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Distinct Pharmacologic Properties of Neuromuscular Blocking Agents on Human Neuronal Nicotinic Acetylcholine Receptors

A Possible Explanation for the Train-of-four Fade

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Background: **Nondepolarizing neuromuscular blocking agents (NMBAs) are extensively used in the practice of anesthesia and intensive care medicine. Their primary site of action is at the postsynaptic nicotinic acetylcholine receptor (nAChR) in the neuromuscular junction, but their action on neuronal nAChRs have not been fully evaluated. Furthermore, observed adverse effects of nondepolarizing NMBAs might originate from an interaction with neuronal nAChRs. The aim of this study was to examine the effect of clinically used nondepolarizing NMBAs on muscle and neuronal nAChR subtypes.**

Methods: ***Xenopus laevis*** **oocytes were injected with messenger RNA encoding for the subunits included in the human $\alpha_1\beta_1\varepsilon\delta$, $\alpha_3\beta_2$, $\alpha_3\beta_4$, $\alpha_4\beta_2$, and α_7 nAChR subtypes. The interactions between each of these nAChR subtypes and atracurium, cisatracurium, d-tubocurarine, mivacurium, pancuronium, rocuronium, and vecuronium were studied using an eight-channel two-electrode voltage clamp setup. Responses were measured as peak current and net charge.**

Results: **All nondepolarizing NMBAs inhibited both muscle and neuronal nAChRs. The neuronal nAChRs were reversibly and concentration-dependently inhibited in the low micromolar range. The mechanism (*i.e.*, competitive *vs.* noncompetitive) of the block at the neuronal nAChRs was dependent both on subtype and the NMBA tested. The authors did not observe activation of the nAChR subtypes by any of the NMBAs tested.**

Conclusions: **The authors conclude that nondepolarizing NMBAs concentration-dependently inhibit human neuronal nAChRs. The inhibition of the presynaptic $\alpha_3\beta_2$ nAChR subtype expressed at the motor nerve ending provides a possible molecular explanation for the tetanic and train-of-four fade seen during a nondepolarizing neuromuscular block.**

NONDEPOLARIZING neuromuscular blocking agents (NMBAs) are extensively used in the practice of anesthesia and intensive care medicine to facilitate tracheal intubation and mechanical ventilation and to improve surgical conditions.

Although it is well established that nondepolarizing NMBAs block the postsynaptic $\alpha_1\beta_1\varepsilon\delta$ nicotinic acetylcholine receptor (nAChR) subtype at the muscle endplate, the effect on the presynaptic motor nerve ending has not been clarified (for a review, see Vizi and Lendvai[1,2] and Bowman *et al.*[2]). It is believed that the mechanism behind tetanic and train-of-four (TOF) fade during neuromuscular block by a nondepolarizing NMBA arise from an interaction with presynaptic cholinergic autoreceptors at the motor nerve ending.[1,3] However, the affinity of nondepolarizing NMBAs to such presynaptic autoreceptors has not been investigated at the molecular level. Further, it has recently been shown that an inhibition of the presynaptic $\alpha_3\beta_2$ nAChR subtype at the motor nerve end[4] induces tetanic fade.[5] Based on this, it seems likely that the tetanic fade phenomenon seen during nondepolarizing neuromuscular block is due to an inhibition of the $\alpha_3\beta_2$ nAChR subtype.

The $\alpha_1\beta_1\varepsilon\delta$ and the $\alpha_3\beta_2$ nAChRs are members of the same neurotransmitter-gated ion channel superfamily. They are composed of five transmembrane subunits with a central cation pore, and the stoichiometry and identity of subunits determines each receptor's unique properties.[6] To date, 17 nicotinic subunits have been cloned in vertebrates: the muscle α_1, β_1, δ, γ, and ε subunits and the neuronal α_{2-10} and β_{2-4} subunits.[7] Although there are many potential combinations of neuronal nAChRs, only a few have as yet been found to be of biologic importance.[8,9] The neuronal nAChRs are found both presynaptically and postsynaptically in neurons of the central ($\alpha_4\beta_2$, $\alpha_3\beta_2$, α_7)[9,10] and peripheral nervous system ($\alpha_3\beta_4$, $\alpha_3\beta_2$, α_7)[9,11,12] as well as in extraneuronal tissues and cells, such as keratinocytes, muscle, lymphocytes, macrophages, carotid bodies, and neurosecretory cells.[6,7,13]

Interactions between NMBAs and neuronal nAChRs may cause serious cardiovascular and respiratory side effects. It has been shown that nondepolarizing NMBAs reduce hypoxic ventilatory response in partially paralyzed humans,[14,15] and the mechanism behind this depression might be interference with nicotinic chemotransduction of the carotid bodies.[16,17] At the molecular level, d-tubocurarine, pancuronium, atracurium, and its degradation product laudanosine have been shown to

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block neuronal nAChR subtypes expressed in *Xenopus* oocytes.[18–22] Interestingly, some reports indicate that NMBAs can act as partial agonists at $\alpha_1\beta_1\gamma\delta$, $\alpha_3\beta_4$, and $\alpha_4\beta_2$ nAChR subtypes[21,23]; however, other studies could not demonstrate any agonism by NMBAs.[20,24]

The α_7 nAChR subtype plays a key role in the cholinergic reflex involved in inflammatory conditions such as sepsis,[25,26] and it can be speculated whether NMBAs used in intensive care settings might interact with the inflammatory response to sepsis. Furthermore, although highly charged, NMBAs can under certain conditions cross the blood–brain barrier,[27–29] thus having the potential to interact with central cholinergic receptors and the synaptic transmission[30] and cause seizures.[31,32]

Because most nondepolarizing NMBAs were developed before cloning and isolation of their target proteins, the precise modes of action have not been examined in detail. A better understanding of the molecular mechanisms of action of clinically used nondepolarizing NMBAs on human neuronal nAChR subtypes is needed. In addition, for future drug design, it is essential to define potential interactions with human neuronal nAChRs.

The aim of this study was therefore to investigate the potency and functional affinity of clinically used nondepolarizing NMBAs on acetylcholine-induced responses on human muscle and neuronal nAChRs heterologously expressed in *Xenopus* oocytes. In addition, potential activation of nAChRs by nondepolarizing NMBAs was also investigated.

Materials and Methods

Clones

The human nAChR subunits α_1, α_{3-4}, α_7, β_1, β_2, β_4, δ, and ε were cloned from a human complementary DNA (cDNA) library. GenBank (Bethesda, MD) access numbers for the cDNA nucleotide sequences are as follows: NM 000079 (α_1), HSU62432 (α_3), L35901 (α_4), Y08420 (α_7), NM 000747 (β_1), Y08415 (β_2), NM 000750 (β_4), NM 000751 (δ), and NM 000080 (ε). The cDNAs were subcloned into different expression vectors, pKGem (AstraZeneca, Wilmington, DE) (α_1, α_3, β_1, β_2, δ and ε), pBluescript II SK (−) (Stratagene, La Jolla, CA) (α_7), and pBSTA (University of California, Irvine, CA) (α_4 and β_4). Messenger RNA (mRNA) was transcribed *in vitro* using the mMessage mMachine® T7 kit (Ambion, Austin, TX) and analyzed using a bioanalyzer (Agilent Technologies, Palo Alto, CA).

Xenopus *Oocyte Injection*

The study was approved by the local animal ethics committee at Karolinska Institutet, Stockholm, Sweden. Preparation and injection of oocytes and the electrophysiologic recordings were conducted as previously described.[33] Briefly, *Xenopus laevis* oocytes were isolated by partial ovariectomy from frogs anesthetized with 0.2% Tricaine (Sigma, St. Louis, MO). The ovaries were mechanically dissected to smaller lumps and digested in OR-2 buffer (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH adjusted to 7.5 with NaOH) containing 1.5 mg/ml collagenase (type 1A; Sigma) for 90 min to remove the follicular epithelia from the oocytes. After 1–24 h, the oocytes were injected with 0.2–18 ng mRNA in a total volume of 30–40 nl/oocyte. Multiple subunit combinations were injected at a 1:1 ratio ($\alpha_1\beta_1\varepsilon\delta$ or $\alpha_x\beta_y$), except for $\alpha_4\beta_2$, where the injection ratio was 1:9. The oocytes were maintained in Leibovitz L-15 medium (Sigma) diluted 1:1 with Millipore filtered double distilled H_2O (Billerica, MA) and 80 μg/ml gentamicin, 100 U/ml penicillin, and 100 μg/ml streptomycin added. Oocytes were incubated at 18°–19°C for 2–7 days after injection before being studied.

