BJP BRITISH JOURNAL OF PHARMACOLOGY
BPS BRITISH PHARMACOLOGICAL SOCIETY

BJP British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2010.00904.x
www.brjpharmacol.org

RESEARCH PAPER

Post-junctional interactions between neuromuscular blocking agents and ethanol at the mouse neuromuscular junction

TJ Searl and EM Silinsky

Department of Molecular Pharmacology and Biological Chemistry (MPBC), Northwestern University Medical School, Chicago, IL, USA

Correspondence
TJ Searl, Department of Molecular Pharmacology and Biological Chemistry (MPBC), Northwestern University Medical School, 303 East Chicago Avenue, Chicago, IL 60611 USA. E-mail: tjs026@northwestern.edu

Keywords
vecuronium; cisatracurium; neurotransmission; non-depolarizing blockers

Received
26 February 2010
Revised
21 April 2010
Accepted
27 April 2010

BACKGROUND AND PURPOSE
Ethanol is known to have both pre-synaptic and post-synaptic effects at a range of loci in the mammalian nervous system, including the neuromuscular junction. However, the effects of ethanol on evoked synaptic transmission have not been previously studied at the mouse neuromuscular junction. Here, we report on the effects of ethanol on evoked neuromuscular transmission and the interaction of ethanol with non-depolarizing blocking drugs.

EXPERIMENTAL APPROACH
Electrophysiological techniques to measure synaptic potentials and synaptic currents were employed in this study.

KEY RESULTS
Ethanol (≥100 mM) produced increases in the amplitudes of both spontaneous and evoked synaptic events. Under conditions in which neuromuscular transmission was blocked by (+)-tubocurarine, ethanol (12–100 mM) produced greater increases in evoked response amplitude than in spontaneous response amplitude recorded in the absence of (+)-tubocurarine. Ethanol (100 mM) did not affect evoked neurotransmitter release in low-calcium/high-magnesium solutions. With respect to the clinically used neuromuscular blocking drugs, ethanol (100 mM) interfered with the blocking action of vecuronium, but not cisatracurium.

CONCLUSIONS AND IMPLICATIONS
Under these conditions, the stimulant effect of ethanol on neuromuscular transmission is exclusively on the post-junctional elements, both to enhance transmission through nicotinic receptors and also via interactions with neuromuscular blocking agents. These actions of ethanol on neuromuscular transmission may affect the dosage of neuromuscular blockers required in patients who have imbibed significant amounts of alcohol.

Abbreviations
EPC, end-plate current; EPP, end-plate potential; MEPP, miniature end-plate potential

Introduction

Early studies on the effects of ethanol on neuromuscular transmission revealed that low concentrations of ethanol were capable of increasing neuromuscular transmission by 'decurarizing' neuromuscular junctions (Feng and Li, 1941). Gage (1965) found that a combination of pre-junctional and post-junctional effects contributed towards the effects of ethanol on neuromuscular transmission in the rat, with increases in both miniature end-plate potential (MEPP) amplitudes (a post-junctional effect) and increases in evoked ACh release (a pre-junctional effect) being reported. The pre-junctional effect was reflected as increases in end-plate potential (EPP) amplitudes relative to MEPP amplitudes (i.e. as

increases in the number of quanta released). Gage (1965) also reported that increases in the input resistance of the muscle membrane contributed to post-junctional potentiation of neuromuscular transmission by ethanol. Ethanol also had effects on muscle nicotinic receptors, increasing the time constant of decay of miniature end-plate currents (MEPCs), but without anti-cholinesterase activity (Gage, 1965; Gage *et al.*, 1975; Bradley *et al.*, 1984). This increase in the MEPC time constant of decay is mediated by increases in the burst length of nicotinic receptor channel openings and has been suggested to contribute towards the increases in MEPP and EPP amplitudes produced by ethanol. It has also been reported that the potentiation of MEPC amplitudes by ethanol might be greater in preparations where MEPCs have been diminished by nicotinic receptor blockade (Linder *et al.*, 1984), although the precise mechanism underlying this effect is not clear.

As a consequence of differing experimental conditions and ethanol concentrations, the proportionate contributions made by the pre-junctional and post-junctional effects of ethanol on neuromuscular transmission are not known. Interpretations of the effects of ethanol on neuromuscular transmission may also be confounded by potential interactions between ethanol and neuromuscular blocking agents (Linder *et al.*, 1984). Such interactions between ethanol and neuromuscular blockers may have clinical, as well as experimental, implications. Indeed, despite the widespread use of mouse models to study synaptic transmission, no studies on the effects of ethanol on neurally evoked neuromuscular transmission have been reported in the mouse. Thus, the purpose of this paper was to examine the effects of ethanol on neuromuscular transmission at the mouse neuromuscular junction with a view to addressing these issues. These results suggest that low concentrations of ethanol produce exclusively post-junctional effects on evoked neuromuscular transmission, as well as complex interactions with selected neuromuscular blocking agents that are in widespread clinical use.

