Antagonistic postsynaptic and presynaptic actions of cyclohexanol on neuromuscular synaptic transmission and function

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Edited by: Kim Barrett & Samuel Young

The peer review history is available in the Supporting Information section of this article (https://doi.org/10.1113/JP281921#support-information-section).

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Abstract  Intentional ingestion of agricultural organophosphorus insecticides is a significant public health issue in rural Asia, causing thousands of deaths annually. Some survivors develop a severe, acute or delayed myasthenic syndrome. In animal models, similar myasthenia has been associated with increasing plasma concentration of one insecticide solvent metabolite, cyclohexanol. We investigated possible mechanisms using voltage and current recordings from mouse neuromuscular junctions (NMJs) and transfected human cell lines. Cyclohexanol (10–25 mM) reduced endplate potential (EPP) amplitudes by 10–40% and enhanced depression during repetitive (2–20 Hz) stimulation by up to 60%. EPP decay was prolonged more than twofold. Miniature EPPs were attenuated by more than 50%. Cyclohexanol inhibited whole-cell currents recorded from CN21 cells expressing human postjunctional acetylcholine receptors (hnAChR) with an IC50 of 3.74 mM. Cyclohexanol (10–20 mM) also caused prolonged episodes of reduced-current, multi-channel bursting in outside-out patch recordings from hnAChRs expressed in transfected HEK293T cells, reducing charge transfer by more than 50%. Molecular modelling indicated cyclohexanol binding (−6 kcal/mol) to a previously identified alcohol binding site on nicotinic AChR α-subunits. Cyclohexanol also increased quantal content of evoked transmitter release by ~50%. In perineurial recordings, cyclohexanol selectively inhibited presynaptic K+ currents. Modelling indicated cyclohexanol binding (−3.8 kcal/mol) to voltage-sensitive K+ channels at the same site as tetraethylammonium (TEA). TEA (10 mM) blocked K+ channels more effectively than cyclohexanol but EPPs were more prolonged in 20 mM cyclohexanol. The results explain the pattern of neuromuscular dysfunction following ingestion of organophosphorus insecticides containing cyclohexanol precursors and suggest that cyclohexanol may facilitate investigation of mechanisms regulating synaptic strength at NMJs.

(Received 24 May 2021; accepted after revision 1 November 2021; first published online 8 November 2021)

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Abstract figure legend  Cyclohexanol, the principal metabolite of a common agricultural insecticide solvent, cyclohexanone, acts both presynaptically and postsynaptically to impair neuromuscular transmission. (1) Cyclohexanol binds to sites in the pore of voltage-sensitive K+ channels that also bind the K+ channel blocker tetraethylammonium (TEA), with the effect of reducing presynaptic motor nerve terminal K+ currents and increasing quantal content of evoked transmitter release. (2) Simultaneously, cyclohexanol binds allosterically to an alcohol binding site on postsynaptic nicotinic acetylcholine receptors, altering ligand-gating characteristics and reducing charge transfer. The combined, overall effects of (1) and (2) result in reduced amplitude, prolonged depolarization and enhanced synaptic depression of endplate potentials (EPPs), weakening tetanic tension responses and causing fade in twitch responses to low frequency nerve stimulation. These effects mimic myasthenia or paralysis that occur in self-harming individuals who have swallowed significant quantities of organophosphorus insecticide, a significant public health issue in rural Asia. The present findings therefore provide an explanation for these clinical signs and indicate potential utility of cyclohexanol in further study of mechanisms of neuromuscular synaptic homeostasis.

Key points

- Intentional ingestion of agricultural organophosphorus insecticides is a significant public health issue in rural Asia, causing thousands of deaths annually. Survivors may develop a severe myasthenic syndrome or paralysis, associated with increased plasma levels of cyclohexanol, an insecticide solvent metabolite.

- Analysis of synaptic transmission at neuromuscular junctions in isolated mouse skeletal muscle, using isometric tension recording and microelectrode recording of endplate voltages and currents, showed that cyclohexanol reduced postsynaptic sensitivity to acetylcholine neurotransmitter (reduced quantal size) while simultaneously enhancing evoked transmitter release (increased quantal content).
Introduction

Self-inflicted poisoning by ingestion of agricultural insecticides is a significant public health issue in large areas of rural Asia. This behaviour has led to over 14 million deaths since the advent of the agricultural Green Revolution in the 1960s and continues to cause tens of thousands of deaths per year (Eddleston, 2000; Gunnell et al. 2007; Vale & Lotti, 2015; Karunarathne et al. 2019). Many of these deaths are associated with acute toxicity of the organophosphorus (OP) constituents of these insecticides. However, some individuals who survive the acute toxicity develop either transient, acute neuromuscular paralysis or, after a 1–3 day delay, a more enduring myasthenic syndrome, not seen with other forms of organophosphorus poisoning, and diagnosed clinically as ‘intermediate syndrome’ (Senanayake & Karalliedde, 1987; He et al. 1998). The cellular and molecular mechanisms have still not been established.

OP compounds inhibit acetylcholinesterase (AChE) and thus prolong the action of neurotransmitter at cholinergic synapses, including neuromuscular junctions (NMJs) in skeletal muscle. In animal models, this can lead to desensitization of acetylcholine receptors (AChR), muscle paralysis and death, although the significance of these factors in poisoned humans is less clear (Katz & Miledi, 1973; Burd & Ferry, 1987; Thiermann et al. 2010; Seeger et al. 2012; Cetin et al. 2019). However, myasthenic signs following insecticide ingestion cannot completely be explained by inhibition of AChE (Karalliedde et al. 2003; Bird et al. 2016) and recent investigations have therefore been directed to the importance of insecticide solvents as potential co-factors (Eddleston et al. 2012). For instance, we found in a previous study, utilizing anaesthetized pigs, that gavage of an agricultural insecticide containing the OP compound dimethoate dissolved in cyclohexanone resulted in acute failure of neuromuscular function, replicating the characteristic signs of myasthenia or paralysis in insecticide-poisoned patients; but gavage of dimethoate alone did not have this effect (Eddleston et al. 2012; Hulse et al. 2014).

Following its ingestion and intestinal absorption, cyclohexanone is reduced in the liver to cyclohexanol by reverse action of alcohol dehydrogenase (Mráz et al. 1994; Buratti & Testai, 2007). We found that intravenous injection of cyclohexanol in anaesthetized pigs mimicked the myasthenic effects of insecticide gavage (Eddleston et al. 2012; Dissanayake et al. 2021). Specifically, muscle force recordings in vivo showed that plasma concentrations of cyclohexanol exceeding 10 mM caused a progressive, temperature-sensitive decrease in contractile force and ‘fade’ in twitch responses to low frequency train-of-four (TOF) stimulation. These characteristics were replicated, also in temperature sensitive fashion, when cyclohexanol (10–25 mM) was added to medium bathing isolated nerve–muscle preparations from mice (Dissanayake et al. 2021). Neither cyclohexanone nor its metabolite cyclohexanol showed substantial anticholinesterase activity. However, complex responses (at lower concentrations) were found when cyclohexanol was added after first poisoning AChE with omethoate, a potent OP metabolite of dimethoate. Specifically, TOF twitch force and duration were initially enhanced and prolonged, but this was followed by progressive neuromuscular block (Dissanayake et al. 2021). Thus, our working hypothesis was that multiple, simultaneous postsynaptic and presynaptic actions of cyclohexanol explain this unusual combination of functional effects.

In the present study, we show that cyclohexanol impairs neuromuscular transmission, evidently by selectively interfering with proteins that determine both the magnitude of transmitter action and the amount of vesicular release. Specifically, cyclohexanol antagonized postsynaptic nicotinic AChRs, reducing but prolonging endplate responses to ACh. Simultaneously, cyclohexanol inhibited voltage-sensitive presynaptic K\(^+\) currents, thereby transiently increasing the quantal content of evoked ACh release from motor nerve terminals. Increases in EPP duration and quantal content initially offset or enhanced neuromuscular responses, but longer exposure to ACh and cyclohexanol when AChE activity was also eliminated accelerated the desensitization of AChR. This combination of effects is consistent with the pattern of failure of neuromuscular function observed following ingestion of agricultural OP insecticides. The data also extend understanding of mechanisms of action of alcohols on synaptic function and suggest that cyclohexanol may have utility as a chemical tool for...
investigation of homeostatic mechanisms that regulate synaptic strength.

Methods

Ethics, animals and tissues

Experiments were carried out on isolated tissues from wild-type mice bred and maintained in University of Edinburgh animal care facilities under conditions closely monitored by appointed Veterinary Officers and regularly inspected under institutional licence by the UK Home Office. The mice were killed by isoflurane anaesthetic overdose (>5% in air) and cervical dislocation, in accordance with approved UK Home Office Schedule 1. Flexor digitorum brevis (FDB) or triangularis sterni (TS) muscles with attached nerve supplies were rapidly dissected and immersed in mammalian physiological saline (MPS) of composition (mM): NaCl (158), KCl (5), CaCl$_2$ (2), MgCl$_2$ (1), glucose (5), HEPES (5), adjusted to pH 7.2–7.4 with droplets of NaOH (1 M). Solutions were bubbled with air for at least 20 min. Experiments were conducted at room temperature (18–25°C). Almost all the experiments reported here were carried out using tissue dissected from mice of the C57Bl6/Wld$^S$ strain and all the experiments reported here were carried out using CD01 strains. There were no systematic or discernible differences in the responses of nerve–muscle preparations made from these mice in the present study.

Drugs and toxins

Cyclohexanol and other drugs (all Sigma-Aldrich, Gillingham, UK) were added to MPS either directly or from aqueous stock solutions to give the concentrations required. Either aliquots (10–100 μl) were pipetted directly into MPS and thoroughly mixed in the recording chamber (volume 10 ml), or solutions containing the required concentration in 50 ml volumes were rapidly exchanged using coupled back-to-back 20–50 ml syringes connected to ports at opposite ends of the chamber. Baffles built into the chamber facilitated laminar flow, and complete solution exchange was achieved within 10–30 s. For electrophysiological recording from NMJs, some nerve–muscle preparations were pre-incubated for 20–30 min in MPS containing 0.5–2 μM μ-conotoxin GIIB (μ-CTX-GIIB; Sigma-Aldrich or Peptide Institute Inc., Osaka, Japan) to block Na$_V$1.4 channels and thus abolish muscle action potentials and contractions (Gillingwater et al. 2002; Ribchester et al. 2004).

Muscle force recording

Tibial nerve–FDB muscle preparations were pinned by their distal tendons to the Sylgard lining of the chamber and the proximal tendon was connected by 6/0 silk suture to an MLT0202 force transducer (ADInstruments, Oxford, UK). The preparations were bathed in 10 ml of MPS and the tibial nerve was aspirated into a glass suction electrode. Nerve stimuli (nominally up to 10 V, 0.1–0.2 ms duration, 2–20 Hz) were delivered via a DS2 stimulator (Digitimer, Welwyn Garden City, UK) triggered by the PowerLab 26T interface (ADInstruments). Force recordings were captured and digitized at 1 kHz using LabChart 7 software (ADInstruments) running on a Macintosh iMac computer.