Electrophysiologic Recordings

All recordings were performed at room temperature (20°–22°C). During recording, the oocytes were continuously perfused with ND-96 (96.0 mM NaCl, 2.0 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.0 mM HEPES, pH 7.4 adjusted with NaOH). Oocyte recordings were performed using an integrated system that provides automated impalement of up to eight oocytes, studied in parallel with two-electrode voltage clamp, and current measurements were automatically coordinated with fluid delivery throughout the experiment (OpusXpress 6000A; Molecular Devices, Union City, CA). Electrodes were made from 1.5-mm borosilicate tubes (World Precision Instruments Inc., Sarasota, FL) and filled with 3 M KCl (0.5–2.5 MΩ resistance). The oocytes were voltage clamped at −60 mV, because it has previously been shown that inhibition of nAChRs by nondepolarizing NMBAs is voltage independent at holding voltages from −100 to −40 mV.[20,23]

Protocol

Oocytes were continuously perfused with ND-96 at a rate of 2 ml/min in a 150-μl chamber. Drugs were delivered from a 96-well plate using disposable tips and administered at a rate of 2 ml/min for the first 2 s, and thereafter at 1 ml/min. Concentration–response curves for acetylcholine were constructed, before and after the addition of 10 μM antagonist in each oocyte, for the neuronal nAChRs. To determine whether nondepolarizing NMBAs activate and furthermore inhibit acetylcholine-induced currents, nondepolarizing NMBAs were applied for 55 s before a 20-s coapplication of both antagonist and acetylcholine. Two different concentrations of acetylcholine were applied on each neuronal receptor subtype. The concentrations 1 and 10 μM (for $\alpha_4\beta_2$), 50 and 300 μM (for $\alpha_3\beta_2$ and $\alpha_3\beta_4$), and 30 and 100 μM (for α_7) were chosen to represent concentrations below and above the EC_{50} for each receptor subtype. The muscle nAChR ($\alpha_1\beta_1\varepsilon\delta$) was used as a reference,

and therefore only one acetylcholine concentration (10 μM) was studied. Between each drug application, there was a 6-min washout period to allow clearance of the drugs and to avoid desensitization of the channels. Before and after each concentration–response experiment, three control responses were recorded at EC_{50} acetylcholine concentration to exclude desensitization. Experiments were rejected if the postcontrol response was less than 80% of the precontrol response. To adjust for the level of channel expression, the responses in acetylcholine concentration–response experiments were normalized to the peak response in each individual oocyte. For inhibition experiments, responses in each oocyte were normalized to the mean of the second and third acetylcholine precontrols.

Drugs

Acetylcholine and d-tubocurarine were purchased from Sigma. Atracurium and cisatracurium were provided by GlaxoSmithKline (Barnard Castle Durham, United Kingdom). Mivacurium (Mivacron®) was purchased from GlaxoSmithKline (Mölndal, Sweden). Org NC 97 (pancuronium), Org NC 45 (vecuronium), and rocuronium were provided by Organon (BH Oss, The Netherlands). Chemicals used in buffers were purchased from Sigma unless otherwise stated. Stock solution of 1mM acetylcholine in ND-96 was prepared and frozen. Nondepolarizing NMBAs were prepared fresh each day and stored at +4°C. All drugs were then diluted in ND-96 immediately before use.

Data Analysis and Statistics

Off-line analyses were made using Clampfit 9.2 (Molecular Devices). Changes in currents were studied both as peak and net charge responses (area under the curve); however, for the α_7 subtype, only net charge analysis was used, as previously described.[33–35] The baseline current immediately before drug application was subtracted from the response, and the analysis region for peak and net charge analysis was 20 s, *i.e.*, during the time of agonist application. Concentration–response relations for acetylcholine were fitted by nonlinear regression (Prism 4.0; GraphPad, San Diego, CA) to the four-parameter logistic equation

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{\left[1 + \left(\frac{x}{EC_{50}}\right)^{nHill}\right]},$$

where Y is the normalized response, x is the logarithm of concentration, and EC_{50} is the logarithm of the concentration of agonist eliciting half-maximal response. When NMBA-induced inhibition was studied, the same equation was used, and EC_{50} was replaced by IC_{50}, which is the concentration of antagonist eliciting half-maximal inhibition, Bottom = 0, Top = 1. Unless otherwise stated, data are given as mean ± SEM or 95% confidence interval (CI). Differences between fitted curves were analyzed using an F test, followed by a *t* test (Prism 4.0). A *P* value of less than 0.05 was considered significant.

Results

Acetylcholine Concentration–Response Relations for Muscular and Neuronal nAChRs

Acetylcholine produced a concentration-dependent inward current in voltage clamped oocytes injected with mRNA encoding muscle- and neuronal-type nAChRs, whereas uninjected oocytes did not respond to acetylcholine (data not shown). The responses to acetylcholine in terms of kinetics and EC_{50} values at the nAChR subtypes were consistent with previous reports[18,33,36] (fig. 1 and table 1), thus confirming the receptor expression model. However, kinetics can also be determined using net charge analysis. Unfortunately, there is a lack of published data for comparison of net charge in human nAChRs, except for the α_7 nAChR subtype.[35] As shown in figure 1, at $\alpha_3\beta_4$ and $\alpha_4\beta_2$ nAChR concentration–response relations based on net charge analysis correlate well with peak currents, with almost identical EC_{50} and Hill coefficients (appendix 1). However, the α_7 subtype nAChR displays unique properties, with very fast desensitization kinetics (fig. 1A), which gives a different concentration–response relation depending on whether peak response or net charge was measured (appendix 1). At the $\alpha_3\beta_2$ subtype, which has an initial rapid desensitization, there was a small difference between the EC_{50} values.

Inhibition of Muscle nAChRs by Nondepolarizing NMBAs

Because the adult muscle ($\alpha_1\beta_1\varepsilon\delta$) nAChR is the clinical target for nondepolarizing NMBAs, this receptor subtype was used as reference in the oocyte setup. Atracurium, cisatracurium, d-tubocurarine, mivacurium, pancuronium, rocuronium, and vecuronium all concentration-dependently inhibited 10 μM acetylcholine–induced currents in oocytes expressing the human $\alpha_1\beta_1\varepsilon\delta$ nAChR (fig. 2 and table 2). The IC_{50} values were in the nanomolar range and were comparable with a similar study investigating nondepolarizing NMBAs, using mouse cRNA.[37]

Neuronal nAChRs Are Inhibited by Nondepolarizing NMBAs

Atracurium, cisatracurium, d-tubocurarine, mivacurium, pancuronium, rocuronium, and vecuronium reversibly and concentration-dependently inhibited all of the neuronal nAChR subtypes tested (*i.e.*, $\alpha_3\beta_2$, $\alpha_3\beta_4$, $\alpha_4\beta_2$, and α_7) with IC_{50} values in the micromolar range (figs. 3 and 4, table 2, and appendix 2).

