

Block of postjunctional muscle-type acetylcholine receptors *in vivo* causes train-of-four fade in mice

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Abstract

Background: Train-of-four (TOF) fade during nerve-mediated muscle contraction is postulated to be attributable to inhibition of prejunctional nicotinic $\alpha 3\beta 2$ acetylcholine receptors (nAChRs), while decrease of twitch tension is attributable to block of postjunctional muscle nAChRs. The validity of these presumptions was tested using specific prejunctional and postjunctional nAChR antagonists, testing the hypothesis that fade is not always a prejunctional phenomenon.

Methods: Pentobarbital anaesthetized mice had TOF fade measured after administration of: either 0.9% saline; the prejunctional $\alpha 3\beta 2$ nAChR antagonist, dihydro- β -erythroidine (DH β E); the postjunctional nAChR antagonists, α -bungarotoxin (α -BTX) or α -conotoxin GI; and a combination of α -BTX and DH β E; or a combination of α -conotoxin GI and DH β E.

Results: Saline caused no neuromuscular changes. Administration of muscle nAChR antagonists, α -BTX or α -conotoxin GI caused significant decrease of twitch tension and TOF fade compared with baseline ($P < 0.01$). DH β E alone caused no change of twitch tension or fade even after 90 min, but its coadministration with α -BTX or α -conotoxin GI significantly accelerated the onset of paralysis and degree of fade compared with α -BTX or α -conotoxin GI alone ($P < 0.01$).

Conclusions: Occupation of postjunctional nAChRs alone by α -BTX or α -conotoxin GI causes fade. As the prejunctional effects of DH β E on fade became manifest only when co-administered with α -BTX or α -conotoxin GI, specific inhibition of prejunctional nAChR alone is not necessary and sufficient to cause fade. Fade observed during repetitive nerve stimulation can be because of block of either postjunctional nAChRs alone, or block of prejunctional and postjunctional nAChRs together.

Key words: acetylcholine; neuromuscular monitoring; neuromuscular block; neuromuscular transmission; receptors; nicotinic

Editor's key points

- The fade of train-of four (TOF) is thought to be a pre-synaptic effect.
- Specific pre- and post-junctional nicotinic acetylcholine receptor (nAChRs) antagonists were used in mouse model.
- Blocking post-junctional nAChRs caused fade.
- Fade is not always a prejunctional phenomenon; block of postjunctional nAChRs can also cause fade.

Neuromuscular relaxants are used extensively in operating and emergency rooms and intensive care units.^{1–3} After the use of

relaxants, assessment of neuromuscular function is pivotal because relaxant-induced residual paralysis increases morbidity and mortality.^{4–6} Typically, the train-of-four (TOF) fade in muscle during 2 Hz nerve stimulation is used to assess neuromuscular function.^{6,7}

Fade in muscle during repetitive nerve stimulation is postulated to be a presynaptic phenomenon, while depression of twitch tension can be either prejunctional in nature because of decreased release of acetylcholine (ACh) or a postjunctional consequence because of decreased availability of muscle nicotinic acetylcholine receptors (nAChR).^{7–9} Some investigators have posited that the prejunctional nAChRs enhance mobilization of

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ACh by positive feedback to maintain tension at the same level during repetitive nerve stimulation; block of the prejunctional nAChRs by neuromuscular blocking agents attenuates ACh mobilization leading to fade.^{7–9} Based on *ex vivo* phrenic-diaphragm and oocyte expression studies, the putative receptor mediating enhanced mobilization of ACh or fade is said to be the $\alpha 3\beta 2$ nAChRs.^{7 10 11} Oocyte expression studies have confirmed that several clinically used relaxants do inhibit $\alpha 3\beta 2$ nAChRs.¹⁰ The relationship of this inhibition of $\alpha 3\beta 2$ to TOF fade, however, has not been demonstrated. Immunohistochemistry has demonstrated the presence of $\alpha 3$ containing nAChR isoforms prejunctionally.¹²