Methods

Animals and electrophysiological methods

Experiments were performed on isolated mouse neuromuscular junctions. Mice (B6129F2J, 20–30 g in weight) were humanely anaesthetized with 5% isoflurane for 3–5 min, until unresponsive to touch, followed by cervical dislocation and exsanguination. This method is in accordance with guidelines laid down by our institutional animal welfare committee and the National Institutes of Health. The phrenic nerve–hemidiaphragm was isolated and pinned out in a recording chamber. Solutions were delivered by superfusion with a peristaltic pump and removed by vacuum suction. Experiments were carried out at room temperature (22–24°C). Intracellular recordings were made using microelectrodes filled with 3 M KCl with resistances 3–10 MΩ. Recordings were made with an Axoclamp-2A amplifier (Axon Instruments, Sunnyvale, CA, USA) using either single-electrode voltage recordings for potentials or conventional two-electrode voltage clamp techniques for currents. Iontophoresis was performed using 0.5 M ACh in the iontophoretic pipette. Both the pulse duration and the strength of iontophoretic pulses were varied between 1 and 7 ms and 50–150 nA, depending on individual electrode placement, in order to obtain consistent depolarizations (ranging from 3 to 5 mV in control) that were below threshold for muscle action potential generation. For determining the effects of ethanol on the muscle membrane resistance, a simple two-electrode methodology was adopted. Briefly, a current-injecting electrode and a voltage-recording electrode were placed in the end-plate region of muscle cells under visual control with an approximate 50 μm separation. Square-wave hyper-polarizing current pulses (5 nA; 20 ms duration) were applied through the current electrode, and the potential change was recorded with the voltage-recording electrode. In experiments made at low levels of release (using low-calcium/high-magnesium solutions; see below), the phrenic nerve was stimulated at a frequency of 5 Hz. In normal calcium solutions, the frequency of stimulation was reduced to 0.05 Hz to avoid complications produced by the co-release of endogenous adenosine derivatives with ACh at normal levels of neurotransmitter output (Redman and Silinsky, 1994). Signals were fed into a personal computer using a Digidata 1200 A/D converter (Axon Instruments). Responses were analysed using CDR, WCP and SCAN programs (Strathclyde University Software; John Dempster). The data were analysed using Microsoft Excel, Corel Quattro Pro and Sigma Plot and Sigma Stat software packages (SPSS Inc., Chicago, IL, USA). For all experimental treatments, recordings were made from single end plates with each individual fibre serving as its own control. The number of ACh quanta released by a nerve impulse was calculated by the direct method, namely the ratio of the average EPP amplitude to the average MEPP amplitude (for specific details, see Searl and Silinsky, 2008). In mammalian preparations, the threshold for firing muscle action potentials is quite low (5–10 mV). The concentrations of

neuromuscular blockers used here were chosen such that they reduced muscle EPPs sufficiently below threshold for muscle contraction to allow for increases in EPP amplitude without muscle twitch. For experiments with α-bungarotoxin, preparations were incubated with 1.5 μM α-bungarotoxin for 45–75 min, until muscle contraction was eliminated as visualized under the microscope, and stable sub-threshold EPPs were measurable.

Drugs and solutions

Control physiological saline solution (pH 7.2–7.4) consisting of (in mM): NaCl, 137; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 2; HEPES, 10; dextrose, 11, was used unless otherwise stated. For low-calcium/high-magnesium solution, $CaCl_2$ was 0.35 mM and $MgCl_2$ was 3.5 mM. (+)-Tubocurarine and α-bungarotoxin were purchased from the Sigma Chemical Company (St Louis, MO, USA); vecuronium bromide was from USP (Rockville, MD, USA); and cisatracurium was a gift from Dr Christine Stock, Chairperson of Anesthesiology, Northwestern University. All other chemicals were purchased from VWR (Westchester, PA, USA).

Statistical methods

Comparisons were made by either parametric statistics (e.g. Student's paired *t*-test) or non-parametric statistics (Mann–Whitney rank sum test, see Glantz, 1992). When more than two groups were compared, an ANOVA for the normally distributed data was followed by multiple comparisons using the Bonferroni inequality (see Glantz, 1992, p. 93). For discussion of the results, differences between groups were considered significant when $P < 0.05$. Unless otherwise stated, *n* represents the number of single experiments carried out at single end plate on individual preparations. Data are presented as means ± 1 SEM.

All drug/molecular target nomenclature conforms to the standards stated in the *British Journal of Pharmacology*'s *Guide to Receptors and Channels* (Alexander *et al.*, 2009), where applicable.