All the nondepolarizing NMBAs except mivacurium

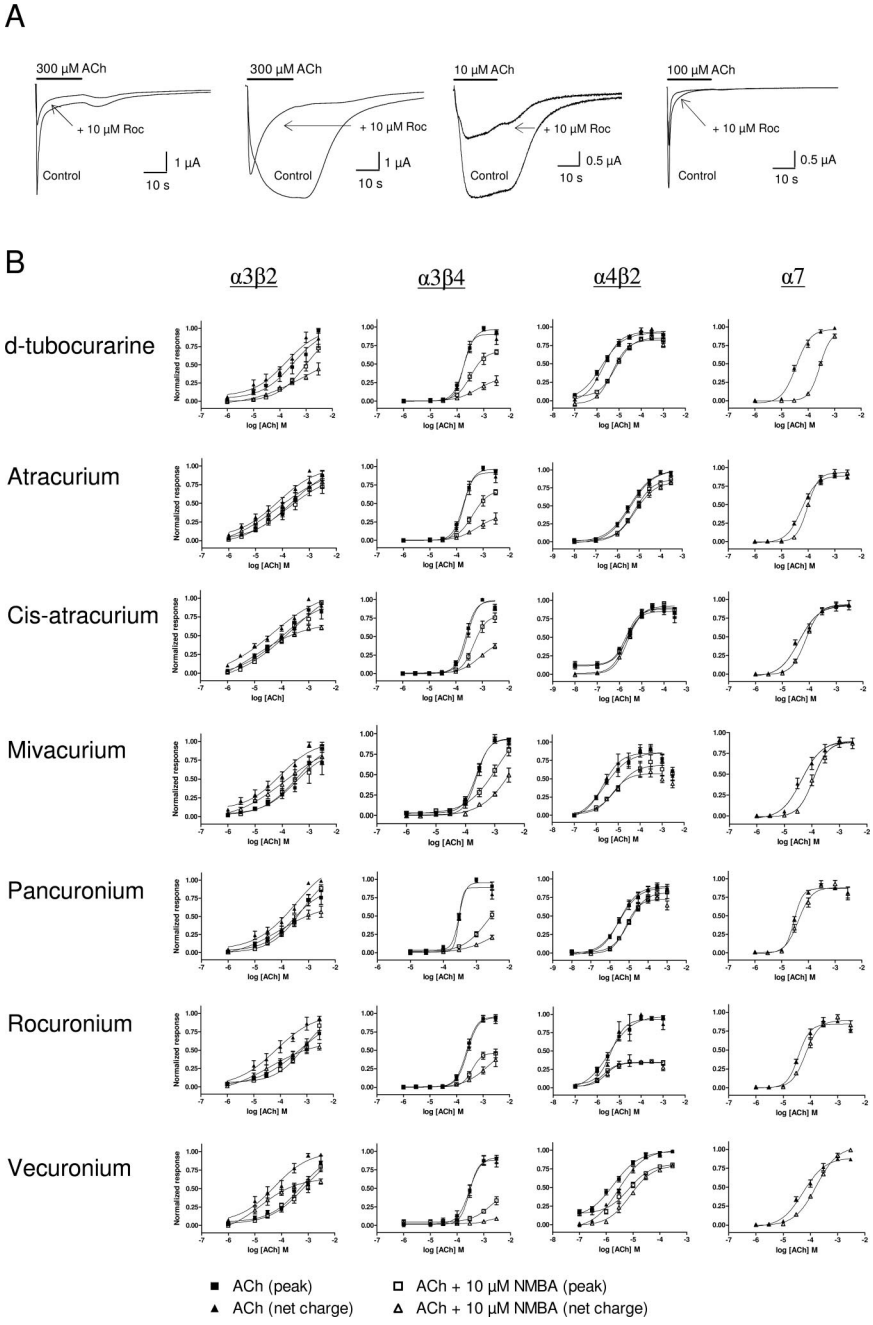

Fig. 1. Effect of 10 μM nondepolarizing neuromuscular blocking agents (NMBAs) on acetylcholine (ACh)–mediated responses in voltage clamped (−60 mV) *Xenopus* oocytes expressing human neuronal nAChRs. (*A*) Representative traces showing the inhibitory effect of 10 μM rocuronium (Roc) on the $\alpha_3\beta_2$, $\alpha_3\beta_4$, $\alpha_4\beta_2$, and α_7 receptor subtypes (displayed in the same order). (*B*) Responses in each oocyte were normalized to the maximal acetylcholine peak current and maximal net charge (α_7) in each oocyte, giving the concentration–response curves. Each *symbol* represents mean ± SEM of 3–14 oocytes. When no *error bars* are seen, they are smaller than the *symbols*.

showed similar affinity at the $\alpha_3\beta_2$ nAChR subtype, with IC_{50} values of 3–20 μM after activation by 50 μM acetylcholine and 1–62 μM at 300 μM acetylcholine. Vecuronium and d-tubocurarine were most potent as inhibitors at the $\alpha_3\beta_2$ subtype independent of acetylcholine concentration, whereas mivacurium had the lowest potency of all the nondepolarizing NMBAs and did not reduce the 300-μM acetylcholine response at concentrations lower than 100 μM. For atracurium, cisatracurium, pancuronium, and rocuronium, an increase from 50 to 300 μM acetylcholine slightly increased the IC_{50} (not significant), suggesting a competitive inhibition or higher affinity to the closed channel. Nondepolarizing NMBAs tended to increase the acetylcholine EC_{50} for the $\alpha_3\beta_2$ nAChR, although the effect was not statistically significant. The peak acetylcholine current was not reduced by 10 μM nondepolarizing NMBAs (fig. 1). Therefore, the inhibition induced by NMBAs at the $\alpha_3\beta_2$ receptor seems mainly to be competitive, except for d-tubocurarine and vecuronium, where there is a noncompetitive component.

All of the nondepolarizing NMBAs concentration-dependently inhibited the $\alpha_3\beta_4$ nAChR subtype, with IC_{50} values from 2 to 20 μM and from 0.3 to 2 μM for 50 and 300 μM acetylcholine, respectively. There was no component of competitive inhibition by the NMBAs at this receptor because the IC_{50} values were unchanged, or even lower, at the higher acetylcholine concentration

Table 1. Pharmacologic Properties of NMBAs on Human Neuronal nAChRs Expressed in *Xenopus* Oocytes and Activated by Acetylcholine

	EC_{50} (95% CI), μM						
	Atracurium	Cisatracurium	d-TC	Mivacurium	Pancuronium	Rocuronium	Vecuronium
$\alpha_3\beta_2$							
ACh	214 (52.18–880)	93.75 (24.75–355)	318 (54.36–1,827)	316 (46.40–2,156)	273 (76.41–978)	548 (15.29–19,630)	551 (65.94–4,597)
ACh + 10 μM NMBA	150 NS (15.64–1,441)	246 NS (26.35–2,301)	988 NS (190–5,127)	345 NS 56.48–2,112)	546 NS (155–1,921)	759 NS (144–3,987)	687 NS (67.85–6,955)
$\alpha_3\beta_4$							
ACh	182 (160–206)	235 (207–268)	218 (195–243)	233 (199–273)	300 (278–324)	236 (205–271)	291 (224–378)
ACh + 10 μM NMBA	408‡ (276–604)	476‡ (343–660)	457† (310–676)	582‡ (357–949)	2,193‡ (233–20,670)	383† (274–534)	1,915* (20.29–180,800)
$\alpha_4\beta_2$							
ACh	3.70 (2.94–4.66)	1.98 (1.49–2.63)	1.62 (1.06–2.47)	2.36 (0.59–9.35)	3.20 (2.06–4.96)	3.52 (2.28–5.44)	1.92 (1.28–2.88)
ACh + 10 μM NMBA	6.44† (4.80–8.64)	2.51 NS (1.94–3.26)	7.15‡ (5.60–9.13)	8.43 NS (2.47–28.74)	10.07‡ (7.19–14.10)	1.96 NS (1.32–2.90)	7.11‡ (4.15–12.17)
α_7							
ACh	57.11 (47.73–68.34)	41.51 (30.26–56.95)	35.65 (29.31–43.35)	42.65 (30.20–60.22)	29.34 (25.40–33.90)	36.65 (30.79–43.63)	53.88 (41.57–69.83)
ACh + 10 μM NMBA	94.83‡ (83.88–107)	73.95† (62.85–87.02)	277‡ (250–307)	111‡ (84.88–145)	42.47* (31.44–57.37)	66.15‡ (54.06–80.93)	164‡ (144–186)

For each receptor subtype, the half activation concentration (EC_{50}) (acetylcholine [ACh]) was compared with the EC_{50} (ACh + neuromuscular blocking agent [NMBA]) using an F test and thereafter a *t* test.

* $P < 0.05$. † $P < 0.01$. ‡ $P < 0.001$.

d-TC = d-tubocurarine; CI = confidence interval; nAChR = nicotinic acetylcholine receptor; NS = not significant.

used (fig. 1 and table 2). Furthermore, addition of the NMBAs to the acetylcholine concentration–response relations both increased the EC_{50} (table 1) and reduced peak acetylcholine responses in presence of a NMBA ($P < 0.05$), independent of concentration. Therefore, the NMBAs seem to inhibit the $\alpha_3\beta_4$ nAChR subtype in a noncompetitive way. All NMBAs showed higher affinity for the closed channel, except cisatracurium and mivacurium.