Specific antagonists used to study $\alpha 3\beta 2$ nAChRs *ex vivo* include dihydro- β -erythrodine (DH β E) or α -conotoxin MII.^{11 13 14} When DH β E or α -conotoxin-MII was used to block $\alpha 3\beta 2$ nAChRs in phrenic-diaphragm preparation of rat, fade was demonstrated, but only when the safety margin of neuromuscular transmission was concomitantly decreased with high magnesium concentrations. On the other hand, specific block of postjunctional muscle nAChRs with α -bungarotoxin (α -BTX) alone also induced TOF fade in rats.¹⁵ Thus, the exact role of prejunctional vs postjunctional nAChRs in the mediation of fade is unclear. The response to nAChR antagonists can differ between species.¹⁶ For example, waglerin-1, a potent antagonist of muscle nAChRs in mice has minimal effects in rats.¹⁶ Therefore, fade observed during postsynaptic nAChRs block¹⁵ may be restricted to rats, and not observed in another rodent species.

This study in mice, using specific postjunctional nAChR antagonists, α -conotoxin GI (from conus snail *Conus geographus*) and α -BTX (from cobra *Bungarus multicinctus*), tested the hypothesis that fade is not always a prejunctional phenomenon and that decreased availability of muscle nAChRs, is necessary and sufficient to cause fade. In separate experiments, the role of prejunctional, $\alpha 3\beta 2$ nAChRs was tested using the specific antagonist, DH β E, alone and in combination with the specific postjunctional antagonists, α -BTX or α -conotoxin GI.

Methods

Animals

The study protocol was approved by the institutional animal care committee (approval number 2011N000013). Relevant aspects of the ARRIVE guidelines were followed. Male C57/Black 6 mice (Jackson laboratory, MA), weighing 23–30 g, were used. After at least one week of acclimatization at our animal facility, 7 groups of mice ($n=5-6$ per group) were randomly allocated to receive one of the following drug regimens during nerve-evoked gastrocnemius-soleus muscle contractions: (1) 0.9% normal saline (NS), (2) the specific postsynaptic muscle nAChR blocker (α -BTX 0.175 $\mu\text{g g}^{-1}$ EMD Chemicals, Inc. Gibbstown, NJ), or (3) the specific postsynaptic muscle nAChR blocker (α -conotoxin GI 30 ng g^{-1} American Peptide Company, Sunnyvale, CA), (4) the specific presynaptic $\alpha 3\beta 2$ nAChR blocker (DH β E 1.0 $\mu\text{g g}^{-1}$ Tocris Bioscience, Ellisville, MO), or a combination of presynaptic and postsynaptic nAChR antagonists: (5) α -BTX 0.175 $\mu\text{g g}^{-1}$ and DH β E 0.5 $\mu\text{g g}^{-1}$, (6) α -BTX 0.175 $\mu\text{g g}^{-1}$ and DH β E 1.0 $\mu\text{g g}^{-1}$, or (7) α -conotoxin GI 30 ng g^{-1} and DH β E 1.0 $\mu\text{g g}^{-1}$.

Neuromuscular function study

For the measurement of twitch tension and fade, the mice were anaesthetized with pentobarbital (50–70 mg kg^{-1} intraperitoneally (i.p.)). Supplemental intermittent doses of pentobarbital

10–20 mg kg^{-1} i.p., were empirically administered every 20–30 min to maintain adequate anaesthesia, ascertained by the withdrawal response to a nociceptive stimulus. Tracheostomy was performed for mechanical ventilation with air at 110 breaths per minute with a tidal volume of 6–8 ml kg^{-1} (MiniVent Type 845, Harvard Apparatus, Holliston, MA). A venous catheter (MRE-025, Braintree scientific, Braintree, MA) was inserted into the right jugular vein and temperature was monitored with a rectal probe and maintained at 36–38°C using a heating pad.