Intracellular EPP recordings

Isolated FDB or TS nerve–muscle preparations were stretched and pinned at slightly more than their resting lengths in a Sylgard-lined chamber containing MPS. Rodent FDB muscles are well-suited to measurement of EPPs because the muscle fibres are only about 500 μm in length and therefore electrotonically isopotential (Bekoff & Betz, 1977; Ribchester et al. 2004). Thus, EPP amplitude and time course are unaffected by the intracellular location of the recording electrode tip. However, continuous, stable recording during bath solution changes proved quite difficult to achieve consistently in this muscle. By contrast, TS is a thin muscle comprising longer muscle fibres than those of FDB but NMJs in TS are readily discernible with brightfield, differential interference or fluorescence optics (McArdle et al. 1981; Ribchester et al. 1994). These features facilitated both positioning of microelectrodes in the vicinity of motor endplates and maintaining stable recording during multiple bathing solution exchanges (Ribchester et al. 1994; McArdle et al. 2005). Muscle fibres were impaled with glass microelectrodes pulled using a P87 puller (Sutter Instruments, Novato, CA, USA) and filled with either 3 M KCl or 4 M potassium acetate. Electrode resistances were typically between 20–35 MΩ. Electrodes were micromanipulated using Sutter MP-285 electric or Huxley-type MP-85 manipulators (WPI-Europe, Hitchin, UK). Single-electrode voltage recordings of EPPs or miniature EPPs (MEPPs) were made using an Axoclamp 2B amplifier (Molecular Devices, San Jose, CA, USA), digitized at 20 kHz (50 μs per point) using either CED micro-1401 (Cambridge Electronic Design, Cambridge, UK) or Digidata 1322A/1550B (Molecular Devices) interfaces, and then analysed using CED Spike-2, WinWCP 4.2/4.8 (Strathclyde Electrophysiology Software, Glasgow, UK), Clampex/pCLAMP 9.0/10.6 software (Molecular Devices) or Mini Analysis (Synaptosoft, Atlanta, GA, USA). In most of these experiments, action potentials in muscle fibres were blocked by pre-incubation of the muscles in MPS containing μ-CTX-GIIB (see above). Recordings from FDB fibres with initial resting membrane potentials less negative than −45 mV or those in which resting potential deteriorated by more than 30 mV/min...
were discarded. EPP amplitudes were otherwise corrected to a resting potential of −70 mV, to compensate for drift in the voltage driving force \((E_m - E_r)\), assuming a constant reversal potential \(E_r\) of −5 mV for action of ACh on its postsynaptic junctional receptors (Harris & Ribchester, 1979). Thus, for an observed EPP amplitude measured at the recorded resting membrane potential \(E_m\), the corrected EPP amplitude, \(E_{PC}\), was given by \(E_{PC} = E_{PO} \times -65/(|E_m - 5|)\). In some experiments, muscle fibre input resistance and membrane time constant were estimated by passing rectangular 1–5 nA, 30–50 ms current pulses through the recording electrode, using the bridge balance circuit built into the Axoclamp 2B amplifier, and recording the voltage response (Ribchester et al. 2004).

**Two-electrode voltage-clamp**

Two-electrode voltage-clamp (TEVC) was used to measure endplate currents in TS muscle fibres. The muscles were pinned in a Sylgard-lined Petri dish fitted with an integral suction electrode and mounted on the stage of an Axioscop 2 FS+ (Carl Zeiss Ltd, Cambridge, UK) upright microscope fitted with x10 and x40 water-dipping objectives. Voltage and current electrodes were mounted on Sutter MP-285 electric manipulators and muscle fibres were impaled in the endplate region with a tip separation of less than 100 µm. Voltage electrodes typically had resistances between 10 and 20 MΩ and current passing electrodes had resistances of 5–10 MΩ. Capacitative coupling between voltage and current electrodes was reduced by wrapping a grounded aluminium foil shield around the shaft of the microscope objective. The feedback gain and phase of the Axoclamp 2B amplifier were adjusted so that escape from voltage-clamp was less than 10% of the unclamped MEPP/EPP response. It proved difficult to provide sufficient feedback gain without oscillation for adequate, stable voltage clamp when the ambient temperature in our Edinburgh laboratory was above 25°C, however (Stiles et al. 1999). Thus, most TEVC experiments were performed outside summer months, when the laboratory temperature was usually between 18 and 22°C. EPPs and EPCs were evoked by connecting the suction electrode to a DS2 stimulator and triggered using Spike2, WinWCp4.2, Clampex, or Digitimer D4030 programs. EPPs, EPCs, MEPPs and MEPCs were measured and analysed using appropriate software tools in pCLAMP 10.6, WinWCp 4.8 or Mini Analysis.

**Cell culture**

CN21 cells were cultured as described previously (Ring et al. 2015). Briefly, cells were derived from the human rhabdomyosarcoma cell line by a stable transfection of the ε-subunit. Cells of this line thus expressed both the fetal muscle (α1, γ, α1, β1, δ subunits) and adult neuromuscular junctional (α1, ε, α1, β1, δ subunits) forms of the human muscle nicotinic ACh receptor (hnAChR). The cells were grown using standard cell culture techniques in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 50 µg/ml streptomycin, 2 mM Glutamax and 0.5 mg/ml geneticin, and kept in a 37°C humidified incubator gassed with 5% CO2. After washing with Ca2+/Mg2+-free phosphate buffered saline, cells were harvested using TrypLE Select and plated into flasks at ratios of 1:5–1:15 for proliferation.

HEK293T cells were maintained at 37°C and 5% CO2 in high glucose DMEM supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic (Thermo Fisher Scientific, Loughborough, UK). Cells were passaged twice weekly and plated onto 13 mm glass coverslips, then transfected with plasmids containing cDNA for adult hnAChR subunits α, β, ε, δ, mixed in a 2:1:1:1 ratio (Webster et al. 2012). A vector carrying the cDNA for enhanced green fluorescent protein (eGFP) was included as a marker of transfection. DNA (2 µg) and Lipofectamine 2000 (6 µl; Thermo Fischer Scientific ) were added to 150 µl of OptiMEM. The mix was incubated for 5 min at room temperature and then added to the cells and returned to the 37°C incubator. The medium was exchanged after 4–6 h and cells left for 48 h. Moderately fluorescent cells were selected for patch recording from small clusters of AChRs. The cells were transfected with cDNA for the adult form of nicotinic AChR (nAChR) found at human NMJs, containing ε-subunits but no γ-subunits.

**Whole-cell recording**

Whole-cell patch-clamp of CN21 cells was performed using an automated system (QPatch; Sophion Bioscience, Ballerup, Denmark). Cells were harvested using TrypLE Select and maintained in CHO serum-free media (Sigma-Aldrich) on the Q-patch platform at a concentration of 2 × 10⁶ cells/ml. After seal formation and cell perforation, cell membrane potential was clamped at −70 mV throughout the recording. The extracellular medium contained (in mM): NaCl (145), KCl (4), CaCl₂ (2), MgCl₂ (1), HEPES (10), and glucose (10), adjusted to pH 7.4 with 1 M NaOH. The intracellular medium contained KF (120 nM), EGTA (10 mM), HEPES (10 mM), and KCl (20 mM), adjusted to pH 7.2 with KOH (1 M). For initial characterization of the hnAChR responses, ACh was applied at concentrations ranging from 0.05–33 µM. To determine the effect of cyclohexanol, ACh (10 µM) was applied to evoke a maximal response then ACh and cyclohexanol were applied...
simultaneously, at concentrations ranging from 0.25 to 25 mM. GraphPad Prism, version 6.0 for Windows or 7.0 for Macintosh computers (GraphPad Software Inc., La Jolla, CA, USA), was used to measure the area under the response and to calculate the exponential decay time constants from non-linear least squares curve fits. Dose–response curves (log [inhibitor] vs. normalized response with a variable slope) were fitted to the normalized data also using routines built into GraphPad Prism.

**Outside-out patch recording**

Successfully transfected HEK293T cells were identified by their eGFP fluorescence. Recordings were made from small clusters of hnAChRs, pulled from cells showing moderate fluorescence, using the outside-out patch recording technique (Hamill et al. 1981). The recording chamber was perfused at 5 ml/min (VC-6 switchable perfusion system, Warner Instrument, Hamden, CT, USA) at room temperature with an extracellular solution comprising (in mM): NaCl (140), KCl (2.8), MgCl₂ (2), CaCl₂ (2), glucose (10), HEPES (10), and adjusted to pH 7.2 with NaOH (1 M). Patch pipettes were made from thick-walled borosilicate glass (GC150F-10; Harvard Apparatus, Cambridge, UK) using a P-97 puller (Sutter Instruments) and fire-polished to give a resistance of 2–8 MΩ when filled with the intracellular solution, containing (in mM): NaCl (5), MgCl₂ (2), potassium gluconate (130), EGTA (5), ATP (0.1), HEPES (10), and adjusted to pH 7.2 with KOH (1 M). Currents were recorded using an Axopatch 200B amplifier (Molecular Devices). Data were filtered at 2–5 kHz (−3 dB) using the inbuilt low-pass Bessel filter and digitized at 50–100 kHz via a Digidata 1322A analog–digital interface (Molecular Devices) connected to a PC running Clampex 9/10.3 software (Molecular Devices). Patch currents were recorded at a holding potential of −80 mV. Channel currents were evoked from patches containing small clusters of AChRs by exchanging the bath solution with physiological saline containing ACh (100 nM). After 1 min of recording, the bathing solution was switched to one containing the same concentration of ACh with added cyclohexanol (10 mM). After a further minute of recording, the perfusion was switched back to the original solution containing ACh. Patch current activity was measured from negative deflections exceeding a threshold set at −1.5 pA. Mean amplitude and area of the suprathereshold deflections (related to channel openings but not characterized as such) during 30 s epochs when the effects of ACh and cyclohexanol had evidently stabilized were measured. Charge transfer (Neher, 1983; Tattersall, 1990) was measured using a custom script written in Python 3.6 (https://www.python.org/), referencing pyabf (https://pypi.org/project/pyabf/), scipy (https://www.scipy.org) and other standard Python supplementary mathematical function libraries. For this analysis, baseline subtraction was applied using a windowed arithmetic function. Switching artefacts were removed manually, and the missing data were substituted with medians of six data points preceding and following the split. The data were then integrated using a trapezoid integration algorithm, split into 1–5 s blocks and expressed as a percentage of maximum response.

**In silico modelling**

Binding of cyclohexanol to nAChRs and voltage-sensitive K⁺ channels was estimated from models based on published sequences. The crystallographic structure of the human nAChR expressed at NMJs has not been suitably determined so we searched for candidate cyclohexanol binding sites and estimated binding energies using a model based on the known crystallographic structure of AChR in Torpedo (Unwin & Fujiyoshi, 2012). Torpedo nAChR structure (Berman et al. 2000) was downloaded from the RCSB PDB database (https://www.rcsb.org/; PDB ID: 4AQ5, Accession ID: 4aq9). The dominant voltage sensitive K⁺ current in motor nerve terminals is probably mediated by Kv3 channels (Tabti et al. 1989; Brooke et al. 2004). Since, to our knowledge, no suitable crystallographic structures of Kv3 isoforms have been published, we investigated candidate binding sites for cyclohexanol to the rat voltage-activated, Kv1.2/2.1 paddle chimera channel model (Matthies et al. 2018). The sequence and structure were downloaded from the RCSB PDB database (PDB ID : 6EBM). BLAST sequence alignment of these channels with human Kv3 channels confirmed sequence homology in the pore region, including the tetraethylammonium (TEA) binding domain.

Ligand models (cyclohexanol, ethanol and TEA) were downloaded from ChemDB (http://chemdb.ics.uci.edu/). Polar hydrogens were added onto the Torpedo receptor and Kv1.2/2.1 chimeric model and the torsional bonds of the ligands were relaxed using relevant modules in AutoDock tools (Morris et al. 2009), downloadable from http://autodock.scripps.edu/. Candidate binding sites were sought using Autodock Vina version 1.1.2 (Trott & Olson, 2009), downloaded from http://vina.scripps.edu/ and run on a Hewlett-Packard ZBook 17 G4 laptop computer fitted with a NVIDIA Quadro M2200 4GB graphics card. For nAChR, a ‘blind’ docking protocol was adopted (covering the entire protein) with the exhaustiveness parameter, which determines the number of concurrent parallel runs required to reach convergence (Jaghoori et al. 2016), set to 100. For the Kv channel model we used both a blind docking search and a targeted search, applying a
search window with dimensions $14 \times 22 \times 42$ Å ($x$, $y$, $z$) positioned in the region of the pore. The default output of Autodock Vina yields nine poses of a ligand with respect to target sites identified on the protein receptor with the highest affinities (lowest binding free energies, $\Delta G_b$) expressed in kcal/mol. We took the poses with the lowest (most negative) binding energies to be the most likely functional binding sites for cyclohexanol with our models of nAChR and Kv channels.