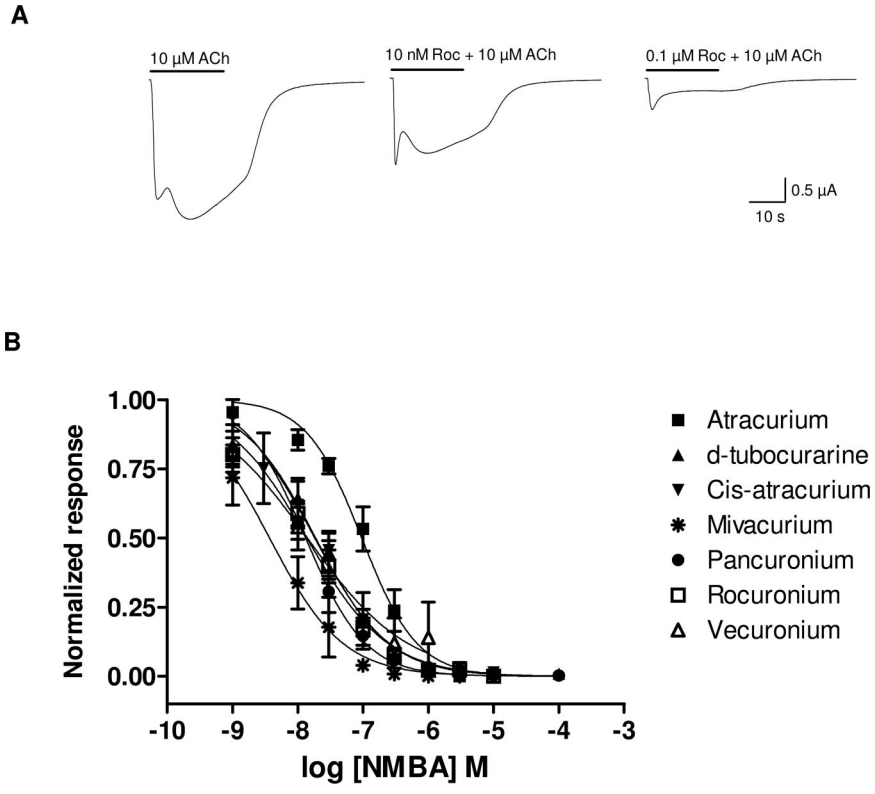

Fig. 2. Nondepolarizing neuromuscular blocking agents (NMBAs) concentration-dependently inhibit the 10 μM acetylcholine (ACh)–induced current response in *Xenopus* oocytes expressing the human muscle ($\alpha_1\beta_1\varepsilon\delta$) nicotinic acetylcholine receptor. (*A*) Representative traces from one oocyte. (*B*) Current responses in each oocyte were normalized to the 10 μM acetylcholine control responses in each oocyte as described in the Materials and Methods. Each *symbol* represents mean ± SEM of 4–7 oocytes. When no *error bars* are seen, they are smaller than the *symbols*. Roc = rocuronium.

Table 2. Pharmacologic Properties of NMBAs as Inhibitors of Acetylcholine-induced Activation of Human Neuronal nAChRs Expressed in *Xenopus* Oocytes

	IC_{50} (95% CI), μM						
	Atracurium	Cisatracurium	d-TC	Mivacurium	Pancuronium	Rocuronium	Vecuronium
$\alpha1\beta1\varepsilon\delta$							
10 μM ACh	96.65 nM (73.89–123.8)	18.19 nM (12.64–26.16)	18.73 nM (13.87–25.30)	3.69 nM (2.22–6.15)	13.17 nM (7.88–22.02)	13.76 nM (10.57–17.91)	10.74 nM (5.48–21.06)
$\alpha3\beta2$							
50 μM ACh	4.99 (3.08–8.09)	12.68 (5.16–31.16)	4.78 (2.93–7.81)	69.04 (46.41–103)	13.71 (6.92–27.17)	8.30 (3.54–19.48)	3.55 (2.32–5.43)
300 μM ACh	9.24 NS (3.72–22.97)	28.34 NS (10.23–78.45)	2.55 NS (1.42–4.55)	Do not fit	22.67 NS (7.51–68.44)	14.63 NS (5.87–36.46)	2.52 NS (0.97–6.57)
$\alpha3\beta4$							
50 μM ACh	11.65 (7.63–17.79)	1.69 (1.21–2.36)	19.78 (13.18–29.70)	3.71 (2.56–5.39)	7.06 (5.07–9.83)	4.12 (2.80–6.08)	1.60 (1.35–1.91)
300 μM ACh	0.94‡ (0.64–1.38)	1.94 NS (1.27–2.98)	2.14‡ (1.27–3.62)	4.58 NS (2.73–7.69)	1.92† (0.94–3.93)	0.46‡ (0.32–0.66)	0.29‡ (0.15–0.56)
$\alpha4\beta2$							
1 μM ACh	7.89 (6.38–9.76)	13.24 (7.61–23.06)	1.77 (1.47–2.14)	1.52 (1.10–2.10)	3.34 (3.06–3.65)	1.11 (0.85–1.44)	1.30 (0.88–1.90)
10 μM ACh	21.07‡ (15.84–28.02)	66.74‡ (28.38–156.9)	5.06‡ (4.31–5.94)	Do not fit	14.70‡ (12.52–17.26)	5.37* (0.83–34.89)	4.98‡ (3.65–6.79)
$\alpha7$							
30 μM ACh	5.56 (4.18–7.40)	6.19 (5.36–7.16)	1.52 (1.31–1.77)	2.90 (2.22–3.78)	13.33 (10.40–17.09)	7.55 (6.24–9.14)	7.70 (6.05–9.78)
100 μM ACh	13.03‡ (8.96–18.94)	14.62 NS (8.77–24.37)	38.11‡ (28.61–50.76)	9.92‡ (8.00–12.30)	11.76 NS (9.70–14.26)	5.06‡ (4.35–5.89)	8.38 NS (7.29–9.63)

For each receptor subtype, the half inhibition concentration (IC_{50}) values for each neuromuscular blocking agent (NMBA) was tested using an F test and thereafter a *t* test.

* $P < 0.05$. † $P < 0.01$. $P < 0.001$.

ACh = acetylcholine; CI = confidence interval; d-TC = d-tubocurarine; nAChR = nicotinic acetylcholine receptor; NS = not significant.

By contrast, the $\alpha_4\beta_2$ nAChR subtype was competitively blocked by most of the NMBAs. That is, increasing the concentrations of acetylcholine from 1 to 10 μM increased the IC_{50} from 1–13 μM to 5–67 μM. In addition, NMBAs generally right-shifted the acetylcholine concentration–response relations without reducing the peak response at the $\alpha_4\beta_2$ nAChR (fig. 1), further suggesting a competitive mode of inhibition. However, 10 μM rocuronium significantly reduced the peak response to all concentrations of acetylcholine tested (fig. 1), and thus, its inhibition is noncompetitive. Further, rocuronium seemed to desensitize the $\alpha_4\beta_2$ receptor because the control responses after both the acetylcholine concentration–response experiment with rocuronium and after the rocuronium inhibition experiment with 10 μM acetylcholine did not return to 80% of the control response in most of the series, which were therefore excluded (see Material and Methods, Protocol). Interestingly, rocuronium inhibition experiments with 1 μM acetylcholine did not show this pattern. Rocuronium therefore inhibits the $\alpha_3\beta_4$ and $\alpha_4\beta_2$ subtypes noncompetitively and with variable state dependency.[38]

All NMBAs concentration-dependently right-shifted the acetylcholine concentration–response curve for the α_7 nAChR subtype, with increased EC_{50} values (table 1), but did not reduce peak responses (not significant). In general, the inhibition of 30 and 100 μM acetylcholine at the α_7 nAChR subtype by NMBAs was concentration dependent (table 2), suggesting that NMBAs inhibit the α_7 nAChR subtype in a competitive manner. However, for rocuronium, the IC_{50} decreased with increased acetylcholine concentration (table 2), indicating a noncompetitive component in the action of rocuronium at the α_7 nAChR subtype.