Neuromuscular transmission and function were monitored by evoked mechanomyography using a Grass Stimulator 88, along with a Grass force transducer and software (Grass Technologies, West Warwick, RI). For this purpose, mice were placed in the dorsal recumbent position. The gastrocnemius-soleus muscle group tendons were tied with non-compliant silk and attached to separate Grass FT03 force displacement transducers. Both sciatic nerves were exposed at the thigh and tied with ligatures. Distal to the ligatures, stimulation electrodes (Subminiature Electrode, Harvard Apparatus, Holliston, MA) were attached for nerve-mediated stimulation of the muscle. The knees were stabilized rigidly with a clamp. A baseline tension of approximately 10 g was applied to the tendon of each muscle, which yielded optimal evoked tensions. The nerve-evoked muscle tension was recorded via a Grass P122 amplifier and displayed using the Grass Polyview Software.

During supramaximal stimuli, tetanic stimulation (50 Hz, 5 s) was applied and baseline mechanomyographic responses were stabilized over a period of 15 min using the TOF stimulation pattern (2 Hz per sec), every 20 s. At the end of this period the evoked muscle tension and fade developed during TOF stimulation were recorded as pre-drug administration (baseline) value. Subsequently, the test drugs alone or in combination were administered i.v. using a Hamilton syringe (Hamilton, Reno, NV). The drugs were reconstituted with 0.9% sterile saline and adjusted to a volume of 50 μl . After drug administration, 30 μl 0.9% sterile saline was administered to flush the drug through the venous catheter. The control group received 80 μl of saline. TOF fade was calculated as the T4/T1 ratio, where T4 and T1 are the fourth and first twitch tensions in the same train. Single twitch tension was defined as first twitch tensions during TOF stimulation. All animals were killed at the end of the experiments with an overdose of pentobarbital (200 mg kg^{-1}).

Data and statistical analyses

Statistical analyses were performed using Mann–Whitney test when two groups were compared. The Kruskal–Wallis test or the Friedman test followed by Dunn's multiple comparison test was used when more than two groups were compared. The results are expressed as median (range). *P*-values of <0.05 were considered significant.

Results

In the control group, intravenous injection saline did not change twitch tension or TOF fade compared with the pre-administration value for up to 90 min after injection (data not shown). Administration of DH β E alone also had no significant effect on twitch depression or TOF fade for up to 90 min, the end of the observation period (Fig. 1). This lack of neuromuscular effect after DH β E also confirms the stability of the preparation for up to 90 min. In the next group of mice, after stabilization of twitch tension, intravenous administration of α -BTX caused twitch depression within a few minutes. At 75, 50, and 25% of baseline twitch