**Perineurial recording**

Perineurial recordings were made for small nerve branches in TS muscles, in similar fashion to methods described previously (McArdle et al. 1981; Brigant & Mallart, 1982; Penner & Dreyer, 1986; Braga et al. 1992; Ding et al. 2001). Briefly, TS muscles were paralysed by incubation in 10 $\mu$g/ml of tetramethylrhodamine isothiocyanate (TRITC)-conjugated $\alpha$-bungarotoxin ($\alpha$-BTX), which within about 20 min abolished EPPs and muscle twitching in response to nerve stimulation. Glass microelectrodes filled with 1 M NaCl, tip resistance 5–10 MΩ, were manipulated to within 100–200 $\mu$m of one or more NMJs, confirmed by the endplate fluorescence of bound TRITC-$\alpha$-BTX, and the tip inserted by applying slight pressure and gentle tapping on the perineurial sheath of the small bundle of axons supplying these NMJs. The relevant intercostal nerve was stimulated via a suction electrode with 0.05–0.2 ms pulses, 0.5–5 V in amplitude. Perineurial current responses were averaged (10 successive sweeps evoked at 1 Hz) using pCLAMP 10.6 software. Stability of the Na\(^{+}\) component of the perineurial waveform and reversibility of the K\(^{+}\) current responses to cyclohexanol and TEA were indicators of the stability and integrity of the motor nerve terminals throughout the recordings.

**Statistics**

Electrophysiological properties of muscle fibres in any given nerve–muscle preparations are influenced by several variables including motor endplate size, synaptic strength per unit area, muscle fibre diameter and input resistance (Harris & Ribchester, 1979; Costanzo et al. 1999; Ribchester et al. 2004; Jones et al. 2016). We therefore averaged recordings from several NMJs in each muscle and tested for significance of differences in mean values, assuming mice to be the independent variable. Data in some figures are plotted, nevertheless, showing both numbers of muscle fibres sampled ($n$) and averaged data based on numbers of mice ($N$). In some instances, where $N = 1$ or 2 mice, we took either muscles or muscle fibres as the independent variable. Summary data are expressed as means ± SD unless otherwise indicated. For avoidance of doubt, we use the term 'standard deviation of the mean' (SDM) rather than 'stand error of the mean' (SEM) when referring to the standard statistical measure of variability in estimating the true population means, calculated from the variance of means of samples between mice. However, statistical analysis of data comparing averages based on numbers of mice or on the numbers of muscle fibres sampled, did not yield any systematic or qualitatively different conclusions.

Data were analysed and graphed using Microsoft Excel and Prism 7 for Mac. Decay time constants of synaptic potentials were also determined using built-in functions either in pCLAMP 11, Prism 7 or in Mini Analysis software. A two-sided Student's $t$-test or ANOVA was used to assess significance of mean differences of continuous parametric data between two groups or multiple groups, respectively, assuming Gaussian distributions in the test and control groups, and applying post hoc evaluations as appropriate in those instances for which ANOVA indicated significant differences between groups. Kruskal–Wallis or Mann–Whitney $U$-tests were used to assess non-parametric data or data for which Gaussian distributions were not assumed. Linear and non-linear regression fits and coefficients were calculated using functions built into Prism 7.

**Results**

In a previous study of anaesthetized pigs, we found that within 6–12 h of gavage of the agricultural insecticide formulation 'dimethoate EC40', plasma concentrations of cyclohexanol increased to 4–7 mM (Eddleston et al. 2012). This was associated with a progressive decrease in nerve-evoked muscle force and 'fade' during TOF stimulation. Subsequently, we showed that cyclohexanol administered either intravenously in vivo or added to medium bathing isolated murine nerve–muscle preparations mimicked the impairment of neuromuscular function associated with insecticide gavage, in a temperature-sensitive fashion (Dissanayake et al. 2021). Our aim in the present study was to establish the synaptic mechanisms underlying these myasthenic effects of cyclohexanol. Our recordings were made from murine tissue or human cellular preparations at ambient room temperature, which normally required concentrations of cyclohexanol in the 10–20 mM range to evoke effects comparable to those observed in pigs at 37–39°C in vivo (Dissanayake et al. 2021).

For the present study, we report first the main qualitative effects of cyclohexanol on neuromuscular function and transmission. We then present quantitative evaluation of the hypotheses arising from these index experiments in more detail, summarizing data from preparations in most cases with respect to mean values.
calculated per mouse. Together, the main conclusions arising from this analysis are that cyclohexanol selectively and simultaneously impairs the function of both postsynaptic ACh receptors and presynaptic voltage-sensitive K⁺ channels. These effects appear to explain the myasthenic actions of cyclohexanol at tissue and cellular levels.

**Qualitative description**

**Cyclohexanol inhibits muscle contraction.** The myasthenic effects of cyclohexanol (20 mM) are illustrated by the muscle force recordings from an isolated mouse tibial nerve–FDB muscle preparation shown in Fig. 1A and B. As reported in our previous study (Dissanayake et al. 2021) and replicated in the recordings shown in Fig. 1A, cyclohexanol reversibly impaired nerve-evoked (indirect) twitch and tetanic tension responses to train-of-four (TOF) nerve stimulation at 2 Hz. Characteristic fade during the TOF becomes even more conspicuous when the preparation was stimulated at 20 Hz (Fig. 1B), a frequency that normally produces sustained tetanic responses in mouse FDB muscles (Ribchester et al. 2004; Gilley et al. 2017). Normal TOF and tetanic responses were restored almost immediately (within seconds to minutes) after washing cyclohexanol from the recording chamber, restoring normal MPS bathing medium (Fig. 1A and B). Fade in TOF and tetanic response are well-established characteristics of progressive neuromuscular transmission block (Bowman et al. 1988; McGrath & Hunter, 2006). For instance, Fig. 1C and D shows qualitatively similar, reversible effects on TOF and tetanic tension responses in the same FDB nerve–muscle preparation, after adding the nicotinic AChR (nAChR) antagonist d-tubocurarine (2 μM), instead of cyclohexanol, to the bathing medium.

**Cyclohexanol potentiates then blocks effects of anti-AChE.** The response to cyclohexanol after inhibiting AChE was more complex. Fig. 1E–H shows muscle force recordings, from the same preparation, after AChE activity was maximally inhibited by adding the carbamate AChE antagonist neostigmine (5 μM), replicating the anticholinesterase effects of the insecticide OP metabolite omethoate (Dissanayake et al. 2021). One hour after adding neostigmine (or omethoate), tetanic stimulation produced powerful muscle responses, notably including a prolonged post-tetanic ‘aftercontraction’, lasting several seconds (Fig. 1E). Similar effects were observed after incubation of muscles in other carbamate or organophosphorus anti-AChE compounds (Chang & Hong, 1986; Burd & Ferry, 1987; Dissanayake et al. 2021). Remarkably, TOF twitch contractions were initially enhanced and prolonged after adding cyclohexanol (Fig. 1F). However, over the following several minutes neuromuscular responses again showed fade and block (Fig. 1G and H), as occurred after incubating muscles in cyclohexanol alone (Fig. 1A). The magnitude and duration of the tetanic response and aftercontraction were also substantially reduced (Fig. 1H). Normal twitch and tetanic responses were restored after washing the preparation with normal MPS (records not shown). Direct observation of NMJs showed that twitch potentiation in the presence of both anti-AChE and cyclohexanol is due to prolonged local contractures of the motor end-plate region of muscle fibres (Dissanayake et al. 2021), associated with localized increases in endplate Ca²⁺ (R. Redman, R. R. Ribchester et al. in preparation).

**Cyclohexanol attenuates and prolongs EPPs.** The effects of cyclohexanol on muscle force and endplate contractures were reflected in intracellular recordings from NMJs (Fig. 2). Figure 2A and B shows intracellular recordings from muscle fibres in two different FDB nerve–muscle preparations, following incubation for about 20 min in 20 mM cyclohexanol dissolved in MPS. Visual inspection confirmed that muscle twitching in response to low frequency (1 Hz) tibial nerve stimulation was substantially weakened. Several recordings show evoked EPPs that occasionally triggered either full action potentials or reduced ‘abortive’ action potentials, indicating that cyclohexanol reduced the amplitude of EPPs, sufficient to prevent at least half of them from reaching threshold for action potential generation in muscle fibres. Specifically, after adding 20 mM cyclohexanol, EPPs triggered action potentials in 25/89 muscle fibre recordings (N = 5 muscles), whereas in MPS stimulation normally triggers action potentials in all fibres. In 19 other fibres, the first evoked EPP triggered attenuated (‘abortive’) muscle action potentials (see for example, Fig. 2A trace 1). Thus, cyclohexanol (20 mM) rendered altogether about half of the muscle fibres sampled (45/89) sub-threshold. The mean amplitude of these subthreshold responses, corrected to a resting potential of −70 mV, was 11.86 ± 1.38 mV (mean ± SDM, n = 45 muscle fibres, N = 4 mice), depolarizations just below the firing threshold for most muscle fibres (Wood & Slater, 1995, 1997). As expected, after washing cyclohexanol from the recording chamber, action potentials were evoked by nerve stimulation in all muscle fibres recorded. These muscle action potentials were then abolished after 20–30 min incubation in μCTX-GIIIB, leaving residual EPPs that were 27.85 ± 2.44 mV in amplitude (mean ± SDM, n = 19 muscle fibres, N = 4 mice); that is, 2–3 times the depolarization normally required to trigger action potentials (Wood & Slater, 2001). The main conclusion from these preliminary recordings was that the myasthenic effect of cyclohexanol apparent in tension recordings is not due to block of...
Figure 1. Cyclohexanol reduces muscle twitch and tetanic force

Representative isometric force recordings from an isolated mouse tibial nerve–FDB muscle preparation showing reversible effects of cyclohexanol on nerve-evoked responses. A, after 10 min incubation in 20 mM cyclohexanol (C6OL), twitch tension during TOF stimulation (2 Hz) or 2 s trains at 20 Hz showed marked depression (‘fade’). Sustained twitch amplitudes were restored, within minutes, after washing with MPS. B, marked tetanic fade with stimulation for 2 s at 20 Hz was also restored after washing cyclohexanol from the recording chamber with MPS. C, adding d-tubocurarine (dTC, 2 μM) caused similar, reversible fade in TOF twitch responses. D, tetanic responses in d-tubocurarine showing a similar pattern of decrementing force (fade) that reverted to a normal, sustained tetanic force response within 30 min of washing tubocurarine from the recording chamber. E, TOF and tetanic responses in the same preparation as that in Fig. 1A-D, after subsequent pre-incubation for ~60 min in 5 μM neostigmine, a concentration sufficient to completely inhibit junctional AChE. TOF stimulation caused twitch responses that were augmented compared with control (compare with scale bars in Fig. 1A,C). In addition, stimulation at 20 Hz for 2 s produced a forceful, sustained tetanus followed by slow relaxation over the following 10 s (‘aftercontraction’). Dashed vertical red line indicates the end of tetanic stimulation. F, within seconds of exchanging the bathing medium containing neostigmine for MPS containing both neostigmine and cyclohexanol (20 mM), TOF responses were rapidly augmented. G, over the following 10–20 min, TOF responses became progressively attenuated. Gaps between traces represent 30 s intervals with no stimulation. H, TOF twitch responses evoked 20 min after adding cyclohexanol. Note change of scale. I, tetanic response/aftercontraction was also attenuated within 20 min of adding cyclohexanol. Note the difference in the force calibration compared with A. Dashed red line indicates the end of tetanic stimulation.
Figure 2. Cyclohexanol depresses EPPs

A and B, intracellular recordings from FDB muscle fibres in two different muscles after 20 min incubation in 20 mM cyclohexanol, without pre-incubation in μCTX-GIIIB. The numbered traces are consecutive records obtained during stimulation at 1 Hz; displaced vertically and horizontally for clarity. In A, the first EPP triggered an attenuated (‘abortive’) action potential and the subsequent two stimuli evoked subthreshold EPPs. In B, the first and third responses triggered action potentials but the second stimulus evoked an EPP that failed to trigger a full sized action potential; a small additive positive, subthreshold inflexion (‘ abortive spike’) was triggered at the peak of the EPP instead. About half of the recordings in 20 mM cyclohexanol showed either action potentials or abortive spikes like these.