Nondepolarizing Neuromuscular Blocking Agents Do Not Activate Human nAChRs

Application of 1 nM to 100 μM atracurium, cisatracurium, d-tubocurarine, mivacurium, pancuronium, rocuronium, or vecuronium to oocytes expressing human muscle ($\alpha_1\beta_1\varepsilon\delta$) or neuronal ($\alpha_3\beta_2$, $\alpha_3\beta_4$, $\alpha_4\beta_2$, and α_7) nAChRs did not result in receptor activation (data not shown).

Discussion

This study shows that nondepolarizing NMBAs inhibit neuronal nAChRs and that the inhibitory mechanism differs between individual receptor subtypes and NMBAs. In addition, we found no evidence that the nAChR subtypes tested were activated by any of the nondepolarizing NMBAs.

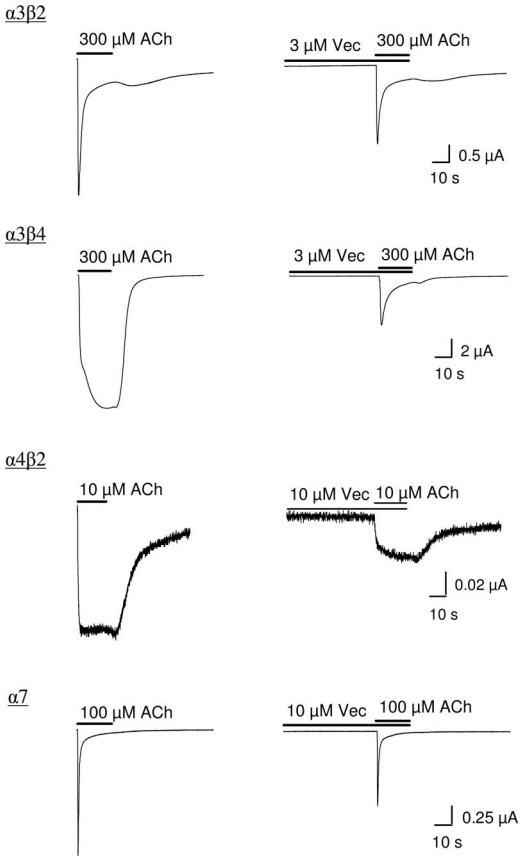

Fig. 3. Representative traces of vecuronium-induced inhibition of acetylcholine (ACh) currents in *Xenopus* oocytes expressing the human $\alpha_3\beta_2$, $\alpha_3\beta_4$, $\alpha_4\beta_2$, and α_7 nicotinic acetylcholine receptor subtypes. Vecuronium (Vec) was preapplied for 55 s before a 20-s coapplication with acetylcholine as indicated with the *horizontal bars*. For each receptor subtype, traces shown in each row are from the same oocyte.

All the nondepolarizing NMBAs reversibly and concentration-dependently inhibited the neuronal $\alpha_3\beta_2$, $\alpha_3\beta_4$, $\alpha_4\beta_2$, and α_7 nAChR subtypes in the micromolar range. Hence, the block occurs at clinically relevant concentrations.[39] In general, nondepolarizing NMBAs block the $\alpha_4\beta_2$, $\alpha_3\beta_2$, and α_7 subtypes in a competitive manner; the exception is the $\alpha_3\beta_4$ subtype, where the block seems noncompetitive. However, the nondepolarizing NMBAs have individual action profiles on different receptors. Mivacurium had rather low potency at the $\alpha_3\beta_2$ nAChR, whereas the other NMBAs showed similar IC_{50} values across the nAChRs tested.

Nondepolarizing NMBAs have higher functional affinity for the $\alpha_1\beta_1\varepsilon\delta$ nAChR subtype than for the neuronal nAChR subtypes, as determined by current amplitude measurements. For the muscle nAChR, the IC_{50} values (nanomolar range) presented here as well as in other studies using the *Xenopus* oocyte expression system[20,23,37] contrast with the micromolar concentrations of NMBAs needed to reduce the nerve-evoked twitch by 50% in isolated rat nerve-muscle preparations[40] and in the clinical setting.[39] This apparent discrepancy probably reflects the large safety factor in the neuromuscular transmission, where approximately 75% of the receptors must be occupied by a nondepolarizing NMBA before there is any reduction in twitch tension, and 90% occupancy is required for full paralysis.[41] This safety factor has not been reported for the neuronal nAChRs as far as we know.

In addition to inhibiting nAChRs, nondepolarizing NMBAs have also been described as partial agonists at both muscle and neuronal nAChR subtypes.[21,23,32] Atracurium-induced activation of the $\alpha_4\beta_2$ nAChR subtype occurs at very low concentrations, even lower than those required for inhibition.[21] However, the reports are contradictory, because Garland *et al.*[20] were unable to show any activation of the $\alpha_1\beta_1\gamma\delta$ or $\alpha_1\beta_1\varepsilon\delta$ nAChR subtype induced by d-tubocurarine or pancuronium. Here, we did not observe activation of the nAChRs by any of the seven nondepolarizing NMBAs studied. One possible explanation for this discrepancy might be that our study, in contrast to the previous one,[21] did not use atropine in the perfusion buffer. A low concentration of atropine (*i.e.*, 0.5 μM) has commonly been used to prevent activation of putative endogenous muscarinic receptors at the epithelial layer of the *Xenopus* oocyte surface.[42,43] However, during recent years, it has become clear that there is no endogenous surface expression of muscarinic receptors in *Xenopus* oocytes themselves,[44] and furthermore, it has been shown that atropine can both inhibit and activate nAChR expressed in the *Xenopus* oocyte.[44] Notably, the $\alpha_4\beta_2$ subtype is activated by atropine; therefore, we suggest that the activation of the $\alpha_4\beta_2$ subtype previously attributed to atracurium might instead have been elicited by atropine. For the muscle-type nAChR, we could not record any activation of the $\alpha_1\beta_1\varepsilon\delta$ nAChR subtype, thus confirming observations reported in previous studies.[20,23,24]

Many of the nondepolarizing NMBAs tested have breakdown products with potential to block muscle nAChRs[39]; however, most of the nondepolarizing NMBAs are not degraded *in vitro*. Atracurium and cisatracurium can to some extent undergo spontaneous degradation to laudanosine by Hofmann reaction *in vitro*,[45] depending mainly on temperature and pH, and although we controlled these parameters, we cannot exclude some contamination of laudanosine. Laudanosine has been shown to block the neuronal $\alpha_3\beta_4$, $\alpha_4\beta_2$, and α_7 in a noncompetitive manner with IC_{50} values of 8–38 μM.[21,22] Because both atracurium and cisatracurium inhibit the $\alpha_4\beta_2$ and α_7 nAChRs in a competitive way, we consider it unlikely that there is any substantial involvement of laudanosine under our experimental conditions.

To date, only one study investigating the effect of clinically used nondepolarizing NMBAs on nAChRs has used human DNA[21]; all of the others have used rat and mouse DNA.[20,24,37] Although there is more than 80% homology between the human and rodent nAChR sub-