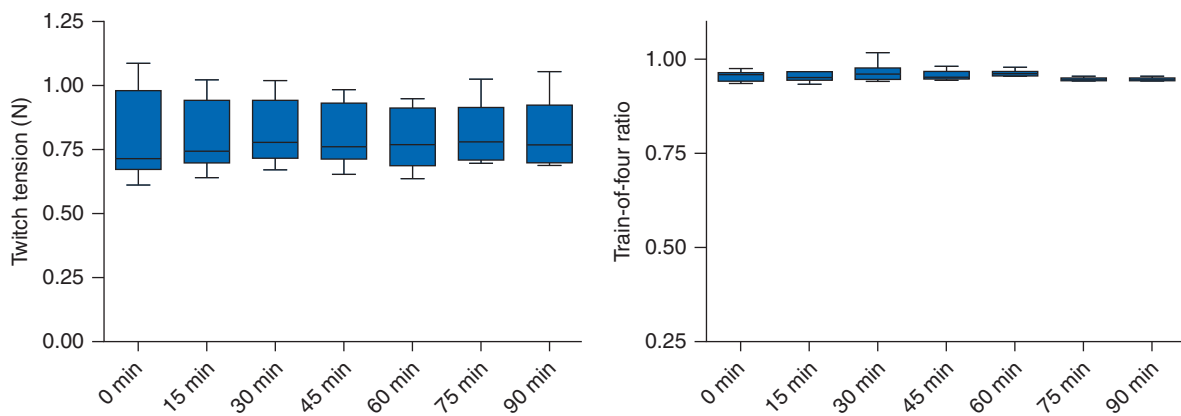


Fig 1 Intravenous administration of DH β E alone causes no significant neuromuscular effect on twitch tension or fade. Following intravenous administration of 1.0 $\mu\text{g g}^{-1}$ DH β E, twitch tension and train-of-four (TOF) ratio were measured for 90 min. No significant change in twitch tension (N: Newton) and train-of-four ratio ($n=6$). Box and whisker plots show median, 25th and 75th percentiles and full ranges.

tension (25, 50 and 75% depression) of the first twitch of TOF (T1) after α -BTX, the fade associated with these twitch depressions were 0.85 (0.81–0.89), 0.76 (0.71–0.81), and 0.69 (0.64–0.71), respectively; all except the TOF at 75% of baseline twitch tension were significantly decreased compared to the pre-administration value ($P=0.425$, $P=0.009$, and $P<0.0001$, respectively; Fig. 2). This dose of α -BTX caused 100% twitch depression over the time course, and the recovery of twitch tension was not observed during the observation period of 60 min after administration.

In another set of mice, intravenous DHE 0.5 or 1.0 $\mu\text{g g}^{-1}$ was administered together with α -BTX. With the administration of DHE 1.0 $\mu\text{g g}^{-1}$ together with α -BTX, the fade was significantly accentuated at 75, 50, and 25% of baseline tension compared to α -BTX alone ($P<0.001$, $P=0.003$, and $P=0.003$, respectively; Fig. 2). There was significant dose-dependent potentiation of the TOF fade by DH β E (linear trend; $P<0.001$ at 75% T1, $P<0.001$ at 50%T1, and $P<0.001$ at 25% of baseline (T1) tension; Fig. 2).

In mice given α -conotoxin GI alone, significant twitch depression and TOF fade was seen compared with pre-administration values at 50% of baseline tension (TOF fade = 0.58 (0.54–0.72); $P=0.010$) and at 25% of baseline tension (TOF fade = 0.55 (0.48–0.68); $P=0.002$; Fig. 3). The administration of DH β E (1.0 $\mu\text{g g}^{-1}$) together with α -conotoxin GI significantly potentiated the TOF fade caused by α -conotoxin GI alone ($P=0.008$ at 75% of baseline tension, $P=0.008$ at 50% of baseline tension, and $P=0.008$ at 25% depression of baseline tension; Fig. 3).

In view of the potentiation of fade by DH β E when co-administered with α -BTX or α -conotoxin GI the time course of the onset of the effects to decrease of tension to 75, 50 and 25% of baseline tension were examined. The administration of α -BTX alone caused a decrease of tension to 75, 50, and 25% of baseline tension in approximately 10, 15 and 24 min, respectively. The combined administration of α -BTX and DH β E (1.0 $\mu\text{g g}^{-1}$) resulted in a faster onset of effect of 7 min ($P=0.008$), 10 min ($P=0.040$), and 18 min ($P=0.716$) for decrease of baseline tension to 75, 50, and 25% of baseline tension compared with a α -BTX alone. With α -conotoxin GI alone and in combination with DH β E, the onset of effect for decrease of tension to 75, 50, and 25% of baseline was approximately 4, 5, 6 min alone, vs 3 min ($P=0.004$), 4 min ($P=0.009$), 5 min ($P=0.030$), respectively, for the combination (Table 1).