Results

Post-junctional effects of ethanol: effects on MEPP and MEPC amplitudes

A useful measure for changes in post-junctional sensitivity at the neuromuscular junction is the amplitude of the spontaneous MEPPs. As shown in Figure 1, application of ethanol resulted in highly significant concentration-dependent increases in the amplitude of MEPPs. At 100 mM ethanol, MEPP amplitudes were 124 ± 7.2% of control, and at 400 mM ethanol MEPP amplitudes were increased to 175 ± 11.9% ($n = 5$) of control (see Figure 1A). These effects on MEPP amplitudes were entirely post-synaptic and not due to pre-junctional changes in the size of the ACh quantum as depolarizing responses to the iontophoretic application of ACh were increased to a similar extent by 400 mM ethanol (201 ± 10.8% of control, $n = 6$). The use of two-electrode voltage clamp techniques eliminates both the effects of membrane resistance and

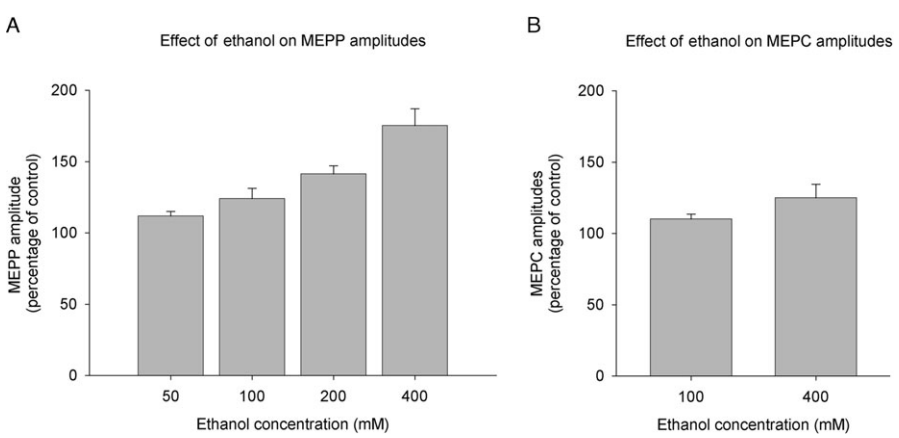

Figure 1

Comparison of the effects of ethanol on MEPP and MEPC amplitudes. The effects of ethanol on (A) MEPP and (B) MEPC amplitudes. The membrane potentials for experiments in (A) were between –60 and –70 mV. In (B), the membrane potential was held at –60 mV. Increases in MEPP amplitude were significantly greater than those for MEPCs, such that at 400 mM ethanol MEPP amplitudes were 175 ± 11.9% ($n = 5$) of control versus 124.9 ± 9.5% ($n = 6$) of control for MEPC amplitudes ($P = 0.009$). In addition to its effect on MEPP amplitudes, ethanol also increased MEPP frequencies. Specifically, MEPP frequency was increased from 0.69 ± 0.18 Hz in control to 1.09 ± 0.28 in 100 mM ethanol ($n = 5$; $P = 0.04$).

membrane capacitance on post-junctional measurements. The effects of ethanol on voltage-clamped MEPCs were thus examined. Under these conditions, the increases in MEPC amplitudes were 110.1 ± 3.4% of control in 100 mM ethanol, and 124.9 ± 9.5% of control in 400 mM ethanol (see Figure 1B). In addition to effects on MEPC amplitudes, ethanol prolonged the MEPC time constant of decay (τ). The MEPCτ was 1.03 ± 0.09 ms in control; 1.41 ± 0.08 ms in 100 mM ethanol; 3.64 ± 0.15 ms in 400 mM ethanol ($P < 0.05$ for each). These are similar to the values reported by Linder *et al.* (1984) for the mouse neuromuscular junction.

It had previously been reported that changes in the input resistance of the muscle cell membrane contribute towards increases in MEPP amplitude (Gage, 1965; 1976). The electrical properties of the muscle membrane may be described as a cable with both input resistance and capacitance (i.e. as an RC circuit; see e.g. Hubbard *et al.*, 1969). Using a two-electrode technique (see Methods), no significant effect of 400 mM ethanol on the muscle input resistance was found. Specifically, the input resistance during ethanol treatment was 102.6 ± 8.8% of control ($n = 5$ experiments, $P = 0.89$). Thus, a more likely explanation for the difference in effects of ethanol between voltage-clamped and -unclamped muscle is the effect of the capacitance properties of the muscle membrane on the voltage signal, as this property would also be eliminated by voltage clamping. Specifically, the brief current transients associated with the MEPC and EPCs are not able to fully charge the membrane capacitance of the unclamped muscle. Prolongation of the underlying EPC by ethanol allows for greater charging of the RC circuit of the unclamped muscle membrane, and hence produces a greater effect on the potential change than might be predicted from the underlying current (Gage and McBurney, 1973; see also Steinbach, 1968, Figure 4 for an illustration of this effect).