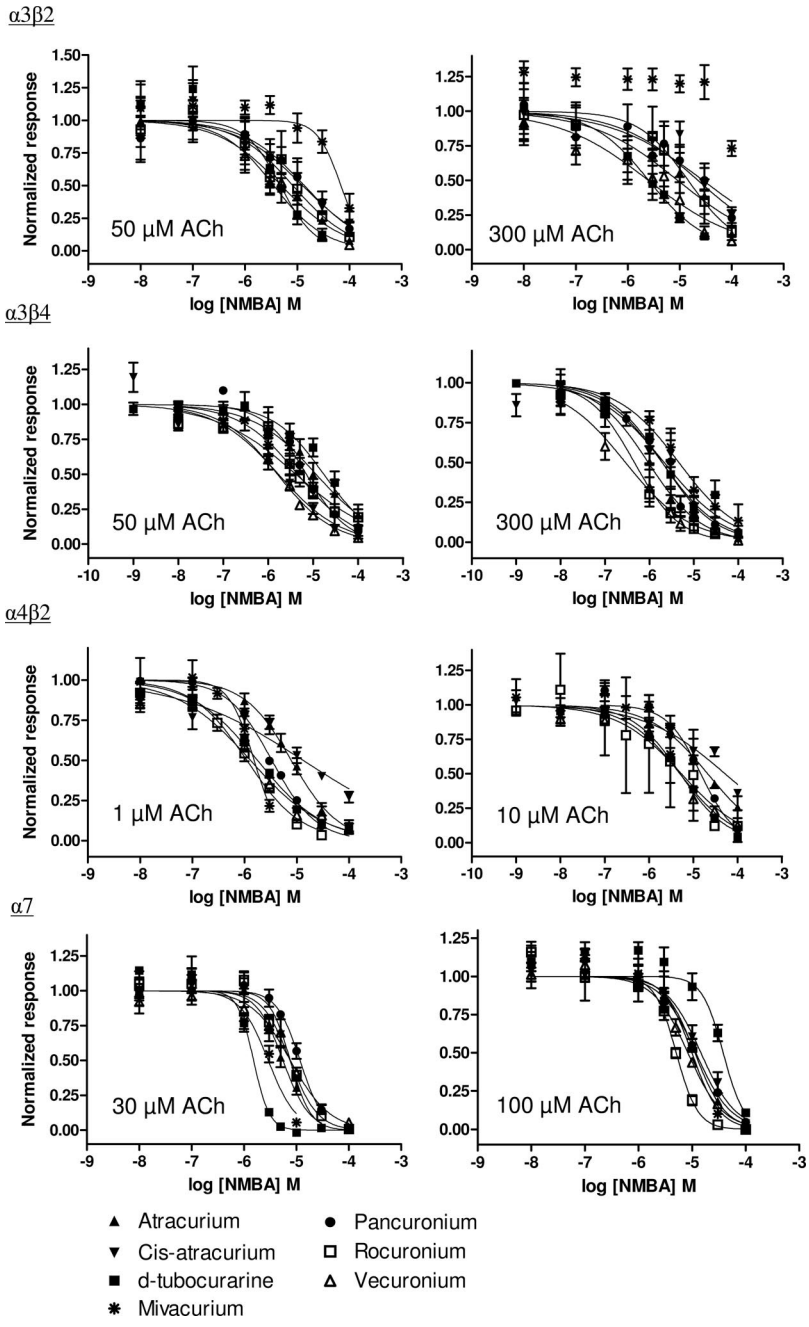

Fig. 4. Concentration–response curves of the inhibition of acetylcholine (ACh)–mediated response by nondepolarizing neuromuscular blocking agents (NMBAs) in human $\alpha_3\beta_2$, $\alpha_3\beta_4$, $\alpha_4\beta_2$, and α_7 nicotinic acetylcholine receptor expressed in *Xenopus* oocytes. For each receptor subtype, two acetylcholine concentrations were applied, one lower and one higher than the EC_{50}: 50 and 300 μM for $\alpha_3\beta_2$ and $\alpha_3\beta_4$, 1 and 10 μM for $\alpha_4\beta_2$, and 30 and 100 μM for α_7. Control acetylcholine peak current or net charge (α_7) responses in each oocyte were normalized to the acetylcholine response with respective nondepolarizing NMBA added, yielding the concentration–response relations. For each receptor subtype, 4–11 oocytes were studied. Data are presented as mean ± SEM. When no *error bars* are seen, they are smaller than the *symbols*.

unit DNA,[6] a small difference in amino acid sequence can cause significant changes in biophysical and pharmacologic properties of the receptors.[18] Therefore, we believe that our study directly comparing the effect of the clinically used NMBAs at the human receptors in the same system adds to the current knowledge on basic pharmacologic properties of nondepolarizing NMBAs.

The mechanisms of the block of neuromuscular transmission by nondepolarizing NMBAs are likely dual; the most important is a postsynaptic block at the $\alpha_1\beta_1\varepsilon\delta$ nAChR subtype, but there is also an inhibition of presynaptic cholinergic receptors.[2,3,46] It has become evident that the tetanic and TOF fade phenomena induced by nondepolarizing relaxants most likely arise from a block of the presynaptic $\alpha_3\beta_2$ nicotinic receptor.[1,2,5] Interestingly, recent data indicate that adenosine and adenosine triphosphate interacting with purinergic receptors also are important in mobilization and release of acetylcholine from the motor nerve ending.[47,48] Here, we can for the first time show that clinically used nondepolarizing NMBAs inhibit the $\alpha_3\beta_2$ nAChR subtype in the micromolar concentration range, thus providing a molecular explanation for the tetanic and TOF fade seen during neuromuscular blockade by nondepolarizing NMBAs. However, mivacurium, which had a lower affinity for the $\alpha_3\beta_2$ nAChR, nonetheless elicited fade, indicated that a block of the $\alpha_3\beta_2$ nAChR is probably not the only mechanism behind tetanic and TOF fade. The reduction in

peak tension and TOF fade seen during neuromuscular monitoring are likely caused by two separate events,[1–3,46] namely the presynaptic and the postsynaptic inhibition by nondepolarizing NMBAs. This also explains the clinical observations that nondepolarizing NMBAs differ in the degree of twitch reduction *versus* tetanic and TOF fade.[49] Furthermore, animal studies of the twitch tension and tetanic fade clearly show that these events are due to two separate mechanisms. Hexamethonium produced a complete tetanic fade without any twitch depression; pancuronium produced tetanic fade in doses that also produced pronounced twitch depression; α-bungarotoxin did not produce tetanic fade but elicited a pronounced twitch depression.[50] Comparing this study to our results, it is clear that nondepolarizing NMBAs have a much higher affinity for the $\alpha_1\beta_1\varepsilon\delta$ nAChR subtype compared with the $\alpha_3\beta_2$ subtype, but still, the IC_{50} values for the $\alpha_3\beta_2$ nAChR subtype are in a clinically relevant range and furthermore correspond roughly to the concentrations that produce a 50% neuromuscular block in *in vitro* animal experiments (1.68–12.3 μM).[40,50] In addition, we have recently shown that succinylcholine, which does not produce tetanic or TOF fade at normal dosage, does not block the $\alpha_3\beta_2$ nAChR subtype at clinically relevant concentrations.[33] The fact that nondepolarizing NMBAs do block the $\alpha_3\beta_2$ nAChR subtype in clinically relevant concentrations, and the fact that succinylcholine does not, strongly support the concept that the clinically observed tetanic and TOF fade are due to a block of the presynaptic $\alpha_3\beta_2$ nAChR subtype.

Based on our results, nondepolarizing NMBAs have the potential to inhibit neuronal nAChRs present in peripheral autonomic ganglia.

It has previously been shown that nondepolarizing NMBAs reduce hypoxic ventilatory response in humans[14,15] and furthermore impair both the hypoxic and nicotine-induced carotid body chemoreceptor response.[16,17,51,52] Neuronal nAChRs have been found to be present and functional in the carotid body and its afferent system.[53–55] We believe that the affinity of NMBAs to the human neuronal subtypes of nAChRs is a key component behind the interaction between nondepolarizing NMBAs and regulation of breathing during hypoxia. We also speculate whether the block of the α_7 nAChR subtype may have an impact on the cholinergic inflammatory reflex mediated *via* the vagus nerve[25,26] and thus on outcome for patients with inflammatory conditions such as sepsis.

In summary, neuronal nAChRs are widespread in the central and peripheral nervous system, as well as in extraneuronal tissues, and a block of these receptors by nondepolarizing NMBAs might interfere with important vital functions.

We conclude that nondepolarizing NMBAs concentration-dependently inhibit human neuronal nAChRs expressed in *Xenopus* oocytes and that the inhibition mechanisms vary between different receptor subtypes and NMBAs. The inhibition of the presynaptic $\alpha_3\beta_2$ nAChR subtype at the motor nerve end provides a possible molecular explanation for the tetanic and TOF fade seen during a nondepolarizing neuromuscular block.