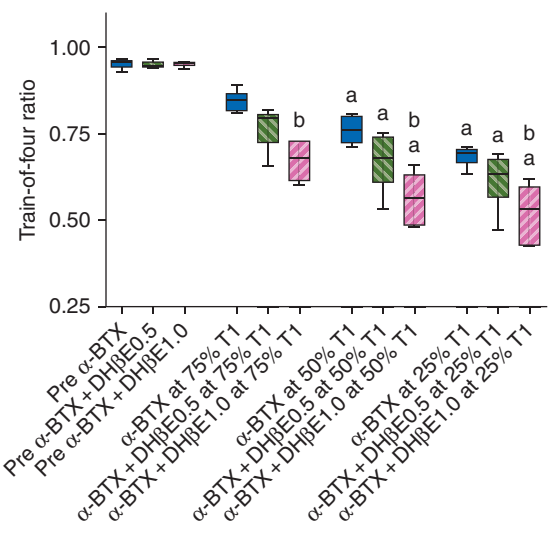


Fig 2 Postsynaptic block of muscle nAChRs by α -BTX causes train-of-four fade which was potentiated by DH β E. Following intravenous administration of α -BTX 0.175 $\mu\text{g g}^{-1}$ with or without DH β E, the train-of-four (TOF) ratio was measured. The postjunctional block of nAChRs by α -BTX alone produced significant fade ($n=6$). This fade was potentiated by block of presynaptic $\alpha 3\beta 2$ nAChR by DH β E [0.5 $\mu\text{g g}^{-1}$ ($n=6$) or 1.0 $\mu\text{g g}^{-1}$ ($n=6$)]. Box and whisker plots show median, 25th and 75th percentiles and full ranges. ^a $P<0.01$ vs pre-drug administration value of TOF ratio. ^b $P<0.01$ vs TOF fade by α -BTX at same degree of T1 depression.

Discussion

These studies in mice demonstrate that block of the postsynaptic nAChRs by i.v. administration of α -BTX or α -conotoxin GI, *per se*, is capable of inducing fade. Injection of the presynaptic nAChR ($\alpha 3\beta 2$) antagonist, DH β E, alone does not cause decrease of twitch tension or TOF fade but when administered with α -BTX or α -conotoxin GI, potentiates the TOF fade produced by the postsynaptic antagonists. DH β E with α -BTX or α -conotoxin GI also accelerated the onset of neuromuscular paralysis.

α -BTX has postjunctional effects only,^{17,18} and does not bind to $\alpha 3\beta 2$ nAChRs,^{14,19} or to any prejunctional nAChRs, as demonstrated by the lack of modulation of [³H]-ACh release during high frequency nerve stimulation in the rat phrenic nerve-hemidiaphragm.^{11,20} Thus, the fade observed after α -BTX is postjunctional phenomenon and is not because of any prejunctional effects. Some previous studies with α -BTX, however, have failed to show fade during *ex vivo* experiments.^{11,21} Concordant with our current observations, some other *ex vivo* experiments did show fade with prolonged incubation with α -BTX.²² This discrepancy in the lack of fade in some studies might be explained by the very slow onset time (150 min) when lower concentrations of α -BTX are used *ex vivo*.²³ Regardless of the lack of fade in some of the *ex vivo* experiments, no study has documented any prejunctional effects of α -BTX.