Thus, the greater effect of ethanol on MEPP amplitudes compared with MEPC amplitudes may be attributed to the effect of ethanol on slowing the decay rate of the underlying current.

Potential pre-junctional effects: the effects of ethanol on (+)-tubocurarine-blocked EPPs

The effects of ethanol on evoked responses were investigated in an attempt to determine if there are any pre-junctional effects of ethanol at normal levels of ACh release. For these initial experiments, (+)-tubocurarine (3.5 μM) was used to reduce the EPP to below threshold and allow recording of EPPs unencumbered by superimposed action potentials. As shown in the example in Figure 2, application of ethanol increased the average EPP amplitude in (+)-tubocurarine. The lowest concentration at which an effect on EPP amplitudes was detectable was 12 mM, but large effects were observed at 50 mM (Figure 2). In all experiments with (+)-tubocurarine, 50 mM ethanol increased EPPs to 166.8 ± 9.4% of control levels ($n = 5$), and EPP amplitudes were increased to 218.4 ± 14.5% of control by 100 mM ethanol ($n = 5$). The increases in EPP amplitude caused by 100 mM ethanol were significantly greater than the increases in MEPP amplitudes ($P < 0.001$). As the ratio of the EPP to the MEPP is a reliable indicator of the level of quantal ACh release, the results using this approach with (+)-tubocurarine-blocked preparations suggest that, in addition to post-junctional effects, there is also a pre-junctional component in the actions of ethanol. For the remainder of the study, 100 mM ethanol was used for comparison with our other results.

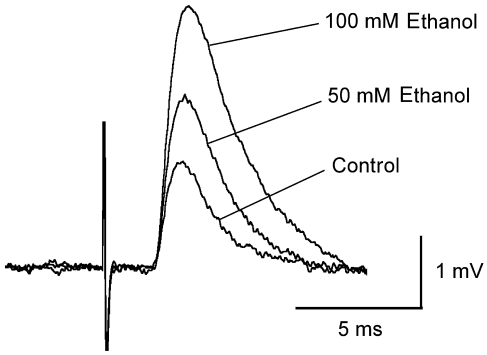

Figure 2

Effect of ethanol on EPPs in preparations paralysed with (+)-tubocurarine. Shown here are averaged records ($n = 8$) of EPPs recorded in the presence of (+)-tubocurarine in control, 50 and 100 mM ethanol, from a representative experiment (in the example shown, 50 mM ethanol increased EPP amplitude to 154% of control ($n = 8$ stimuli averaged), and 100 mM ethanol increased EPP amplitudes to 241% of control ($n = 8$ stimuli averaged; see text for further details). Note that these increases in EPP amplitude are much greater than those predicted from the effects of these concentrations of ethanol on MEPP amplitudes. The resting membrane potential was between −66 and −68 mV throughout the duration of the experiment.

Potential pre-junctional effects: the effects of ethanol on low-calcium/high-magnesium EPPs

In order to assess potential pre-junctional effects of ethanol on neurotransmitter release, a low-calcium/high-magnesium physiological solution was used. This solution reduces the level of evoked neurotransmitter release such that changes in the number of quanta released, as calculated from the ratio of the EPP amplitudes to MEPP amplitudes, can be measured simultaneously at the same end plate.

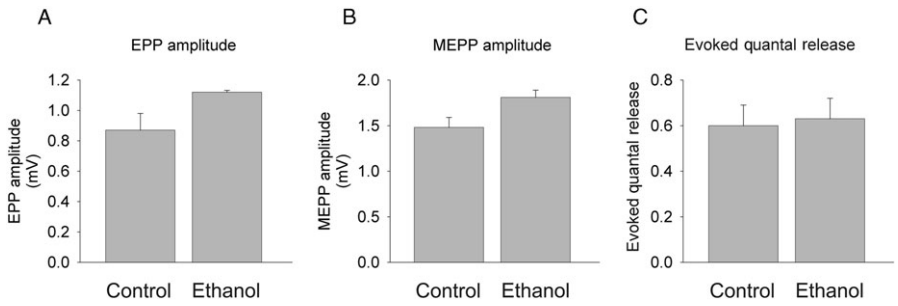

Figure 3

Absence of effects of ethanol (100 mM) on quantal release of acetylcholine. Effects of ethanol on EPP amplitudes (A), MEPP amplitudes (B) and the mean number of ACh quanta released (evoked quantal release, C) are depicted in the graphs. EPP amplitudes were increased from 0.87 ± 0.11 mV in control to 1.12 ± 0.13 mV in 100 mM ethanol ($P = 0.04$; $n = 5$). The average MEPP amplitudes were increased from 1.48 ± 0.11 mV in control to 1.81 ± 0.08 mV in 100 mM ethanol ($P = 0.021$; $n = 5$). The number of evoked quanta released was unchanged (0.60 ± 0.09 quanta in control vs. 0.63 ± 0.09 quanta in 100 mM ethanol ($n = 5$).