C–E, EPP trains evoked by tibial nerve stimulation at 20 Hz during a continuous recording from an FDB muscle fibre before, during and after exchanging the bathing medium with MPS containing 20 mM cyclohexanol (C6OL). EPPs were rapidly and reversibly attenuated, prolonged and depressed during the stimulus train; these effects were reversed within 1–3 min of restoring normal bathing medium (E). Muscle action potentials in this preparation were blocked by preincubation for 30 min in μCTX-GIIIB (2 μM). F, EPP recorded from an FDB muscle fibre after 20 min pre-incubation in neostigmine (5 μM). As expected, the EPP was prolonged about threefold compared with most EPPs in recorded in MPS alone; half-decay time was about 8 ms. G, EPP recording from the same muscle fibre as shown in F, 1 min after replacing the bathing medium with MPS containing neostigmine and 20 mM cyclohexanol. EPP decay time was further extended, by more than a factor of 2; half-decay time was about 20 ms. H, data from sequential records of successive EPPs evoked at 1 Hz from the same muscle fibre as the traces shown in F and G. Each point is the half-decay time for one EPP before, during and after removing cyclohexanol, with neostigmine present throughout. Half-decay time reversibly increased more than twofold on adding cyclohexanol. I, averaged spontaneous MEPPs (>30 successive MEPPs) recorded in a TS muscle fibre in control solution (MPS containing 5 μM neostigmine) and after progressively increasing the concentration of cyclohexanol in the bathing medium. Cyclohexanol reduced average MEPP amplitude and prolonged MEPP duration. These effects were reversed on washing cyclohexanol from the chamber with control MPS solution.

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axonal action potentials but, rather, to a decrease in the safety factor for synaptic transmission at NMJs.

Inspection of EPP responses in cyclohexanol revealed other notable effects. Figure 2C and D shows continuous recordings from an FDB muscle fibre in a different preparation, in response to a 1 s, 20 Hz train of EPPs before and after exchanging the normal bathing medium (MPS) with MPS containing 20 mM cyclohexanol. Muscle action potentials in this preparation were blocked by pre-incubating the muscles in μCTX-GIIIIB. Cyclohexanol reduced the amplitude of the first EPP by about 30% in this muscle fibre but also markedly enhanced short-twitch synaptic depression (the amplitude of subsequent EPPs compared to the first). Specifically, in continuous recordings from three muscle fibres (in muscles from different mice) in control MPS solution, the tenth nerve-evoked EPPs (corrected to –70 mV to compensate for slight drift in resting membrane potential during the recordings) were reduced in amplitude from 27.7 ± 2.12 to 17.64 ± 1.52 mV, a decrease of about 35%. But after adding cyclohexanol (20 mM), similar stimulation in the same fibres depressed EPP amplitude by about 60%, from 24.3 ± 2.81 to 9.83 ± 1.52 mV (P = 0.0136, paired t-test, N = 3 mice). EPP decay was also markedly and consistently prolonged (see below). All three effects (reduced EPP amplitude, enhanced high-frequency depression and prolonged EPP duration) were almost completely reversed within seconds of rinsing cyclohexanol from the recording chamber, restoring MPS (Fig. 2E).

Intracellular recordings made after inhibiting junctional AChE with neostigmine (5 μM) revealed additional features, commensurate with the tension responses observed after adding cyclohexanol (see Fig. 1F). We showed previously that 20 mM cyclohexanol has only a weak effect on AChE activity (Dissanayake et al. 2021) but 5 μM neostigmine was sufficient to completely inhibit AChE activity in FDB muscle homogenates (R. Chou and R. R. Ribchester, unpublished data). Figure 2F and G shows EPP responses in a continuous recording from an FDB muscle fibre incubated in 5 μM neostigmine, before and after adding 20 mM cyclohexanol. As expected, neostigmine alone markedly prolonged EPP decay, due to persistence of unhydrolysed synaptic ACh. But EPP decay was rapidly and reversibly prolonged, by an additional factor of 3, after adding cyclohexanol (Fig. 2H). Thus, the result of this individual experiment was consistent with the initially protracted twitch responses during TOF stimulation shown in Fig. 1F. Since AChE was already completely inhibited by neostigmine in this experiment, the additional prolongation of EPPs by cyclohexanol could not be explained by inhibition of AChE enzymic activity. Finally, cyclohexanol also reversibly attenuated and prolonged spontaneous MEPPs. Figure 2I shows averaged MEPPs (>30 records) from a single TS muscle fibre incubated in 5 μM neostigmine, then after increasing the concentration of cyclohexanol to 10–20 mM, followed by washing with MPS.

In sum, the results of these illustrative pilot experiments defined, provisionally, the principal characteristic physiological effects of cyclohexanol on NMJs, motivating our more detailed investigations reported below. Our working hypothesis was that cyclohexanol impairs neuromuscular transmission by altering either postsynaptic sensitivity to neurotransmitter, transmitter release or both. We used a combination of voltage recording, two-electrode voltage-clamp, whole-cell recording, outside-out patch-clamp and perineurial current recording from isolated NMJs to determine the postsynaptic and presynaptic loci of action of cyclohexanol. We also modelled the binding of cyclohexanol to nAChR and voltage-sensitive K+ channels (Kv channels). Our analysis led to two firm conclusions: first, that cyclohexanol antagonizes nicotinic ACh receptor function postsynaptically, and second, it simultaneously inhibits voltage-sensitive, TEA-sensitive K+ channel function presynaptically. We identified candidate binding sites for cyclohexanol on nAChR and Kv channels that seem likely to have mediated these effects. The mechanisms we have identified are consistent with the description and measurement of the myasthenic effects of cyclohexanol on muscle force following either gavage of an OP compound dissolved in a cyclohexanol metabolic precursor (cyclohexanone) or intravenous infusion of cyclohexanol in vivo (Eddleston et al. 2012; Dissanayake et al. 2021).

Post synaptic effects of cyclohexanol

Cyclohexanol attenuates EPPs. We first measured the attenuating effect of cyclohexanol on EPP amplitude. For this analysis we preincubated preparations for 20–30 min in μCTX-GIIIIB, to block muscle action potentials while preserving physiological levels of evoked transmitter release (Wood & Slater, 1995; Ribchester et al. 2004). Representative recordings from two different muscle fibres showing responses to TOF stimulation at 2 Hz, before and after adding cyclohexanol (20 mM) are shown in Fig. 3A and B.

Cyclohexanol did not significantly affect resting membrane potentials (Fig. 3C and D; MPS: −63.5 ± 4.4 mV, N = 9 mice; 5–25 mM cyclohexanol: −62.1 ± 3.4 mV, N = 4 mice; P = 0.5827, ANOVA, F[5,19] = 0.774). The effects on EPP amplitude were variable between muscle fibres but mean values were consistent between mice (Fig. 3E and F). Corrected EPP amplitude in MPS was 30.78 ± 8.46 mV (mean ± SDM; N = 8 mice). At concentrations up to 15 mM, cyclohexanol had little effect on the amplitude of the first recorded EPP (P = 0.71, ANOVA). However, 20 mM cyclohexanol reduced
EPP amplitude by about 30%, to 18.92 ± 3.03 mV (N = 6 mice; P = 0.007, Dunnett’s post hoc test). EPP amplitude was reduced further within a few minutes of adding 25 mM cyclohexanol to one muscle but neuromuscular transmission was completely abolished at this concentration in three other FDB preparations, consistent with complete muscle paralysis observed in most muscle force recordings at this concentration (Dissanayake et al. 2021).

Additional attenuation of EPPs was evaluated from the ratio of the fourth EPP to the first, evoked by TOF stimulation at 1–2 Hz. Overall, this short-term synaptic depression was more sensitive to the concentration of cyclohexanol than initial EPP amplitude (Fig. 3G and H). In MPS the ratio of the fourth EPP (P4/P1) was 90.5 ± 2.4% (mean ± SDM; n = 39 muscle fibres, N = 4 mice).

In 15 mM cyclohexanol this ratio decreased to 79.45 ± 3.82% (n = 38 muscle fibres, N = 4 mice; Kruskal–Wallis statistic 11.53, df = 3,16; P = 0.043, Dunn’s post hoc test), which decreased slightly but significantly to 75.06 ± 3.69% in 20 mM cyclohexanol (n = 30 muscle fibres; N = 3 mice; P = 0.0098, Dunn’s post hoc test).

Thus, cyclohexanol reduced the safety margin for neuromuscular transmission by both attenuating initial EPP amplitude and enhancing synaptic depression during repetitive stimulation. The nature and amount of EPP depression were consistent with the observed fade and block in muscle twitch and tetanic tension responses.

**Cyclohexanol increases EPP duration.** Prolonged duration was a consistent and robust feature of EPP

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**Figure 3. Cyclohexanol depresses EPPs at low stimulation frequencies**

A and B, intracellular recordings from FDB muscle fibres showing successive EPPs in response to TOF stimulation at 2 Hz. Traces are laterally displaced for clarity and numbers beneath each trace refer to the relative time (s) of stimulation with reference to the first EPP. A, EPPs recorded in MPS after blocking muscle action potentials with μCTX-GIIIB; B, EPPs in a different muscle fibre in the same muscle preparation, a few minutes after adding 20 mM cyclohexanol. Note the decrease in EPP amplitude, enhanced depression in response to TOF stimulation and the prolonged duration of the EPPs. C–H, summary data from recordings like those shown in A and B. Graphs on the left (C, E, G) show results of all muscle fibre recordings, where each point represents one muscle fibre. The graphs on the right (D, F, H) summarize data with respect to each mouse, where each point represents the mean ± SDM. The data show: C and D, no significant effect of cyclohexanol on resting membrane potential; E and F, attenuation of the first EPP in the TOF that was significant in 20 mM cyclohexanol; G and H, significant depression during the TOF stimulus train, expressing the ratio of the fourth EPP (P4) to the first (P1) as a percentage. Depression in 15 mM cyclohexanol and 20 mM cyclohexanol was significant.
responses in cyclohexanol. Figure 4A illustrates the reversible effect on EPP duration (and amplitude) and after adding cyclohexanol, in a continuous recording from an FDB muscle fibre pre-incubated in μCTX-GIIIB. Figure 4B shows continuous measurements of EPP rise time and half-decay time during this experiment.

Summary data showing the dependence of rise time, half-decay time and time to 90% decay, in samples of muscle fibres in preparations dissected from different mice are shown in Fig. 4C–E. Rise time increased by 50–80% over a range of concentrations from 10 to 25 mM (Fig. 4C; \( P = 0.0002 \), ANOVA). For instance, EPP rise time in MPS was \( 1.48 \pm 0.14 \) ms (mean \( \pm \) SDM; \( N = 9 \) mice) and \( 2.16 \pm 0.32 \) ms in 20 mM cyclohexanol (\( N = 3 \) mice; \( P = 0.0002 \), Dunnett’s test). Half-decay time was even more substantially increased (Fig. 4D; \( P < 0.0001 \), ANOVA). For

Figure 4. Cyclohexanol reversibly prolongs EPPs
A, representative EPPs evoked with low frequency (1 Hz) tibial nerve stimulation in a single FDB muscle fibre before, during and after exchanging the bathing medium with MPS containing 20 mM cyclohexanol. Muscle action potentials were blocked by preincubation in μCTX-GIIIB (2 μM). In this fibre the EPP amplitude was reversibly attenuated by about 25% but half-decay time was prolonged about threefold. B, continuous data from a recording from a different muscle fibre where each point shows the rise time (red) or half-decay time (black) before, during and after perfusing 20 mM cyclohexanol dissolved in MPS through the recording chamber. Rise time was increased by about 30% but half-decay time almost doubled in this recording. C, summary data of EPP rise time as a function of cyclohexanol concentration. Each point is the mean rise time based on recordings from 5–10 muscle fibres in each preparation dissected from one mouse. Lines show the mean \( \pm \) SDM of these data. Cyclohexanol (20 mM) increased rise time by about 50%. D and E, summary data on EPP half-decay and time to 90% decay as a function of cyclohexanol concentration for the same EPPs and muscle as analysed in C. Cyclohexanol (15 mM) significantly increased half-decay time and at a concentration of 20 mM, half-decay time increased about threefold. F, decay time showed dependence on EPP amplitude. Each point shows the EPP amplitude and corresponding 90% decay time (\( T_{90} \)) recorded in each muscle fibre from the preparations summarized in C–E. Continuous curve is a non-linear least squares best fit with 95% confidence limits (dashed lines; \( r^2 = 0.425 \)) of a hyperbola with the derived parameters (\( T_{90, max}, K_d \) as indicated.