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Appendix 1: Pharmacologic Properties of Human Neuronal nAChRs Activated by Acetylcholine and with the Addition of NMBAs

	$\alpha_3\beta_2$			$\alpha_3\beta_4$			$\alpha_4\beta_2$			α_7		
	EC_{50}, μM	n_H	n	EC_{50}, μM	n_H	n	EC_{50}, μM	n_H	n	EC_{50}, μM	n_H	n
Atracurium												
ACh	214 (52.18–880)	0.60 ± 0.23	12	182 (160–206)	2.20 ± 0.23	6	3.70 (2.94–4.66)	0.83 ± 0.08	8	210 (88.35–498)	1.24 ± 0.58	4
	67.39 (26.67–170)	0.59 ± 0.21	12	183 (145–232)	2.59 ± 0.51	5	3.99 (2.92–5.45)	0.82 ± 0.11	8	57.11 (47.73–68.34)	1.53 ± 0.17	5
ACh + atracurium	150 (15.64–1,441)	0.49 ± 0.31	12	408 (276–604)	1.47 ± 0.35	6	6.44 (4.80–8.64)	1.00 ± 0.13	8	1,677 (21–133,700)	0.80 ± 0.51	4
	71.43 (10.58–482)	0.42 ± 0.29	12	606 (117–3,139)	1.11 ± 0.66	5	6.43 (4.67–8.85)	0.92 ± 0.12	8	94.83 (84.88–107)	2.12 ± 0.29	5
Cisatracurium												
ACh	93.75 (24.76–355)	0.54 ± 0.25	7	235 (207–268)	2.28 ± 0.31	12	1.98 (1.49–2.63)	1.48 ± 0.29	8	213 (138–230)	1.35 ± 0.36	6
	46.83 (16.51–133)	0.45 ± 0.17	7	269 (242–299)	2.31 ± 0.31	12	1.79 (1.35–3.38)	1.45 ± 0.28	8	41.51 (30.26–56.95)	1.21 ± 0.20	3
ACh + cisatracurium	246 (26.35–2,301)	0.40 ± 0.18	7	476 (343–660)	1.92 ± 0.42	12	2.51 (1.94–3.26)	1.30 ± 0.21	8	236 (187–298)	1.87 ± 0.37	6
	10.48 (1.44–76.28)	0.46 ± 0.18	7	838 (493–1,422)	1.15 ± 0.19	12	2.32 (1.88–2.85)	1.40 ± 0.19	8	73.95 (62.85–87.02)	1.64 ± 0.18	3
d-TC												
ACh	318 (54.36–1,827)	0.75 ± 0.37	8	218 (195–243)	2.01 ± 0.19	14	1.62 (1.06–2.47)	0.89 ± 0.15	8	177 (133–237)	2.37 ± 0.53	4
	176 (36.96–835)	0.71 ± 0.37	7	197 (177–220)	2.66 ± 0.26	14	1.96 (1.50–2.57)	1.09 ± 0.15	7	35.65 (29.32–43.36)	1.61 ± 0.22	5
ACh + d-TC	988 (190–5,127)	0.79 ± 0.25	8	457 (310–676)	1.41 ± 0.31	14	7.15 (5.60–9.13)	1.18 ± 0.15	8	502 (44.10–57,130)	2.94 ± 9.2	4
	713 (1.70–298,000)	0.44 ± 0.34	7	560 (168–1,866)	1.10 ± 0.51	14	5.03 (3.98–6.37)	1.24 ± 0.15	7	277 (250–307)	2.14 ± 0.24	5
Mivacurium												
ACh	316 (46.40–2,156)	0.74 ± 0.40	5	233 (199–273)	1.91 ± 0.26	8	2.36 (0.59–9.35)	0.79 ± 0.29	5	233 (170–319)	1.56 ± 0.35	8
	85.18 (33.95–214)	0.69 ± 0.25	5	240 (202–286)	2.11 ± 0.36	7	2.03 (0.69–6.00)	1.05 ± 0.41	5	42.65 (30.20–60.22)	1.18 ± 0.22	7
ACh + mivacurium	345 (56.48–2,112)	0.70 ± 0.33	5	582 (347–949)	1.17 ± 0.28	8	8.43 (2.47–28.74)	0.97 ± 0.59	5	290 (217–388)	1.91 ± 0.52	8
	128 (9.39–1,745)	0.46 ± 0.35	5	860 (481–1,538)	1.36 ± 0.45	7	5.27 (1.80–15.42)	1.17 ± 0.61	5	111 (84.88–145)	1.62 ± 0.33	7
Pancuronium												
ACh	273 (76.41–978)	0.80 ± 0.34	8	300 (278–324)	4.26 ± 3.78	5	3.20 (2.06–4.96)	0.84 ± 0.16	13	96.55 (71.42–130)	2.83 ± 2.29	6
	328.3 (71.13–1,515)	0.59 ± 0.20	7	294 (183–474)	6.56 ± 85.40	5	3.12 (2.26–4.30)	0.91 ± 0.13	14	29.34 (25.40–33.90)	2.68 ± 0.75	4
ACh + pancuronium	546 (155–1,921)	0.75 ± 0.22	8	2,193 (233–20,670)	1.20 ± 0.54	5	10.07 (7.19–14.10)	1.00 ± 0.16	13	175 (99.52–309)	2.18 ± 0.95	5
	82.01 (12.28–548)	0.51 ± 0.35	7	3,966 (9.28–169,400)	0.87 ± 0.56	5	6.89 (5.06–9.37)	1.22 ± 0.20	14	42.47 (31.44–57.37)	1.75 ± 0.37	3
Rocuronium												
ACh	548 (15.29–19,630)	0.55 ± 0.33	11	236 (205–271)	2.17 ± 0.30	8	3.52 (2.28–5.44)	0.82 ± 0.14	4	324 (201–522)	1.12 ± 0.24	4
	73.61 (20.65–245)	0.60 ± 0.30	11	255 (219–296)	2.22 ± 0.37	5	4.35 (2.21–8.55)	1.36 ± 0.47	4	36.65 (30.79–43.63)	2.07 ± 0.35	10
ACh + rocuronium	759 (144–3,987)	0.83 ± 0.32	11	383 (274–534)	2.36 ± 0.87	8	1.96 (1.32–2.90)	1.34 ± 0.31	4	279 (191–409)	1.98 ± 0.75	4
	40.13 (7.28–221)	0.48 ± 0.31	11	1,537 (39.7–59,230)	0.95 ± 0.65	5	2.49 (1.11–5.58)	1.56 ± 0.78	4	66.15 (54.06–80.93)	1.76 ± 0.26	8
Vecuronium												
ACh	551 (65.94–4,597)	0.74 ± 0.36	8	291 (224–378)	2.03 ± 056	8	1.92 (1.28–2.88)	0.91 ± 0.16	4	347 (257–469)	1.39 ± 0.24	7
	53.72 (21.63–133)	0.67 ± 0.25	8	290 (239–352)	2.58 ± 0.90	8	2.92 (2.17–3.92)	0.91 ± 0.12	4	53.88 (41.57–69.83)	1.07 ± 0.14	5
ACh + vecuronium	687 (67.85–6,955)	0.69 ± 0.30	8	1,915 (20.29–180,800)	1.17 ± 1.14	8	7.11 (4.15–12.17)	1.04 ± 0.28	4	290 (215–392)	1.82 ± 0.48	7
	16.43 (3.69–73.15)	0.63 ± 0.30	8	1,294 (17.29–96,780)	2.55 ± 11.44	8	8.00 (5.81–11.03)	1.04 ± 0.16	4	164 (144–186)	1.10 ± 0.07	5

Shaded areas = peak, white areas = net charge.
ACh = acetylcholine; CI = confidence interval; d-TC = d-tubocurarine; EC_{50} = Half activation concentration; nAChR = nicotinic acetylcholine receptor; n_H = Hill coefficient; NMBA = neuromuscular blocking agent.