Under these conditions, it was found that 100 mM ethanol had no significant effect on neurotransmitter release. Specifically, as shown in Figure 3A, the averaged EPP amplitudes were increased from 0.87 ± 0.11 mV in control to 1.12 ± 0.13 mV in 100 mM ethanol, and the average MEPP amplitudes (Figure 3B) were increased from 1.48 ± 0.11 mV in control to 1.81 ± 0.08 mV in 100 mM ethanol. The number of quanta of ACh released in response to stimulation was unchanged (0.60 ± 0.09 quanta in control vs. 0.63 ± 0.09 quanta in 100 mM ethanol; Figure 3C).

The lack of a measurable pre-junctional effect of ethanol in these experiments was not immediately reconcilable with the results previously found in (+)-tubocurarine-blocked preparations. One possibility is that pre-junctional effects of ethanol only occur at more physiological levels of release than at the low levels of release measured in these low-calcium/high-magnesium experiments. However, we know of no other agent that can selectively increase the number of evoked quanta released at physiological levels of release without affecting release in magnesium-blocked preparations (see e.g. Ginsborg and Jenkinson, 1976). Alternatively, it seemed possible that the effects of ethanol recorded in the (+)-tubocurarine-blocked preparations might be caused by an interaction between (+)-tubocurarine and ethanol, causing a reduction in the potency or effectiveness of (+)-tubocurarine. These possibilities were tested by comparing the effects of ethanol on EPCs using an array of different neuromuscular blocking agents.

Interactions of ethanol with different post-junctional neuromuscular blockers

In order to eliminate the contribution that muscle capacitance and changes in muscle cell input resistance might make towards the measured responses, voltage clamp techniques were used for these experiments. The effects of ethanol on EPC amplitudes recorded in the presence of (+)-tubocurarine (3.5 μM) were measured first. As previously found in the experiments on EPPs, application of 100 mM ethanol resulted in a substantial increase in EPC amplitude in the presence of (+)-tubocurarine (Figure 4). This increase in EPC amplitude (187.8 ± 5.5% of control, $n = 7$) was significantly greater than that seen for MEPCs recorded in the absence of any neuromuscular blocking agent (Figure 4, first column 110 ± 3.4% of control, $n = 6$, $P < 0.001$), confirming the previous results with EPPs.

The potential for interactions between ethanol and a post-junctional blocking agent is likely to be greatly reduced if an irreversible antagonist at nicotinic receptors is used rather than a reversible competitive inhibitor. The irreversible neuromuscular blocking agent α-bungarotoxin was employed to record EPPs and examine its potential interaction with ethanol. As shown in Figure 4, application of 100 mM ethanol in the α-bungarotoxin-paralysed preparations resulted in a small increase in EPC amplitude (117.2 ± 3.1% of control, $n = 7$), which is not significantly different from that seen for the MEPC amplitudes recorded in the absence of any neuromuscular blocking agent.

This result suggests that the effects of ethanol on nerve-evoked EPC amplitude were dependent on the neuromuscular blocking agent used. Therefore, the effects of ethanol on EPCs recorded in the presence two non-depolarizing blockers in widespread clinical use, vecuronium (2 μM) and cisatracurium (8 μM), were compared (see Figure 4). In the presence of vecuronium, 100 mM ethanol increased the EPC amplitudes to 183.3 ± 12.6% of control ($n = 8$, $P < 0.001$). This increase was similar to that observed

