%)	-5.780 $\pm$ 2.527		<0.001	<0.001	-0.481 $\pm$ 2.620		<0.001	0.052
LP/FP - EPP 150Hz (%)	-7.878 $\pm$ 2.683		<0.001	<0.001	-5.638 $\pm$ 3.698		<0.001	0.183

LP/FP = Last peak (LP)/first peak (FP) EPP amplitude ratio. Compared to control (*), clenbuterol (**), or ω -conotoxin GVIA (***). Statistically significant values are in bold.

Table 6

Arvanil prevents clenbuterol-enhanced neuromuscular transmission

MEPP	Arvanil			Arvanil + Clenbuterol			Arvanil, Same Fiber		Arvanil + Clenbuterol Same Fiber	
	Mean	SEM	p-value (*)	Mean	SEM	p-value (**)	Mean	SEM	Mean	p-value (***)
Amplitude (mV)	1.521	±0.070	0.811	1.342	±0.123	0.021	1.204	±0.031	1.196	±0.018 0.113
Frequency (Hz)	2.373	±0.399	<0.001	1.361	±0.217	0.028	1.988	±0.383	1.746	±0.268 0.611
Time to Peak (ms)	0.730	±0.061	0.163	0.640	±0.072	0.177	0.677	±0.030	0.622	±0.012 0.056
Half decay time (ms)	1.730	±0.091	0.015	1.549	±0.119	0.115	1.641	±0.066	1.530	±0.079 0.167
Duration (ms)	4.878	±0.315	0.200	4.180	±0.419	0.097	3.966	±0.190	3.730	±0.107 0.077
EPP	Mean	SEM	p-value (*)	Mean	SEM	p-value (**)	Mean	SEM	Mean	p-value (***)
	Mean	SEM	p-value (*)	Mean	SEM	p-value (**)	Mean	SEM	Mean	p-value (***)
Amplitude (mV)	34.826	±2.183	0.063	25.629	±1.942	0.003	31.945	±0.550	32.146	±0.335 0.382
Latency (ms)	1.584	±0.114	0.017	1.494	±0.140	0.174	1.260	±0.099	32.146	±0.335 0.120
Time to peak (ms)	2.402	±0.101	0.850	2.421	±0.101	0.625	2.280	±0.104	2.550	±0.151 0.090
Half-decay time (ms)	6.390	±1.080	0.082	5.068	±0.158	0.986	4.970	±0.161	5.060	±0.215 0.374
Quantal content	23.773	±0.965	0.079	22.583	±1.523	0.253	26.174	±0.356	27.086	±0.438 0.208
LP/FP-EPP 2 Hz (%)	-6.310	±1.226	<0.001	-5.652	±1.339	0.759	-5.772	±1.726	-6.652	±1.643 0.361
LP/FP-EPP 10 Hz (%)	-30.142	±0.763	<0.001	-36.593	±4.696	0.957				
LP/FP-EPP 25 Hz (%)	-42.042	±1.997	<0.001	-50.640	±5.185	0.504				
LP/FP-EPP 30 Hz (%)	-41.183	±2.450	<0.001	-51.212	±4.761	0.32				
LP/FP-EPP 50 Hz (%)	-43.658	±2.523	<0.001	-55.729	±5.610	0.551				
LP/FP-EPP 100 Hz (%)	-58.660	±1.903	<0.001	-57.172	±2.735	0.134				
LP/FP-EPP 150 Hz (%)	-64.919	±1.972	<0.001	-67.026	±2.957	0.786				

LP/FP = Last peak (LP)/first peak (FP) EPP amplitude ratio. Compared to control (*), or arvanil (**) in difference fibers, or arvanil (***) in the same fiber. Statistically significant values are in bold.

Table 7

Propranolol prevents clenbuterol-enhanced neuromuscular transmission

MEPP	Propranolol			Propranolol + Clenbuterol		
	Mean	SEM	p-value (*)	Mean	SEM	p-value (**)
Amplitude (mV)	1.371	± 0.050	0.804	1.376	± 0.031	0.686
Frequency (Hz)	0.825	± 0.170	0.813	0.739	± 0.067	0.494
Time to Peak (ms)	1.012	± 0.154	0.215	0.723	± 0.034	0.418
Half decay time (ms)	2.908	± 0.213	0.002	2.883	± 0.045	<0.001
Duration (ms)	8.850	± 1.164	0.004	7.964	± 0.210	0.013
EPP	Pro panolol			Propranolol + Clenbuterol		
	Mean	SEM	p-value (*)	Mean	SEM	p-value (**)
Amplitude (mV)	28.721	± 1.600	0.865	30.444	± 1.548	0.55
Latency (ms)	2.258	± 0.106	0.007	2.070	± 0.034	<0.001
Time to peak (ms)	2.650	± 0.175	0.320	2.343	± 0.050	0.898
Half-decay time (ms)	3.808	± 0.428	0.152	3.089	± 0.075	<0.001
Quantal contents	21.001	± 1.157	0.972	22.194	± 1.092	0.242
LF/FP - EPP 2 Hz (%)	-2.306	± 1.552	0.650	-2.314	± 1.427	0.211
LF/FP - EPP 10 Hz (%)	-21.210	± 1.959	0.028	-14.991	± 1.174	0.949
LF/FP - EPP 25 Hz (%)	-25.080	± 0.791	0.115	-19.948	± 1.875	0.942
LF/FP - EPP 30 Hz (%)	-27.862	± 2.443	0.099	-21.156	± 1.252	0.333
LF/FP - EPP 50 Hz (%)	-32.803	± 1.706	0.143	-26.028	± 1.480	0.405
LF/FP - EPP 100 Hz (%)	-40.129	± 2.074	0.645	-41.372	± 1.971	0.312
LF/FP - EPP 150 Hz (%)	-45.888	± 2.121	0.734	-46.856	± 2.771	0.533
						0.9

LP/FP = Last peak (LP)/first peak (FP) EPP amplitude ratio. Compared to control (*) or propranolol (**). Statistically significant values are in bold.