Appendix 2: Pharmacologic Properties of NMBAs as Inhibitors of Acetylcholine-induced Activation of Human Neuronal nAChRs Expressed in *Xenopus* Oocytes

	Atracurium			Cisatracurium			d-Tubocurarine			Mivacurium		
	IC_{50}, μM	n_H	n	IC_{50}, μM	n_H	n	IC_{50}, μM	n_H	n	IC_{50}, μM	n_H	n
$\alpha_1\beta_1\varepsilon\delta$ (nM)												
10 μM ACh	95.65 (73.89–123.80)	−1.01 ± 0.12	4	18.19 (12.64–26.16)	−0.76 ± 0.10	4	18.73 (13.87–25.30)	−0.78 ± 0.08	5	3.69 (2.22–6.15)	−0.77 ± 0.12	4
	67.36 (47.36–95.80)	−0.82 ± 0.11	4	17.01 (11.95–24.21)	−0.80 ± 0.11	4	15.85 (10.92–23.01)	−0.84 ± 0.12	5	2.96 (2.11–4.17)	−0.78 ± 0.08	4
$\alpha_3\beta_2$												
50 μM ACh	4.99 (3.08–8.09)	−0.72 ± 0.12	4	12.68 (5.16–31.16)	−0.69 ± 0.23	6	4.78 (2.93–7.81)	−1.29 ± 0.46	6	69.04 (46.41–103)	−1.93 ± 0.58	8
	20.42 (11.55–36.12)	−0.96 ± 0.25	4	19.30 (11.31–32.96)	−0.47 ± 0.07	6	4.01 (3.34–4.81)	−1.09 ± 0.12	6	43.59 (22.00–86.34)	−0.42 ± 0.07	8
300 μM ACh	9.24 (3.72–22.97)	−0.52 ± 0.14	2	28.34 (10.23–78.45)	−0.49 ± 0.14	5	2.55 (1.42–4.55)	−0.80 ± 0.21	7	Do not fit		9
	9.61 (6.81–13.55)	−0.65 ± 0.08	3	62.08 (24.89–154.9)	−0.49 ± 0.12	3	1.44 (1.10–1.88)	−0.58 ± 0.05	7	33.31 (23.36–47.51)	−0.66 ± 0.08	9
$\alpha_3\beta_4$												
50 μM ACh	11.65 (7.63–17.79)	−0.61 ± 0.09	6	1.69 (1.21–2.36)	−0.62 ± 0.0.6	10	19.78 (13.18–29.70)	−0.89 ± 0.15	12	3.71 (2.56–5.39)	−0.68 ± 0.11	4
	4.75 (3.12–7.23)	−0.65 ± 0.10	6	0.59 (0.36–1.00)	−0.48 ± 0.05	10	14.30 (9.61–21.26)	−1.07 ± 0.20	12	3.71 (2.56–5.39)	−0.43 ± 0.06	4
300 μM ACh	0.94 (0.64–1.38)	−0.73 ± 0.09	6	1.94 (1.27–2.98)	−0.73 ± 0.10	8	2.14 (1.27–3.62)	−0.59 ± 0.09	9	4.58 (2.73–6.69)	−0.63 ± 0.11	5
	0.32 (0.18–0.57)	−0.63 ± 0.09	4	0.40 (0.22–0.71)	−0.47 ± 0.06	8	0.76 (0.42–1.38)	−0.74 ± 0.14	9	1.04 (0.74–1.46)	−0.79 ± 0.10	5
$\alpha_4\beta_2$												
1 μM ACh	7.89 (6.38–9.76)	−0.96 ± 0.10	6	13.24 (7.61–23.06)	−0.36 ± 0.05	7	1.77 (1.47–2.14)	−0.68 ± 0.04	8	1.52 (1.10–2.10)	−1.82 ± 0.07	3
	5.43 (3.30–8.95)	−0.87 ± 0.22	6	1.98 (0.96–4.10)	−0.30 ± 0.04	7	8.20 (6.44–10.40)	−0.66 ± 0.05	8	1.17 (0.77–1.76)	−2.08 ± 0.08	3
10 μM ACh	21.07 (15.84–28.02)	−0.67 ± 0.07	8	66.74 (28.38–156.9)	−0.43 ± 0.09	7	5.06 (4.31–5.94)	−0.81 ± 0.05	7	Do not fit		4
	10.44 (7.15–15.24)	−0.51 ± 0.06	8	16.00 (7.84–32.65)	−0.42 ± 0.08	7	3.29 (2.60–48.17)	−0.79 ± 0.07	7	Do not fit		4
α_7												
30 μM ACh	5.56 (4.18–7.40)	−1.73 ± 0.45	4	6.19 (5.36–7.16)	−1.27 ± 0.11	5	1.52 (1.31–1.77)	−2.87 ± 0.40	4	2.90 (2.22–3.78)	−1.61 ± 0.33	8
100 μM ACh	13.03 (8.96–18.94)	−1.62 ± 0.35	5	14.62 (8.77–24.37)	−1.34 ± 0.42	6	38.11 (28.61–50.76)	−2.20 ± 0.63	5	9.92 (8.00–12.30)	−1.67 ± 0.26	8

(continued)

Appendix 2: Continued

	Pancuronium			Rocuronium			Vecuronium		
	IC_{50}, μM	n_H	n	IC_{50}, μM	n_H	n	IC_{50}, μM	n_H	n
$\alpha_1\beta_1\varepsilon\delta$ (nM)									
10 μM ACh	13.17 (7.88–22.02)	−0.95 ± 0.22	7	13.76 (10.57–17.91)	−0.70 ± 0.06	7	10.74 (5.48–21.06)	−0.58 ± 0.11	6
	7.11 (4.45–11.36)	−0.78 ± 0.13	7	18.64 (12.06–28.81)	−0.75 ± 0.12	5	8.02 (4.39–14.64)	−0.76 ± 0.15	3
$\alpha_3\beta_2$									
50 μM ACh	13.71 (6.92–27.17)	−0.74 ± 0.20	8	8.30 (3.54–19.48)	−0.78 ± 0.29	7	3.55 (2.32–5.43)	−0.87 ± 0.17	7
	7.84 (4.82–12.74)	−0.43 ± 0.06	8	8.99 (6.38–12.66)	−0.77 ± 0.08	7	3.32 (2.32–4.74)	−0.59 ± 0.07	7
300 μM ACh	22.67 (7.51–68.44)	−0.57 ± 0.21	8	14.63 (5.87–36.46)	−0.91 ± 0.33	6	2.52 (0.97–6.57)	−0.50 ± 0.14	7
	13.88 (7.31–26.38)	−0.39 ± 0.07	8	11.82 (8.74–15.97)	−0.64 ± 0.07	6	1.29 (0.58–2.88)	−0.42 ± 0.08	7
$\alpha_3\beta_4$									
50 μM ACh	7.06 (5.07–9.83)	−0.92 ± 0.15	3	4.12 (2.80–6.08)	−0.49 ± 0.06	6	1.60 (1.35–1.91)	−0.69 ± 0.04	5
	9.60 (5.36–17.21)	−0.90 ± 0.24	4	2.77 (1.83–4.19)	−0.67 ± 0.10	6	0.97 (0.62–1.54)	−0.59 ± 0.07	5
300 μM ACh	1.92 (0.94–3.93)	−0.67 ± 0.16	6	0.46 (0.32–0.66)	−0.86 ± 0.10	5	0.29 (0.15–0.56)	−0.59 ± 0.09	11
	0.53 (0.31–0.92)	−0.84 ± 0.17	6	0.18 (0.13–0.25)	−0.80 ± 0.07	5	0.13 (0.09–0.18)	−1.12 ± 0.20	11
$\alpha_4\beta_2$									
1 μM ACh	3.34 (3.06–3.65)	−1.03 ± 0.05	6	1.11 (0.85–1.44)	−0.83 ± 0.08	5	1.30 (0.88–1.90)	−0.62 ± 0.07	8
	2.64 (2.25–3.10)	−1.09 ± 0.10	6	0.71 (0.47–1.06)	−0.72 ± 0.10	5	0.33 (0.19–0.58)	−0.49 ± 0.06	8
10 μM ACh	14.70 (12.52–17.26)	−1.14 ± 0.08	6	5.37 (0.83–34.89)	−0.61 ± 0.34	3	4.98 (3.65–6.79)	−0.71 ± 0.08	3
	13.29 (10.46–16.88)	−1.33 ± 0.14	6	2.28 (0.42–12.47)	−0.51 ± 0.23	3	2.65 (1.83–3.83)	−0.86 ± 0.13	3
α_7									
30 μM ACh	13.33 (10.40–17.09)	−1.91 ± 0.41	5	7.55 (6.24–9.14)	−1.65 ± 0.22	8	7.70 (6.05–9.78)	−1.16 ± 0.16	9
100 μM ACh	11.76 (9.70–14.26)	−1.32 ± 0.16	8	5.06 (4.35–5.89)	−2.18 ± 0.34	6	8.38 (7.29–9.63)	−1.34 ± 0.12	8

Shaded areas shows pharmacologic data based on peak responses, whereas white areas shows net charge (area under the curve). Negative Hill coefficient (n_H) is a result of inhibition.

ACh = acetylcholine; CI = confidence interval; IC_{50} = half inhibition concentration; nAChR = nicotinic acetylcholine receptor; NMBA = neuromuscular blocking agent.