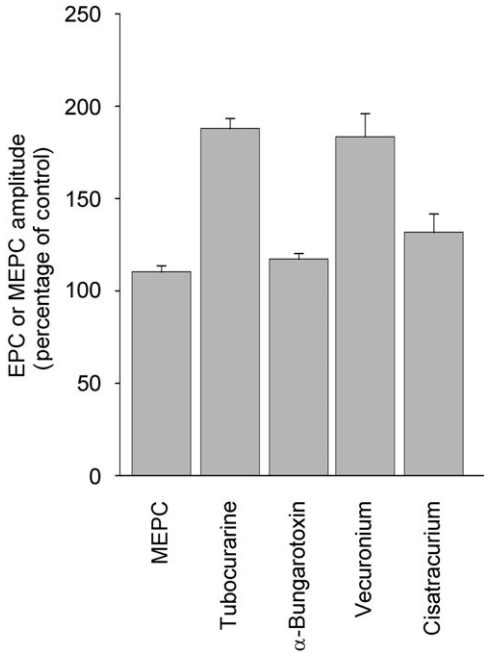

Figure 4

Comparison of the effects of ethanol on EPC amplitudes recorded in the presence of different neuromuscular blocking agents. The MEPC amplitudes (recorded in the absence of neuromuscular blocking agents, first column) and EPCs recorded in the presence of the competitive neuromuscular blocking agents (+)-tubocurarine, vecuronium and cisatracurium, and the irreversible neuromuscular blocking agent α-bungarotoxin. As shown here, in preparations where either cisatracurium or α-bungarotoxin was used to paralyse the muscle, increases in EPC amplitude following application of ethanol (100 mM) were similar to the increase in MEPC amplitude. In preparations with either (+)-tubocurarine or vecuronium, 100 mM ethanol had a much greater effect, resulting in an approximate doubling in EPC amplitude. These results demonstrate that the magnitude of the effect of ethanol on EPC amplitudes is dependent on the neuromuscular blocking agent used. For further details, see text.

when (+)-tubocurarine was used as the neuromuscular blocking agent (187.8%, $P > 0.05$). In contrast, the increase in EPC amplitude seen with cisatracurium was significantly less (127.1 ± 12.5% of control), and statistically indistinguishable from the change seen in α-bungarotoxin-paralysed preparations. Thus, these experiments suggest that the magnitude of the effects of 100 mM ethanol on EPCs in the mouse is dependent on the neuromuscular blocker employed.

Discussion and conclusions

The decurarizing effect of ethanol on neuromuscular transmission was first described by Feng and Li (1941). The suggested mechanism underlying the decurarizing effects of ethanol have included increases in input muscle resistance (Gage, 1965), increases in neurotransmitter release (Gage, 1965) and increases in nicotinic channel open time (Linder *et al.*, 1984). In this study, we attempted to clarify the mechanisms by which moderate concentrations of ethanol affect neuromuscular transmission. As shown here, concentrations of ethanol that have no effect on neurotransmitter release enhance neuromuscular transmission in three separate ways: (i) a modest increase in MEPC amplitude; (ii) increases in the MEPC time constant of decay, an effect consistent with the known effects of ethanol on nicotinic receptor channel burst length; and (iii) increases in EPC amplitudes in preparations blocked by either (+)-tubocurarine or vecuronium. Indeed, in the presence of either of these two agents, a far greater potentiation of EPC amplitudes was observed than would be predicted based on the effects of ethanol on MEPC amplitudes in the absence of these blocking drugs.

When either (+)-tubocurarine (or another neuromuscular blocking drug) is used to reduce the EPP to below threshold for firing an action potential, changes in EPP or EPC amplitude produced by a pharmacological agent are often assumed to reflect changes in neurotransmitter release when MEPP amplitudes are unchanged by this agent (the MEPPs being recorded in the absence of a blocking drug). However, as found here, because of an interaction between the blocking drug and the released neurotransmitter, the effects of 100 mM ethanol on EPC amplitudes in the presence of (+)-tubocurarine were not reflected in changes in the evoked quantal release of high-magnesium/low-calcium concentration-blocked preparations. (Concentrations of ethanol >100 mM increased evoked ACh release; the mechanism of this effect will be described in a subsequent paper.)

Comparison of the effects of ethanol on EPCs in preparations blocked with (+)-tubocurarine, vecuronium, α-bungarotoxin or cisatracurium revealed substantial increases in EPCs by ethanol in those blocked with (+)-tubocurarine or vecuronium. In contrast, the presence of cisatracurium or α-bungarotoxin did not influence the effect of ethanol, as the increases in EPCs produced by ethanol in the presence of these agents were statistically comparable to the increases in MEPC amplitudes observed in the absence of neuromuscular blockade. (+)-Tubocurarine, vecuronium and cisatracurium are competitive, reversible neuromuscular blockers, whereas α-bungarotoxin is a non-competitive irreversible nicotinic blocker. Thus, the level of neuromuscular blockade in α-bungarotoxin-paralysed preparations is unlikely to be affected by ethanol in these experiments, allowing us to measure the effects of ethanol on EPCs without

regard to changes in antagonist potency and at normal levels of ACh release. From these results, it is clear that 100 mM ethanol has no effect on neurotransmitter secretion at physiological levels of ACh release. Indeed, the effects of ethanol on EPCs in preparations paralysed by either (+)-tubocurarine or vecuronium are directed to reducing the potency of these agents as nicotinic receptor blocking drugs.