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instance, half-decay time in MPS increased from 2.95 ± 0.74 ms (mean ± SDM, n = 41 muscle fibres, N = 9 mice) to 8.40 ± 1.09 ms in 20 mM cyclohexanol (n = 29 muscle fibres, N = 4 mice; P < 0.0001, Dunnett’s test). Figure 4E shows that time to 90% decay (T90) was increased even more (P < 0.0001, ANOVA), from 6.42 ± 1.16 ms in MPS (N = 9 mice) to 21.59 ± 2.93 ms in 20 mM cyclohexanol (N = 4 mice; P < 0.0001, Dunnett’s), a three- to fourfold difference. The effect of cyclohexanol on EPP decay also varied with EPP amplitude. Figure 4F shows the best fit, with 95% confidence limits, for hyperbolic non-linear regression of T90 decay against EPP amplitude as the independent variable (r² = 0.43, df = 25, Sy.x = 3.82). The quantitative effects of cyclohexanol on EPP rise time and decay time were corroborated by separate groups of experiments performed to evaluate presynaptic effects of cyclohexanol (see below, Figs 10–12).

Plausible explanations for an increase in EPP duration include changes in muscle fibre membrane time constant or loss of AChE activity (Harris & Ribchester, 1979; Ribchester et al. 2004). However, a two- to fourfold increase in membrane time constant would be required to explain the prolongation of EPP decay in 20 mM cyclohexanol. But neither single microelectrode recordings in FDB muscles (see below) nor twin microelectrode recordings from TS muscle fibres showed any discernible differences in time constant in 20 mM cyclohexanol. Since cyclohexanol has only a weak effect on AChE activity (Dissanayake et al. 2021), inhibition of AChE was also an insufficient explanation of the prolonged EPP decay (see also Figs 2F–H). We also ruled out any non-specific, osmotic effect of cyclohexanol on EPP duration. For instance, when sucrose (30 mM) was added to the bathing medium, instead of cyclohexanol, there were no significant changes in half-decay time (2.40 ± 0.42 ms; mean ± SD; n = 20 muscle fibres in 2 muscles; P = 0.29, unpaired t-test compared with recordings in control MPS solution). Together with measurements of EPC decay (see below), the results of these tests do not support any explanation for prolonged EPP decay based on passive changes in membrane biophysical properties. Thus, a more plausible hypothesis is that cyclohexanol impairs postsynaptic responses to ACh in a fashion that impacts on both amplitude and duration of EPPs. We tested this hypothesis, first by measuring effects of cyclohexanol on MEPPs then by recording AChR currents directly in transfected human cell lines.

Cyclohexanol also attenuates MEPPs. Cyclohexanol substantively reduced the amplitude of spontaneous MEPPs. Figure 5A shows recordings from an FDB muscle fibre in which MEPPs were reduced by more than 50%. Overall, MEPP amplitude was reduced from 0.51 ± 0.15 mV in MPS (mean ± SD, n = 8 muscle fibres) to 0.26 ± 0.06 mV in 20 mM cyclohexanol (n = 10 muscle fibres, N = 1 muscle; P = 0.002, t-test with Welch’s correction). Input resistance was estimated by passing 1–5 nA current pulses through the recording electrode. There was no discernible change in muscle fibre input resistance in the same muscle fibre after adding cyclohexanol (Fig. 5B). Input resistances was not significantly altered in samples of muscle fibres from two other muscles (3.86 ± 1.16 MΩ in MPS, n = 9 muscle fibres in 2 muscles; compared with 3.03 ± 1.22 MΩ in 20 mM cyclohexanol, n = 12 fibres in 2 muscles; P = 0.135, t-test based on number of muscle fibres). Thus, attenuation of MEPPs in cyclohexanol was not readily explained by any concomitant change in input resistance (compare, for example, with Harris & Ribchester, 1979; Ribchester et al. 2004).

Drift in resting membrane potential and difficulties in maintaining stable recordings while bathing solutions were exchanged precluded a more robust analysis of MEPPs in FDB muscles, so we turned to TS nerve–muscle preparations. The larger diameter of muscle fibres in this thin muscle facilitated continuous, stable micro-electrode recording without significant deterioration of resting membrane potential during several bath solution changes.

Figure 5C shows recordings of MEPPs from a TS muscle fibre as the concentration of cyclohexanol was progressively increased. Histograms of MEPP amplitude in this muscle fibre, comparing MPS and 20 mM cyclohexanol, indicated a clear and reversible decrease (Fig. 5D) and the cumulative histogram plot in Fig. 5E shows the dependence of MEPP amplitude in this fibre on cyclohexanol concentration.

Data summarizing the magnitude of MEPP attenuation with increasing cyclohexanol concentration are shown in Fig. 5F (P < 0.0001, ANOVA). For instance, in 20 mM cyclohexanol, MEPP amplitude decreased by 63.8 ± 3.54% (mean ± SDM; N = 5 muscles; P < 0.0001, Dunnett’s test). MEPP amplitude was almost completely restored within 1–2 min of reverting to normal bathing medium. Thus, the attenuation of MEPPs appeared to be greater than the decrease in initial amplitude of evoked EPP responses (see Fig. 3F). This finding prompted a quantal analysis of transmitter release, described later (see Figs 9 and 10).

Consistent with the correlation of EPP duration and amplitude in cyclohexanol (Fig. 5G), changes in MEPP half-decay time were barely discernible in some recordings. Overall, however, exponential curve fits to average MEPP decay showed a significant increase in time constant (time to 37% amplitude), from 2.83 ± 1.08 ms in MPS to 10.44 ± 6.37 ms in 20 mM cyclohexanol (mean ± SDM, N = 5 muscles and fibres; P = 0.035, paired t-test).

Although MEPP frequency discernibly increased during the recording shown in Fig. 5C, recordings from five other preparations showed an increase in MEPP
Figure 5. Cyclohexanol also attenuates MEPPs

A, consecutive traces showing spontaneous MEPPs in an FDB muscle fibre before and after exchanging MPS bathing medium with one containing 20 mM cyclohexanol. MEPP amplitudes were reduced by about 50%. B, representative hyperpolarizations of an FDB muscle fibre in response to rectangular hyperpolarizing current pulses of 1 nA were delivered through the recording microelectrode via a bridge circuit in the recording amplifier, balanced for the electrode resistance, before (upper trace) and after (lower trace) adding 20 mM cyclohexanol to the bathing medium. There was no difference in the muscle fibre input resistance comparable to the change in MEPP amplitude (A). C, continuous records on a slow time base from a muscle fibre in a different TS muscle preparation bathed in MPS during progressive increases in concentration of cyclohexanol in the bathing medium, showing reversible dependence of MEPP amplitude on cyclohexanol concentration. D, histograms of MEPP amplitude from the same muscle fibre shown in G based on measurements and analysis of 1 min long recordings of MEPPs in normal MPS (upper histogram), 20 mM cyclohexanol (middle) and after washing (lower). Histograms remained unimodal with a systematic shift to lower amplitudes. E, cumulative histogram plots from the same muscle fibre as in G, showing progressive, reversible decrease in amplitude of MEPPs with increasing concentration of cyclohexanol. F, summary data from 3–6 muscles, showing the concentration-dependent effect of cyclohexanol on mean MEPP amplitude. Each point shows the percentage change in mean MEPP amplitude in one muscle fibre (one recording per preparation/mouse). Lines show the overall mean ± SD of these values. MEPP amplitude was reduced by 30–60% in 10 and 20 mM cyclohexanol. G, paired data showing significant increase in MEPP decay time constant (Tau) before and after addition of 20 mM cyclohexanol in TS muscle fibre recordings from different muscles.
frequency in three fibres, a small decrease in one and no change in another (mean frequency in MPS: 1.23 ± 0.52/s; 20 mM cyclohexanol: 1.38 ± 1.18/s; P = 0.45; t-test). Thus, overall, there was no consistent change in MEPP frequency.

In sum, cyclohexanol attenuated and prolonged both EPPs and MEPPs with no overt or consistent changes in MEPP frequency, together suggesting that the predominant effect of cyclohexanol was on postsynaptic sensitivity to ACh.

Cyclohexanol enhances desensitization of human nAChR. If cyclohexanol reduces postsynaptic sensitivity then we would predict that transmembrane currents carried through postsynaptic nAChRs would also be reduced. To evaluate this explicitly, we turned to a stably transfected CN21 human cell line expressing both junctional and extrajunctional forms of hnAChR (subunit composition α2βδγ/e; Ring et al. 2015; Cetin et al. 2019). Whole-cell current recordings from these cells were therefore analogous to measurement of nAChR functioning at NMJs in human muscle in the absence of AChE activity. We used a semi-automated system (QPatch) to record whole cell currents at ambient room temperature, evoked by prolonged (3 s) pulses of ACh.

Figure 6A shows the whole-cell current response of a cell to a 3 s-long pulse of ACh (10 μM) and Fig. 6B shows progressive attenuation of this current with increasing concentrations of cyclohexanol (0.25–25 mM). The response to ACh was almost completely restored when the cell was superfused again with solution containing ACh but no cyclohexanol (Fig. 6C). The principal characteristic of the response to ACh was a relatively rapid increase in inward membrane current followed by a slower decline, suggesting desensitization of AChRs. Cyclohexanol attenuated ACh responses (measured as the area under the curve) in concentration-dependent fashion. The best non-linear least squares fit indicated an IC_{50} of 3.74 mM (95% CI: 3.17–4.43 mM, N = 17 cells; \( r^2 = 0.87 \) Fig. 6D). This is similar to the efficacy of cyclohexanol on neuronal nAChRs in Lymne (McKenzie et al. 1995) but less than the plasma concentration associated with neuromuscular block following gavage of insecticide at 37°C in anaesthetized pigs (Eddleston et al. 2012). hnAChR currents were substantially reduced in most experiments at a cyclohexanol concentration of 20 mM. But, in contrast to murine EPP responses, ACh-evoked currents in CN21 cells were not prolonged; rather, they were curtailed more rapidly as the concentration of cyclohexanol was increased. Specifically, the decay time constants of these currents decreased approximately exponentially with increasing concentration of cyclohexanol, from 210 ± 10 ms with ACh alone to 80 ± 20 ms after adding 20 mM cyclohexanol (\( K_D = 4.25 \text{ mM}; 95\% \text{ CI: } 1.98–10.24; \) \( r^2 = 0.491; \) Fig. 6E). Thus, during prolonged (3 s) administration of ACh, cyclohexanol appeared to promote or enhance desensitization of AChRs.

Cyclohexanol has complex effects on EPC decay. To explore further the possible pathophysiological significance of this apparent desensitization, we reverted to isolated mouse TS muscles and recorded EPCs under voltage-clamp. TEVC during repetitive stimulation was technically difficult, due to localized contracture of NMJs when AChE was inhibited with omethoate (Dissanayake et al. 2021), which tended to mechanically displace the voltage recording and/or current passing microelectrodes, damaging the muscle fibres. However, we obtained sufficient TEVC recordings from different groups of muscle fibres to evaluate the effect of brief tetanic stimulation (5 stimuli at 30 Hz) before and after adding either cyclohexanol (20 mM), omethoate (150 μM) or both compounds (Fig. 6F–I). Recording commenced 20 min after adding each compound or combination.