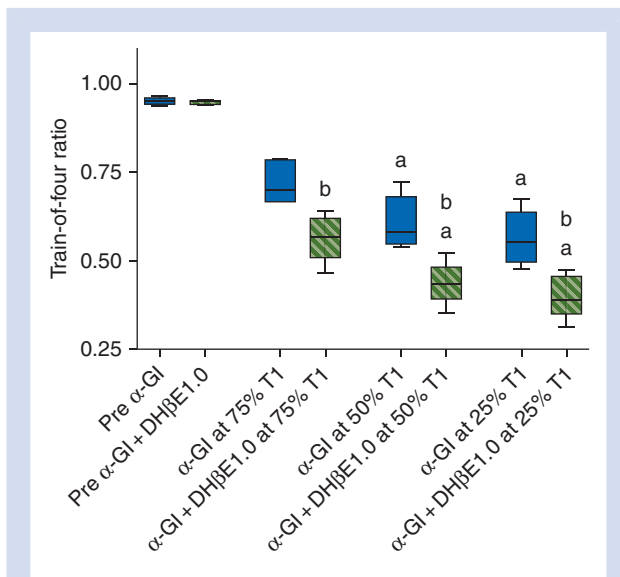


Fig 3 Postsynaptic block of muscle nAChRs by α -conotoxin GI leads to train-of-four fade which was accentuated by DH β E. After intravenous α -conotoxin GI 30 ng g⁻¹ with or without DH β E (1.0 μ g g⁻¹), the TOF ratio was measured. Postjunctional nAChR antagonist, α -conotoxin GI, produced significant fade (n=5), which was potentiated by prejunctional block of nAChRs by DH β E (n=5). Box and whisker plots show median, 25th and 75th percentiles and full ranges. ^aP<0.01 vs pre-drug administration value of TOF ratio. ^bP<0.01 vs TOF fade by α -BTX at same degree of T1 depression.

In order to exclude the possibility that the fade observed with α -BTX was not limited to that drug only, another specific postjunctional antagonist, α -conotoxin GI which has no effects on $\alpha 3\beta 2$ nAChRs, was tested.^{24–26} Previously, the administration of α -conotoxin GI had no effects on arterial blood pressure or heart rate, and did not modulate responses to vagus and preganglionic stimulation, all of which confirmed the absence of any autonomic side-effects.²⁴ Therefore, the twitch depression and TOF fade observed after injection of α -conotoxin GI is a postjunctional effect and not related to autonomic effects. The fade observed with decreased availability of muscle nAChRs induced by α -BTX or α -conotoxin GI is consistent with the observations in the pathological state of myasthenia gravis, a postjunctional disease, where fade is observed because of autoantibody-mediated decrease of muscle nAChRs.^{27,28}

In view of the previous elegant documentations that DH β E (1 μ M) effectively decreased prejunctional ACh release,¹¹ or efficiently blocked ACh-induced currents in $\alpha 3\beta 2$ nAChRs expressed in cells,²⁹ our study did not quantify the effect of DH β E on ACh release. In the phrenic nerve, the DH β E (1 μ M)-mediated decrease of ACh release was similar to that produced by α -conotoxin MII (0.1 μ M) and d-tubocurarine (1 μ M) (~50–60%).¹¹ Despite this decrease of ACh release by DH β E and α -conotoxin MII, no fade was observed until the margin of safety was decreased by very high concentration of Mg⁺⁺ (6–7 mM).¹¹ Normal Mg⁺⁺ concentration is 0.7–1.0 mM. DH β E 1 μ M, which reduced [³H]-ACh release *ex vivo* equals 0.36 μ g ml⁻¹. The intravenous dose of 0.5 or 1.0 μ g g⁻¹ used in our mice weighing ~30 g should result in several fold greater plasma concentrations; yet no fade was seen. The effects of DH β E on prejunctional $\alpha 3\beta 2$ nAChRs of mice became manifest only when the margin of safety was decreased by occupation of muscle nAChRs by α -BTX or α -conotoxin GI.

It is possible that the fade observed during postjunctional block of nAChRs is attributable to excess unbound ACh in synaptic cleft causing desensitization of the prejunctional nAChRs. This scenario seems unlikely as fade with α -BTX and α -conotoxin GI was observed even at 50% depression of baseline twitch tension (Figs 2 and 3), even before deep postjunctional block. In fact, the presence of ACh agonists (nicotine, cytosine and dimethylphenylpiperazinium), mimicking excess ACh, enhances the release of ACh (transmitter) release, and not desensitization.^{30,31} Furthermore, the presence of high concentrations of ACh is highly unlikely because of effective hydrolysis of ACh by acetylcholinesterase enzyme at the neuromuscular junction.