The mechanisms responsible for the reduced potency of (+)-tubocurarine and vecuronium in the presence of ethanol are unknown. One possibility is that ethanol acts on the nicotinic receptor itself to reduce the binding of those antagonists. Indeed, it has been reported that, in addition to effects on the rate constant of decay of the EPC, an increase in the apparent affinity of ACh occurs in the presence of ethanol at the neuromuscular junction (Bradley *et al.*, 1984). As the level of inhibition by a competitive inhibitor is reduced by higher agonist concentrations, there exists a theoretical possibility that the apparent antagonist affinity for the receptor might be modulated in the opposite way by ethanol (see e.g. Linder *et al.*, 1984). Furthermore, the rate of dissociation of the agents that appear to compete with ethanol [(+)-tubocurarine and vecuronium] differs dramatically with those of cisatracurium (Demazumder and Dilger, 2001) and the irreversible agent α-bungarotoxin. Our results might also be attributed to effects on the nicotinic receptor/ion channel complex. For example, current literature suggests that, in addition to competitive receptor blockade, the ion channel itself is blocked by (+)-tubocurarine (Katz and Miledi, 1978; Colquhoun *et al.*, 1979) and vecuronium (Shinozaki and Ishida, 1984), but not α-bungarotoxin or cisatracurium. Thus, actions at the receptor and at the ionic channel incorporated within the receptor could work synergistically to explain the effects of ethanol on the neuromuscular block produced by (+)-tubocurarine or vecuronium. However, it should be noted that the differences seen between the actions of ethanol on the neuromuscular block produced by (+)-tubocurarine, vecuronium and cisatracurium are unlikely to be due to differences in the subunit specificity of these antagonists. Both cisatracurium and (+)-tubocurarine bind preferentially to the interface between the α/ε subunit, whereas vecuronium preferentially binds to the α/δ subunit interface of the mouse adult muscle nicotinic acetylcholine receptor (Liu and Dilger, 2009). Because vecuronium and (+)-tubocurarine produce similar enhancement of the effects of ethanol, yet bind to different loci on the nicotinic receptor, it appears that the subunit binding preferences of the nicotinic antagonists do not correlate with the changes in efficacy of nicotinic receptor blockers resulting from the presence of ethanol. Finally, it is also possible that ethanol interacts directly with (+)-tubocurarine and vecuronium, but not cisatracurium, to reduce the potency of those drugs.

In conclusion, these results thus provide a general cautionary note to evaluating pre-synaptic effects by comparing the effects of an agent on EPP amplitudes in the presence of blocking drugs such as (+)-tubocurarine or vecuronium with its effects on MEPP amplitudes in the absence of a blocking drug. Such results could provide spurious evidence for pre-junctional effects of this agent when in truth the results would represent an interaction with the post-junctional blocking agent.

The enhanced EPPs due to a reduction in vecuronium potency by physiologically relevant concentrations of ethanol (as low as 12 mM), when coupled with the more established mechanisms to enhance post-junctional sensitivity for ACh and hence EPPs (see above), could be of clinical relevance in anaesthesiology. Indeed, when unclamped neuromuscular junctions were studied, 50–100 mM ethanol produced increases in EPPs in vecuronium that were similar to the EPP increases shown in Figure 2 in the presence of (+)-tubocurarine. For example, 100 mM ethanol produced a doubling of EPP amplitudes in vecuronium (n = 9 experiments). It is important to note that individuals attending accident and emergency departments commonly have blood alcohol concentrations exceeding 17.4 mM (the legal limit for driving a motor vehicle in the UK), and the average concentration of those individuals is in the range of 35–40 mM (Holt *et al.*, 1980). Additionally, alcohol-dependent individuals with higher tolerance for ethanol may have ethanol concentrations exceeding 100 mM (Vonghia *et al.*, 2008). Hence, the dosage of vecuronium and other neuromuscular blockers that share its mode of action may need to be adjusted in patients who have consumed alcohol prior to surgical procedures, as our results suggest that ethanol would produce an apparent resistance to a typical clinical dose of vecuronium. Finally, these experiments demonstrate the general potential for ethanol at clinically relevant concentrations to cause unexpected interactions either through actions on drugs directly or through actions on receptors to alter drug potency.

As ethanol is known to affect receptor gating for a large number of receptor and channel types (Liu and Hunt, 1999), this may represent a hitherto unexplored source for interactions between ethanol and therapeutic drugs. In this regard, it is worth pointing out that in addition to potentiating nicotinic receptor-mediated responses, ethanol also potentiates responses mediated by 5-HT_3 (Lovinger *et al.*, 2000), $GABA_A$ (see Wallner and Olsen, 2008)

and strychnine-sensitive glycine receptors (Mascia *et al.*, 1996). It is noteworthy that these receptor channel complexes whose responses are potentiated by ethanol are all members of the Cys-loop family of ligand-gated ion channels. Thus, it may be worth investigating whether antagonists of these receptors are also subject to altered potencies by ethanol. Additionally, it is possible that in other systems, the magnitude of synaptic potentials may similarly be limited by the post-synaptic membrane capacitance properties. If so, then relatively small alterations in the time constant of decay of synaptic currents may translate into much greater changes in the physiologically relevant synaptic potentials than would be predicted from measurements of the underlying current.