EPC amplitude and decay time were quite variable between muscle fibres (Fig. 6J and K) but the changes in amplitude and time course during 30 Hz stimulus trains were more consistent. As expected from EPP trains (see Figs 2C–E and 3H), after adding cyclohexanol EPC amplitude decreased during each stimulus train, but in this sample only by about 20% (Fig. 6L). By comparison, in solutions containing omethoate the amount of depression was not significantly different from that in recordings made in MPS. However, after blocking AChE with omethoate and then adding cyclohexanol, the amplitude and time course of the first EPCs were markedly decreased and prolonged (Fig. 6J and K, compare with the voltage recordings shown in Fig. 2F–H). Specifically, mean amplitude of the first EPC was 488.3 ± 84.58 nA in MPS (mean ± SDM, N = 4 mice), 381.7 ± 155.1 nA (N = 3) in 20 mM cyclohexanol, 248.6 ± 91.0 (N = 3) in 100 μM omethoate, and 129.6 ± 32.47 in omethoate and cyclohexanol together (n = 19 muscle fibres in N = 2 mice; P < 0.001, ANOVA; Dunnnett’s post hoc test, \( P = 0.004, 0.052, 0.0186 \), respectively). EPC_{1} decay time constants were 1.49 ± 0.35 ms in MPS (mean ± SD, N = 3 mice, n = 9 muscle fibres), 2.42 ± 0.66 ms in 20 mM cyclohexanol (n = 19 fibres), 5.28 ± 2.45 ms in omethoate (n = 11 fibres) and 19.40 ± 5.20 ms (n = 11 fibres) in both compounds (\( P < 0.001, \) ANOVA; Tukey’s tests: omethoate vs. cyclohexanol, \( P = 0.040, \) other combinations, \( P < 0.0001 \)).

As shown in Fig. 6L, MEPC amplitude and decay time constant were both significantly depressed during stimulus trains when both omethoate and cyclohexanol were present, albeit on a progressively increasing baseline current. In MPS, the fifth EPC (EPC_{5}) was 92.2 ± 4.05%
Figure 6. Cyclohexanol promotes desensitization of AChRs
A–C, whole-cell currents from a patched CN21 cell showing response to 3 s pulses of 10 μM ACh before (A) and their progressive attenuation with increasing concentration of cyclohexanol (0.25–25 mM, B), followed by recovery in normal MPS (C). D, summary data showing normalized integrated response (area under curve, AUC) with increasing concentration of cyclohexanol, indicating an IC50 of about 3.4 mM. Points show mean ± SD, n = 4–12 cells. E, the decay time constant of ACh-evoked whole-cell currents decreased progressively with increasing cyclohexanol concentration, consistent with an effect of cyclohexanol on desensitization of AChRs. Points are means ± SD from the same cells as in D; curve is the best single exponential fit to the mean data. F–I, representative EPC recordings made under TEVC in TS muscle fibres during trains of five stimuli delivered at 30 Hz: F, MPS; G, cyclohexanol alone (C6OL, 20 mM); H, omethoate alone (Ometh, 150 μM); I, omethoate and cyclohexanol together (Ometh + C6OL). EPC decay was prolonged in either cyclohexanol or omethoate but synergistically extended after adding both compounds, accompanied by a marked decrease in initial EPC amplitude and increased depression of EPC amplitude during the stimulus train. Close inspection indicated that, despite a tonic increase in baseline current (due to temporal summation), individual EPC decay times also decreased during the repetitive trains, suggesting enhanced desensitization of AChRs when both omethoate and cyclohexanol were present together. Vertical calibration, 200 nA for all but the bottom trace, which is 75 nA. Blue dotted line, baseline for reference. J and K, summary data showing distribution of initial EPC amplitude (J) and decay time constant (K) from all muscle fibre recordings in the data set. Each point represents data from one muscle fibre and the bars show means ± SDM of these data. L, relative depression of EPC amplitude during stimulus trains in each of the groups indicated. Each point represents the mean of recordings in preparations dissected from one mouse. Bars indicate mean ± SDM of these values. EPCs were substantially depressed by cyclohexanol when AChE was also inhibited with omethoate (Ometh + C6OL). M, relative change in EPC decay time constant during stimulus trains in each of the groups indicated. Each point represents the mean of recordings in preparations dissected from one mouse. Bars indicate means ± SDM. EPCs were substantially depressed by cyclohexanol during the stimulus train when AChE was also inhibited with omethoate (Ometh + C6OL).
of the first EPC (EPC₁; mean ± SDM, N = 4 mice, n = 7 muscle fibres). In omethoate, the EPC₅/EPC₁ ratio was slightly less, 82.82 ± 5.60% (N = 3, n = 7). In 20 mM cyclohexanol, the EPC₅/EPC₁ ratio was reduced to 69.84 ± 4.00% (N = 3, n = 17) but when combined with omethoate this ratio was further reduced to 30.01 ± 2.2% (N = 2, n = 11; P < 0.0001, ANOVA; P = 0.0395, P < 0.0001, P < 0.0001, Fisher's post hoc test comparing omethoate, cyclohexanol and the combination against MPS, respectively). Similarly, EPC decay was significantly accelerated during the stimulus train when omethoate and cyclohexanol were combined. Specifically, in MPS, the median decay constant of the first EPC was 1.66 ms (IQR 1.19–1.85 ms; n = 9 muscle fibres, N = 4 muscles) and this did not change during 30 Hz stimulation (median 1.64 ms, IQR 1.19–1.79 ms; P > 0.99, Wilcoxon test). Similarly, when AChE was inhibited with omethoate, although the EPC₁ decay time constant increased, it did not change substantively during 30 Hz stimulation (EPC₁ median 4.78 ms, IQR 3.12–9.58; EPC₅ median 4.74 ms, IQR 3.02–7.12 ms, n = 11 muscle fibres, N = 3 muscles; P = 0.25, Wilcoxon test). In cyclohexanol alone, the decay time constant of the first EPC increased to a median of 2.32 ms (IQR 2.08–2.8 ms, n = 19 muscle fibres, N = 3 muscles), but this also did not change significantly during 30 Hz stimulation (median 2.06 ms, IQR 1.86–2.68 ms; P = 0.25, Wilcoxon test). However, in solutions containing both omethoate and cyclohexanol (20 mM), the median decay time constant decreased during the stimulus train from 22.1 ms (IQR 17.34–26.86 ms, N = 2 muscles, n = 5 fibres in one muscle and n = 6 muscle fibres in the other; mean EPC₁ of both muscles = 19.54 ms) to 10.22 ms (IQR 9.42–12.3 ms; mean EPC₅ = 10.86 ms; P = 0.002, Wilcoxon test ranking n = 11 muscle fibres). Thus, cyclohexanol caused EPC decay time constant to decrease by about 50% during repetitive stimulation when junctional AChE was also inhibited.

These effects of cyclohexanol on EPC amplitude and decay during relatively high frequency stimulation therefore suggest that an increase in desensitization as AChRs become saturated, as indicated by the whole-cell recording data, could be of significance in the pathophysiology of NMJs exposed to a combination of OP and solvent metabolites.

Cyclohexanol impairs human nAChR channel gating. We attempted to reconcile the complex effects of cyclohexanol on EPC decay, by outside-out patch recording from small clusters of hnAChRs (comprising 3–8 channels) isolated from transfected HEK293T cells. An illustrative recording is shown in Fig. 7A and B. As predicted from whole-cell current measurements (Fig. 6A-C), within a few seconds of superfusing patches with a solution containing ACh (100 nM), initial channel activity was reduced, consistent with desensitization. Channel activity stabilized over the following 10–20 s into a pattern typical of the simultaneous opening of several channels. However, this settled pattern of channel activity changed markedly after adding cyclohexanol (10 mM). Channel openings became more erratic, with prolonged noisy episodes of inward current interspersed with individual brief openings. These complex effects of cyclohexanol made it almost impossible to distinguish single from multiple channel openings unequivocally but the overall pattern was consistent with a ‘flickery block’ of the AChR channel currents (Auerbach & Akk, 1998; Turner et al. 2011). The pattern of rectangular single and multiple channel events was largely restored on washing cyclohexanol (but retaining ACh) in the bath solution.

Our recordings were not suitable for conventional single-channel analysis of dwell times and bursting, since the patches contained multiple channels and we used relatively high concentrations of ACh (100 nM) to activate them. Nevertheless, a conservative, threshold analysis of four patches showed that the amplitude and area of detected inward current episodes consistently decreased, by about 50%, after adding 10 mM cyclohexanol (Fig. 7C and D; see figure legend for statistical analysis). As an overall measure of the effect of cyclohexanol on patch membrane current we calculated the charge transfer over specific 5 s episodes, before during and after adding cyclohexanol. The data are summarized in Fig. 7E. In patches superfused with 100 nM ACh, 50–55 s after adding 10 mM cyclohexanol charge transfer declined to about 30% of that in ACh alone (ACh median: 23.83% of initial charge transfer, IQ 17.76–29.3%; ACh + cyclohexanol: 6.4%, IQ 5.17–12.93%; N = 4 patches; P = 0.0013, Friedman’s test; P = 0.0037, post hoc Dunn’s test). Reversal of mean peak amplitude, area and charge transfer rate after removing cyclohexanol was incomplete, but most likely due to different rates of recovery of the ion channels in the patches.

Docking analysis suggest multiple binding sites for cyclohexanol on AChR. AChRs in Torpedo have been well characterized and are generally regarded as a good model of nAChR expressed postsynaptically at mammalian NMJs. Analysis of ligand docking using Autodock Vina, applying a ‘blind’ docking approach (Trott & Olson, 2010), identified several candidate binding sites for cyclohexanol. Of the nine highest affinity poses (largest −ΔGb binding energies), three poses, with predicted binding energies (ΔGb) of −6.58 and −5 kcal/mol, were associated with the same site located on the extracellular domain of the α-subunits (Fig. 8A), specifically, a pocket located on the external surface of the extracellular side of the permeability pore comprising amino acid residues Ile-31, Gln-58, Gln-59, Trp-60, Asp-83, Trp-86, Leu-87,
Leu-108, Leu-109, Ile-109, Ile-116, Met-117 and Trp-118 (Fig. 8B–D). This putative binding site is also located in close proximity to the Cys-loop of the receptor, in a region previously implicated in binding–gating coupling of nAChR (Gupta et al. 2017) and previously identified also as an allosteric site for binding of ethanol (Noori et al. 2018). Autodock Vina analysis predicted the binding energy for ethanol at this site (Fig. 8D) to be about −2.7 kcal/mol; that is, less than half the predicted binding energy of cyclohexanol at the same site, consistent with substantially higher concentrations of ethanol required to impair muscle AChR function (see Discussion). Four other candidate, externally-located binding sites of unclear significance were identified, with binding energies for cyclohexanol of about −5 kcal/mol (Fig. 8E–H). Notably, none of the five sites with highest affinity for cyclohexanol were located in the AChR pore region.

Taken together, the electrophysiological data obtained using several different recording techniques (voltage recording, TEVC, whole-cell current and outside-out patch recording) in two different paradigms (mouse muscle and transfected human cell lines) strongly support the hypothesis that cyclohexanol reduces the safety margin for neuromuscular transmission in intact muscle by impairing AChR function. The outside-out patch data also suggest that interference with AChR function is complex, triggering partially synchronized channel bursting. It seems likely that within the concentration ranges (5–25 mM) that cause myasthenia or neuromuscular paralysis in vivo, cyclohexanol affects ligand gating of AChR channels as a result of binding to multiple sites external to the channel pores. Overall, these effects offer a plausible underpinning of the attenuation but prolongation of spontaneous MEPPs and nerve-evoked EPCs, and hence the myasthenic responses observed in muscle force recordings following exposure to concentrations of cyclohexanol relevant to its toxicity in vivo.