The onset of neuromuscular block was slow when postjunctional nAChRs were used alone, and accelerated when DH β E

Table 1 DH β E accentuates the onset of neuromuscular block induced by postjunctional nAChR antagonists, α -BTX, or α -conotoxin GI. Values are expressed as median (range). ^{*}P<0.05 and ^{**}P<0.01 vs values of toxin postjunctional (α -BTX or α GI) when given alone.

Doses of antagonists	Time to 25% inhibition of T1 (min)	Time to 50% inhibition of T1 (min)	Time to 75% inhibition of T1 (min)
α -BTX (0.175 μ g g ⁻¹) (n=6)	10.4 (8.4–12.4)	14.8 (11.3–20.2)	24.5 (12.9–34.1)
α -BTX (0.175 μ g g ⁻¹) +DH β E (0.5 μ g g ⁻¹) (n=6)	9.8 (7.1–11.1)	16.0 (9.2–20.8)	29.8 (15.0–35.7)
α -BTX (0.175 μ g g ⁻¹) +DH β E (1.0 μ g g ⁻¹) (n=6)	6.4 ^{**} (4.8–8.7)	9.7 [*] (6.6–13.8)	18.2 (9.4–28.4)
α -conotoxin GI (30 ng g ⁻¹) (n=5)	3.7 (3.0–4.4)	4.4 (3.8–5.3)	5.7 (4.5–6.7)
α -conotoxin GI (30 ng g ⁻¹) +DH β E (1.0 μ g g ⁻¹) (n=5)	3.0 ^{**} (2.2–3.0)	3.7 ^{**} (2.7–3.9)	4.8 [*] (3.3–5.0)

and α -BTX or α -conotoxin GI were combined together. The faster onset of effects seen when pre- and postjunctional effecting drugs are combined is reminiscent of effects of neuromuscular blocking agents which have combined actions on the pre- and post-junctional nAChRs. The presynaptic and postsynaptic effects of muscle relaxants are essential in contributing to the total efficacy of neuromuscular depression.³² DH β E and α -conotoxin MII, in addition to binding to α 3 β 2 nAChRs, also bind to α 4 β 2 and α 6 containing nAChRs, respectively.^{11 29 33} The presence of the latter nAChRs has not been demonstrated at the motor nerve terminal. Thus the accelerated onset of effect when DH β E is combined with postjunctional AChRs is unrelated to the block of α 6 or α 4AChRs by DH β E.

Our study examined if block of α 3 β 2 nAChRs causes fade. Although decrease of ACh release by DH β E alone may not be necessary and sufficient to cause fade, there could be other receptor-mediated contributory factors that can induce fade. Muscarinic AChRs, in addition to purinergic and adrenergic receptors, are also present prejunctionally.^{8 34} Muscle relaxants do have actions on non-nicotinic (muscarinic) AChRs and also other receptors.^{8 34} It is possible that muscle relaxants may act on other nicotinic, muscarinic and purinergic receptors, in addition to α 3 β 2 nAChR, to cause fade. Nevertheless, our results with two specific postsynaptic ligands, α -BTX and α -conotoxin GI, strongly indicate that decrease of the postjunctional receptors available for neuromuscular transmission, in and of itself, is capable of producing fade. Block of prejunctional nAChRs by DH β E is not necessary and sufficient to cause fade *in vivo*. Therefore, fade is not necessarily a prejunctional phenomenon but possibly also reflects the decreased margin of safety of neuromuscular transmission, which can be postjunctional or combined pre- and postjunctional in origin.

Authors' contributions

M.N.: designed, conducted this study, analysed the data, and wrote the manuscript. T.S. and S.J.S.: helped with the experiments and critically reviewed the manuscript. J.A.J.M.: helped design the study, supervised the work, analysed the data, and edited the manuscript. All authors approved the final manuscript.

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Declaration of interest

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