Acknowledgements

We thank Drs Evan Kharasch and Jay Yeh for their valuable comments on the manuscript and Dr Christine Stock for the generous gift of cisatacurium. We would also like to extend particular thanks to the editors and reviewers of this paper who provided us with insightful comments on this work. This work was supported by a grant from the National Institutes of Health of the USPHS (AA 016513).

Conflicts of interest

None.

References

Alexander SPH, Mathie A, Peters JA (2009). *Guide to Receptors and Channels*, 4th edition. Br J Pharmacol 158: S1–S254.

Bradley RJ, Sterz R, Peper K (1984). The effects of alcohols and diols at the nicotinic acetylcholine receptors of the neuromuscular junction. Br Res 295: 101–112.

Colquhoun D, Dreyer F, Sheridan RE (1979). The actions of tubocurarine at the frog neuromuscular junction. J Physiol 293: 247–284.

Demazumder D, Dilger JP (2001). The kinetics of competitive antagonism by cisatracurium, of embryonic and adult nicotinic actetylcholine receptors. Mol Pharmacol 60: 797–807.

Feng TP, Li TH (1941). Studies on the neuromuscular junction. XXV, Eserinelike actions of aliphatic alcohols and ketones. Chin J Physiol 16: 317–340.

Gage PW (1965). The effects of methyl, ethyl and *n*-propyl alcohol on neuromuscular transmission in the rat. J Pharmacol Exp Ther 150: 236–243.

Gage PW (1976). Generation of end-plate potentials. Physiol Rev 56: 177–247.

Gage PW, McBurney RN (1973). An analysis of the relationship between the current and potential generated by a quantum of acetylcholine in muscle fibers without transverse tubules. J Membrane Biol 12: 247–272.

Gage PW, McBurney RN, Schneider GT (1975). Effects of some aliphatic alcohols on the conductance change caused by a quantum of acetylcholine at the toad end-plate. J Physiol 244: 409–429.

Ginsborg BL, Jenkinson DH (1976). Transmission of impulses from nerve to muscle. In: Zaimis E (ed.). Neuromuscular Junction Handbook of Experimental Pharmacology. Springer Verlag: New York, pp. 229–364.

Glantz SA (1992). Primer of Biostatistics. McGraw Hill Inc: New York, NY.

Holt S, Stewart IC, Dixon JMJ, Elton RA, Taylor TV, Little K (1980). Alcohol and the emergency patient. Br Med J 281: 638–640.

Hubbard JI, Llinas R, Quastel EM (1969). Electrophysiological analysis of synaptic transmission. Monographs Physiological Soc 19: 321–361.

Katz B, Miledi R (1978). A re-examination of curare action at the motor end-plate. Proc R Soc B 203: 119–133.

Linder TM, Pennefather P, Quastel DM (1984). The time course of miniature endplate currents and its modification by receptor blockade and ethanol. J Gen Physiol 83: 435–468.

Liu M, Dilger JP (2009). Site selectivity of competitive antagonists for the mouse adult nicotinic acetylcholine receptor. Mol Pharmacol 75: 166–173.

Liu Y, Hunt WA (1999). Overview of symposium. In: Liu Y, Hunt WA (eds). THE 'DRUNKEN SYNAPSE' Studies of Alcohol-related Disorders. Kluver Academic/ Plenum: New York, pp. 1–15.

Lovinger DM, Sung KW, Zhou Q (2000). Ethanol and trichloroethanol alter gating of 5-HT_3 receptor-channels in NCB-20 neuroblastoma cells. Neuropharmacol 39: 561–570.

Mascia MP, Machu TK, Harris RA (1996). Enhancement of homomeric glycine receptor function by long-chain alcohols and anaesthetics. Br J Pharmacol 119: 1331–1336.

Redman RS, Silinsky EM (1994). ATP released together with acetylcholine as the mediator of neuromuscular depression at frog motor nerve endings. J Physiol 477: 117–127.

Searl TJ, Silinsky EM (2008). Mechanisms of neuromodulation as dissected using Sr^{2+} at motor nerve endings. J Neurophysiol 99: 2779–2788.

Shinozaki H, Ishida M (1984). Inhibitory actions of vecuronium bromide on acetylcholine and glutamate responses at the frog and crayfish neuromuscular junction. Jpn J Pharmacol 34: 299–306.

Steinbach AB (1968). A kinetic model for the action of xylocaine on receptors for acetylcholine. J Gen Physiol 52: 162–180.

Vonghia L, Leggioa L, Ferrullia A, Bertinib M, Gasbarrinia G, Addoloratoa G (2008). Acute alcohol intoxication. Eur J Intern Med 19: 561–567.

Wallner M, Olsen RW (2008). Physiology and pharmacology of alcohol: the imidazobenzodiazepine alcohol antagonist site on subtypes of $GABA_A$ receptors as an opportunity for drug development? Br J Pharmacol 154: 288–298.