**Presynaptic effects of cyclohexanol**

**Cyclohexanol increases quantal content.** As indicated above, there was a quantitative discrepancy in the

![Figure 7. Cyclohexanol impairs hnAChR gating](image)

A, continuous outside-out patch recordings from HEK293T cells transfected with cDNA for human nicotinic junctional AChRs (containing ε subunits), following perfusion with 100 nM ACh (blue bar), then after adding 10 mM cyclohexanol (red), then washing with ACh solution (blue). The trace shows an overt, reversible decrease in patch current, following initial desensitization. B, expanded regions of the recording shown in A, in the regions indicated (approximately) by lettered arrows (a–c). a, 100 nM ACh triggered multiple channel openings in this patch; b, cyclohexanol attenuated the overall responses but induced prolonged clusters of transient channel opening events; c, washing the patch with 100 nM ACh restored the normal pattern of channel openings. Dashed lines indicate the thresholds set for the analysis shown in C and D. C and D, threshold analysis of inward patch current from four outside-out patch recordings like those shown in A. C, mean peak amplitude of detected transient currents; D, area of inward current, during 30 s epochs after responses to ACh and cyclohexanol had stabilized. Summary data (mean ± SD and results of paired one-tailed t-tests): amplitude (C) – ACh: 4.73 ± 0.83 pA, C6OL: ACh + C6OL: 2.82 ± 0.48 pA, P = 0.003; area (D) – 10.09 ± 5.64 pA.ms, ACh + C6OL: 2.67 ± 1.71 pA.ms, P = 0.0175. E, charge transfer in selected 5 s epochs of four patch recordings before, during and after application of 10 mM cyclohexanol to patches superfused with 100 nM ACh, as shown in A. Box and whisker plots showing individual measurements (symbols) and median, interquartile range and overall range of charge transfer, expressed as a percentage of the peak charge transfer during the first 5 s of recording.
effects of cyclohexanol on EPP and MEPP amplitude. For instance, in 20 mM cyclohexanol MEPPs were reduced by more than 50% whereas EPPs evoked by low frequency stimulation (0.5–1 Hz) were reduced by only about 20–30% (see Figs 2, 3 and 5). Studies of neuromuscular block in different vertebrate and invertebrate species have revealed similar discrepancies. For instance, homeostatic up-regulation of transmitter release occurs at glutamatergic larval Drosophila NMJs and cholinergic mouse NMJs after blocking postsynaptic receptors with phosphotin or d-tubocurarine, respectively (Frank et al. 2006; Wang et al. 2016, 2018). Thus, the simplest explanation for the discrepant effect on EPPs and MEPPs is that while cyclohexanol reduces postsynaptic sensitivity to ACh (quantal size) it simultaneously increases evoked vesicular release (quantal content). An increase in quantal content is also consistent with steeper synaptic depression during repetitive stimulation (compare Figs 2D, 3H, 6H and 6L). Repetitive stimulation causes greater depletion of the readily releasable pool of synaptic vesicles and retards recruitment from the reserve pool when release probability at active zones is elevated, for example when extracellular [Ca$^{2+}$] is increased or repolarization of nerve terminals is prolonged by inhibiting presynaptic voltage-sensitive K+ channels (Hubbard, 1963; Saint et al. 1987; Braga et al. 1991; Ruiz et al. 2011; Wang et al. 2016). We therefore carried out a quantal analysis of transmitter release during exposure to cyclohexanol.

In a pilot experiment we made recordings in an FDB muscle preparation bathed in low Ca$^{2+}$/raised Mg$^{2+}$ solution (1 mM Ca$^{2+}$:3 mM Mg$^{2+}$), shown in Fig. 9. The records shown in Fig. 9A–C were obtained in a low Ca$^{2+}$/raised Mg$^{2+}$ solution (1 mM Ca$^{2+}$:3 mM Mg$^{2+}$), shown in Fig. 9. The records shown in Fig. 9A–C were obtained in a

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continuous recording from one muscle fibre in this preparation before and after adding 20 mM cyclohexanol. This modified MPS solution reduced the amplitude and increased the coefficient of variation of EPPs (mean ± SD = 3.53 ± 1.36 mV, n = 58 EPPs evoked at 1 Hz). Almost immediately after adding cyclohexanol, as expected, EPP half-decay time approximately doubled (Fig. 9B and G). However, mean EPP amplitude actually increased slightly, to 4.05 ± 1.07 mV (n = 89 EPPs; P = 0.04, unpaired t-test; Fig. 9H). Simultaneously, MEPP amplitude decreased by 53% (Fig. 9D and E), from 0.55 ± 0.22 mV (mean ± SD, n = 36 MEPPs) to 0.29 ± 0.07 mV (n = 36 MEPPs; P < 0.0001, unpaired t-test). Resting membrane potential was quite stable throughout the experiment (Fig. 9F). Thus, mean quantal content (the ratio of evoked depolarization to uniquantal, spontaneous depolarization) more than doubled, from 6.4 to 13.9 quanta. Quantal content estimated using the variance method (that is, for a coefficient of variation, CV, of EPP amplitudes quantal content, \( m = 1/CV^2 \)) increased from 6.7 quanta in MPS to 14.3 quanta after adding cyclohexanol. After washing cyclohexanol from the recording chamber, mean quantal content partly reverted, to 10.1 quanta calculated directly and 9.9 quanta using the variance method. In the same muscle, we repeated the exchange of solutions and used the variance method to estimate quantal content in three discontinuous groups of recordings (MPS, 20 mM cyclohexanol, Wash; Fig. 9I). Mean quantal content in MPS was 2.67 ± 1.72 quanta (mean ± SD, n = 8 muscle fibres, N = 1 muscle). After adding cyclohexanol (20 mM), mean quantal content increased to 10.24 ± 4.80 quanta (n = 9 different muscle fibres; P = 0.0006, Mann–Whitney test). Washing cyclohexanol from the bathing medium partially reversed this effect, reverting quantal content to 6.16 ± 2.54 quanta (n = 9 different fibres; P = 0.0625, Mann–Whitney test). Thus, in this experiment, cyclohexanol increased quantal content after adding cyclohexanol under conditions of

**Figure 9.** Cyclohexanol increases quantal content in low Ca\(^{2+}\) solution
A–C, superimposed EPPs recorded from an FDB muscle fibre stimulated at 1 Hz, bathed in MPS containing 1 mM Ca\(^{2+}\)/3 mM Mg\(^{2+}\). The muscle was not pre-incubated in μCTX-GIIB. A, MPS; B, after adding 20 mM cyclohexanol; C, after restoring normal MPS bathing medium. D and E, averaged MEPPs (>30) recorded from the same muscle fibre as A before adding cyclohexanol (D) and after adding 20 mM cyclohexanol (E). Red lines indicate exponential curve fits to the MEPP decay, generated by Mini Analysis software. Cyclohexanol reduced average MEPP amplitude by about 40% in this fibre. F–J, continuous data plots for each recorded EPP illustrating: F, stability of resting membrane potential baseline; G, reversible increase in half-decay time after adding 20 mM cyclohexanol (20 mM), a robust indicator of the effect of cyclohexanol on EPPs; H, peak amplitude. Note the discernible, small increase in mean EPP amplitude, despite the decrease in MEPP amplitude, and a decrease in EPP amplitude variance during application of cyclohexanol, consistent with an increase in quantal content. Data capture was paused between solution changes (about 1 min, not indicated) so the apparent stepwise change in EPP decay (in G) does not reflect the rate of change of EPP decay. I, summary data showing reversible increase in quantal content calculated from the variance of EPP amplitudes. Each point represents data from a different muscle fibre in the same muscle as the recordings shown in A–H. Lines indicate means ± SD.
reduced Ca\textsuperscript{2+}/increased Mg\textsuperscript{2+}. We therefore measured the effect of cyclohexanol on quantal content of EPCs under more stringent physiological ionic conditions.

First, we established that the same differential effect of cyclohexanol on EPP and MEPP amplitude occurred in TS muscle fibres. Figure 10A–C shows data from a continuous recording from a TS muscle fibre after blocking muscle action potentials with \(\mu\)-CTX-GIIIB. After adding cyclohexanol (20 mM), EPP amplitude was reduced by only about 20% in this fibre. Simultaneously, the modal MEPP amplitude was reduced by about 50%. These changes were almost completely reversed on restoring control MPS solution to the recording chamber.

We then calculated quantal contents from EPC rather than MEPP recordings, to overcome the potentially confounding effect of non-linear summation of EPPs that normally occurs at physiological levels of release (McLachlan & Martin, 1981). Representative averaged EPC and MEPC recordings are shown in Fig. 10D–G. The initial peak amplitude of evoked EPCs in MPS during low frequency stimulation (2 Hz) was 388.5 ± 58.62 nA (mean ± SDM, \(n = 13\) muscle fibres, \(N = 5\) muscles; Fig. 10H and I).

The initial EPC amplitude in this group of recordings after adding cyclohexanol was not significantly different, 387.4 ± 64.79 nA (\(n = 22\) fibres, \(N = 5\) muscles; \(P = 0.99\), unpaired \(t\)-test). Nevertheless, as expected, cyclohexanol consistently prolonged EPCs as we observed for EPPs (Fig. 10J; time constants, MPS, 1.81 ± 0.12 ms, \(N = 5\); cyclohexanol 2.71 ± 0.19 ms, \(N = 4\); \(P = 0.004\), \(t\)-test).

MEPCs (quantal size, Fig. 10K) recorded in the same traces as the EPCs were reduced in amplitude in this sample by about 40%, from 2.8 ± 0.3 to 1.7 ± 0.2 nA (mean ± SDM, \(N = 5\) muscles, in both samples, \(P = 0.009\), unpaired \(t\)-test). Thus, after adding 20 mM cyclohexanol quantal content estimated from the EPC/MEPC amplitude ratio was increased by approximately 60%, from a median of 147 (IQR 86–168) quanta in MPS to 237 (IQR 98–217) quanta (\(P = 0.011\), Mann–Whitney test; Fig. 10L). These estimates of increased quantal content are likely to be conservative, compared with estimates based on the ratio of EPC to MEPC charge transfer (Wood & Slater, 1997), since the increase in decay time of MEPCs in cyclohexanol was less than that of EPCs. Either way, the increase in quantal content mostly offset the effect of cyclohexanol on quantal size, explaining the discrepancy between the effect of cyclohexanol on EPPs and MEPPs.

Analysis of successive EPCs evoked at a low frequency of stimulation (1 Hz) in control MPS showed that, like the effect on EPPs, repetitive stimulation caused a relatively small amount (about 5–10%) of EPC depression (Fig. 10I and M). The mean amplitude of the fourth EPC (EPC\(_4\)) in MPS was 345.7 ± 52.03 nA (mean ± SDM, \(n = 13\) muscle fibres, \(N = 5\) muscles; \(P = 0.002\), paired \(t\)-test compared with EPC\(_1\)). By contrast, EPC amplitude in 20 mM cyclohexanol declined by about 25%, to 290.9 ± 47.66 nA (\(n = 22\) fibres; \(N = 4\) muscles; \(P < 0.0001\), paired \(t\)-test; Fig. 10M). Correspondingly, the median quantal content of EPC\(_4\) in control MPS decreased by about 15%, to 125 quanta (IQR 78–157 quanta) but in cyclohexanol median quantal content of EPC\(_4\) was reduced by almost twice as much, to 74 quanta (IQR 98–217 quanta; \(P = 0.016\), Mann–Whitney test).

The resulting difference in EPC\(_4\) quantal content, comparing MPS control and cyclohexanol solutions, was small and not quite statistically significant by standard criteria (\(P = 0.053\), Mann–Whitney test). Taken together, these measurements suggest that the initial quantal content of EPCs, or those evoked by low frequency stimulation, is increased in cyclohexanol. However, depression of EPC quantal content during higher frequency stimulation effectively abolished the initial offset of quanta size and quantal content. These effects are consistent with the failure of neuromuscular transmission during TOF stimulation observed in membrane muscle force (Fig. 1A) and voltage recordings (Fig. 3B).

Cyclohexanol inhibits presynaptic K\textsuperscript{+} currents. Increases in quantal content are associated in some circumstances with delayed or prolonged repolarization of motor nerve terminals, for example as a result of pharmacological block of presynaptic K\textsuperscript{+} currents (Datyner & Gage, 1980; Molgó et al. 1980; Murray & Newsom-Davis, 1981; Brigant & Mallart, 1982; Penner & Dreyer, 1986). If the increase in quantal content caused by cyclohexanol was explained by a similar mechanism, we would predict inhibition of presynaptic K\textsuperscript{+} currents in nerve terminals by cyclohexanol. To test this, we made perineural recordings from intramuscular nerve branches in TS muscles, within about 100 \(\mu\)m of NMJs. Postsynaptic responses to nerve stimulation were blocked by pre-incubating the muscles in 10 \(\mu\)g/ml TRITC-\(\alpha\)-bungarotoxin. Some muscles were also pre-incubated in \(\mu\)-CTX (0.5–2 \(\mu\)M).

Figure 11A shows representative perineurally recorded responses to intercostal nerve stimulation in control MPS solution and increasing concentrations of cyclohexanol (5–25 mM). As expected, the principal components of the waveform, after the stimulus artefact, comprised a brisk negative deflection – previously associated with inward Na\textsuperscript{+} current at nodes of Ranvier – followed by a larger negative wave, associated with outward current generated by voltage-sensitive K\textsuperscript{+} channels concentrated in presynaptic nerve terminals (Brigant & Mallart, 1982; Penner & Dreyer, 1986; Searl & Silinsky, 2010a). The axonal Na\textsuperscript{+} current was evidently unaffected by cyclohexanol but the nerve terminal K\textsuperscript{+} current was progressively reduced as cyclohexanol concentration was increased. This effect was partially but rapidly reversed on restoring normal MPS bathing medium. As expected, the large negative wave was also selectively and reversibly inhibited by 10 mM...
Figure 10. Cyclohexanol increases quantal content in normal Ca^{2+} solution

A–C, continuous recordings of EPPs evoked at low frequency (1 Hz; upper traces in each case) and spontaneous MEPPs (lower traces) from a TS muscle bathed in MPS (A); then after adding 20 mM cyclohexanol (B); then restoring normal bathing medium (C). The EPP amplitude was reduced by less than 20% in cyclohexanol, while half-decay increased about twofold. However, the amplitudes of spontaneous MEPPs (occurrences indicated by points under the high gain traces) were reduced by more than 50%. Inset histograms indicate the distributions of MEPP amplitudes in each solution. Calibration: upper traces (EPP) 20 mV, 20 ms; lower traces (MEPPs): 0.5 mV, 50 ms.

D–G, TEVC recordings of averaged nerve-evoked EPCs (D, E; \( n = 20 \) traces) and corresponding spontaneous MEPCs (F, G; average of \( >100 \) events) in the same two representative TS muscle fibres, in normal MPS (D, F) and 20 mM cyclohexanol (E, G). Exponential curves fitted to EPC and MEPC decay by Mini Analysis software are indicated by superimposed red lines. H, peak amplitudes of first (EPC1, filled circles) and fourth (EPC4, open circles) responses to TOF stimulation in MPS and, in a different sample, 20 mM cyclohexanol. Each point represents data from one muscle fibre. I, mean amplitudes of EPCs. Each point represents mean data from a preparation dissected from one mouse. J, cyclohexanol prolonged the decay of EPCs (open bars) but in this sample did not significantly

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alter the decay of MEPCs (hatched bars). Each point represents mean data from one muscle (mouse). Bars indicate means ± SDM of these mean data. K, MEPC amplitudes in the same muscle fibres as those in A (open circles). Filled circles are mean amplitudes from each mouse. Bars represent the means of these mean data and lines show 95% confidence limits. L, quantal content (m) measurements calculated from the ratio of EPC to MEPC amplitudes in the muscle fibres corresponding to the data shown in H and K. Each point represents the mean quantal content, averaged with respect to mice, and bars indicate the overall mean with 95% confidence intervals. The difference between means indicated a significant increase in quantal content of the first EPC (m₁) comparing cyclohexanol (C60L) with control MPS solution. M, depression of quantal content of EPCs, shown as the ratio of the quantal contents of the fourth (m₄) to the first (m₁) and EPCs in TOF responses and expressed as percentage, was also significantly greater (lower m₄/m₁ ratio) in 20 mM cyclohexanol. Each point represents the mean quantal content ratio, averaged with respect to mice, based on the same recordings used to calculate quantal contents (L).

tetraethylammonium (TEA; Fig. 11B), consistent with its identity as a voltage-sensitive K⁺ current (Penner & Dreyer, 1986).

Data summarized from four preparations (N = 4 mice) confirmed that the amplitude of the Na⁺ component (Fig. 11C) was not significantly altered by cyclohexanol (Pearson’s r² = 0.36; 95% CI = −0.9494 to 0.4119; P = 0.21). By contrast, selective attenuation of the K⁺ component showed clear dependence on the concentration of cyclohexanol (Fig. 11D). For instance, the peak K⁺ component was reduced from −7.10 ± 1.24 mV (mean ± SDM, n = 4) in MPS to −3.95 ± 0.78 mV in 15 mM cyclohexanol (P = 0.0015, paired t-test). However, the effect of cyclohexanol on K⁺ current was not as distinct as that caused by similar concentrations of TEA. Notably, as shown in Fig. 11B, TEA (10 mM) unmasked a delayed positive wave attributed previously to inward nerve terminal voltage-sensitive Ca²⁺ current (Brigant & Mallart, 1982; Penner & Dreyer, 1986). In one experiment we observed small (+200 μV) positive, putative Ca²⁺ waves when K⁺ current was reduced by cyclohexanol but this was not evident in preparations from the other mice, even when the cyclohexanol concentration was increased to 25 mM. Rather, the effect of 20–25 mM cyclohexanol was similar to the submaximal effect produced by 1–5 mM TEA (Brigant & Mallart, 1982; Penner & Dreyer, 1986). However, we cannot rule out the possibility of other effects of cyclohexanol on K⁺ channel sub-types that may be expressed in motor nerve terminals, or additional antagonistic effects of cyclohexanol on presynaptic Ca²⁺ currents.

Cyclohexanol and TEA may bind to the same sites in Kv channels. To obtain further insight into the causes of the reduced presynaptic K⁺ current, we examined models of Kᵥ1.2 and a Kv1.2/2.1 chimeric channel, which contains binding sites for TEA in the selectivity filter (Luzhkov et al. 2003; Wacker et al. 2012). Analysis with Autodock Vina using a blind-docking approach identified candidate sites for both cyclohexanol and TEA on surfaces external to the permeability pore, with predicted binding energies of approximately −4 kcal/mol. However, targeting the search region to the pore predicted a binding site with affinity for cyclohexanol located on the four non-polar residues at the base of the permeability filter (Fig. 11E), with a predicted binding energy of −3.8 kcal/mol. These residues are thought to be the same as the principal binding sites for TEA, which in our Autodock Vina simulation predicted a TEA binding energy at the same sites of −3.4 to −3.9 kcal/mol (Fig. 11E). Thus, consistent with the perineurial recording data, cyclohexanol may interfere directly with the conductance and permeability of voltage-sensitive K⁺ channel pores, reducing transmembrane K⁺ ionic flux through the open channel, albeit less effectively than TEA. Perhaps the dimensions and charge distribution on TEA moieties constitute a greater obstacle to ionic flux through the pore than cyclohexanol.

**EPP duration is greater in cyclohexanol than in TEA.** Finally, we considered whether inhibition of K⁺ current by cyclohexanol, by enhancing and prolonging transmitter release, might contribute to the prolonged time course of EPPs. To assess this, we compared recordings of EPP trains evoked at 20 Hz (N = 3 muscles from 2 mice) sampling 2–5 muscle fibres in each preparation before and after adding either 20 mM cyclohexanol or 10 mM TEA (Fig. 12A–F). Data shown in Fig. 12G and H indicate that EPP rise time in cyclohexanol (2.57 ± 0.19 ms, mean ± SDM, N = 3 muscles) was about the same as the increase in TEA (2.13 ± 0.27 ms) and significantly greater than in MPS (1.11 ± 0.21; P = 0.0002, ANOVA; P = 0.0522; P = 0.0002, Fisher’s test; compare also with data presented in Figs 3 and 5). However, overall EPP duration (rise time plus time to 90% decay) in cyclohexanol was 26.75 ± 7.99 ms (mean ± SD, N = 3 muscles), about twice that in TEA (12.74 ± 5.2 ms) and more than four times greater than in MPS (6.72 ± 1.13 ms; N = 3 muscles; P = 0.012, ANOVA; P = 0.0214 and P = 0.0045, post hoc Fisher’s test). By contrast, synaptic depression was more than five times greater in TEA than in MPS and more than three times greater than in 20 mM cyclohexanol (Fig. 12J; MPS: 62.5 ± 14.0%; cyclohexanol: 41.8 ± 8.4%; TEA: 10.9 ± 3.8% initial EPP amplitude; P = 0.0102, repeated measures ANOVA; P = 0.0258, P = 0.0238, respectively, Tukey’s post hoc test).

Thus, although these data comparing effects of TEA and cyclohexanol do not rule out a contribution of prolonged transmitter release to the prolongation of EPPs in
Figure 11. Cyclohexanol selectively inhibits perineurial K currents

A, superimposed traces showing perineurial recordings from TS axons and motor nerve terminals in response to intercostal nerve stimulation (average of 10 responses per trace), in control MPS solution (blue trace), with 5 mM increments in cyclohexanol (5–25 mM, red trace), and after washing with normal MPS (orange trace). Cyclohexanol selectively reduced the K\(^+\) component without systematically altering the Na\(^+\) component of the perineurial waveform. B, similar recording to A, from the same perineurial location, showing the response of the waveform in MPS (blue), before adding 10 mM TEA (red) to the bathing medium (averages of 10 responses per trace). The K\(^+\) component was abolished and unmasked a transient positive component, attributable to nerve terminal Ca\(^{2+}\) current. Washing with normal MPS (orange) restored the control response almost completely.

C, summary data showing the absence of significant effect of cyclohexanol on the amplitude of the initial Na\(^+\) component of the perineurial waveform. Each point represents the response from one preparation. Line shows no significant regression \((r^2 = 0.36; P = 0.21)\).

D, summary data showing progressive inhibition of the K\(^+\) component of the perineurial waveform with increasing concentration of cyclohexanol. Each point represents the response from the same preparations as those in C. Line is the non-linear least squares best fit of a sigmoidal function \((r^2 = 0.84; Sy.x = 0.93)\). E, expanded view of the pore region of a model of the K\(_{V1.2}\) voltage-sensitive K\(^+\) channel, including the four known binding sites for TEA. Poses indicated by Autodock Vina are shown for cyclohexanol (cyan skeleton) and TEA (magenta/blue), which had a slightly lower predicted binding energy than cyclohexanol, bound to TEA-binding motifs (red).
cyclohexanol, a combination of pre- and postsynaptic factors including effects on the gating of postsynaptic nAChR channels, seems more plausible.

Discussion

Overall, the data support the hypothesis that cyclohexanol impairs postsynaptic AChR function at neuromuscular junctions by reducing their sensitivity to ACh (see Figs 6–8). Simultaneously, cyclohexanol depresses presynaptic $K^+$ currents, one consequence of which would be to prolong presynaptic action potentials, thus indirectly enhancing vesicular release of ACh (see Figs 9–11). However, the negative effects of cyclohexanol on AChR function appeared ultimately to dominate, leading to subthreshold EPPs, failure of muscle action potential generation and neuromuscular paralysis, as observed respectively in microelectrode recordings and in muscle force recordings in vitro (see Figs 1–5) and in vivo (Dissanayake et al. 2021). Under pathophysiological conditions, when AChE is also inhibited or absent, cyclohexanol ultimately antagonizes AChRs, impairing channel opening properties, reducing conductance and perhaps promoting desensitization. In parallel, the effects of cyclohexanol on quantal content and duration of EPPs at NMJs both increase and prolong the effect of ACh, potentially exacerbating downstream, $Ca^{2+}$-mediated cytotoxic effects of ACh (Laskowski et al. 1975; Leonard & Salpeter, 1979; Meshul et al. 1985; Fucile et al. 2006; Zayas et al. 2007). Thus, our findings provide novel pathophysiological insight into the causes of acute muscle weakness or paralysis that follow OP insecticide ingestion (Edelsten et al. 2012). Additional studies are required to establish whether solvent toxicity

Figure 12. Cyclohexanol prolongs EPPs more than TEA

A–C, intracellular recordings of EPP trains evoked at 20 Hz from three different muscle fibres in the same FDB muscle bathed in control MPS solution (A); after adding 20 mM cyclohexanol (C6OL) (B); and after substituting cyclohexanol with 10 mM TEA (C). Corresponding records of the first and tenth EPP (insets) are shown in D–F. TEA enhanced the initial EPP but then caused marked depression of EPPs, the expected result of depletion of synaptic vesicles from active zones in response to the prolonged presynaptic action potential and prolonged $Ca^{2+}$ current, caused by inhibition of presynaptic Kv channels (see Results). The duration of EPPs in TEA was also prolonged. However, in cyclohexanol the initial EPP was slightly decreased compared to those of EPP trains recorded in MPS (see also Figs 2–4); synaptic depression was less, but EPP duration was greater than in TEA. D–F, expanded records of the first EPP (EPP$_1$) and tenth EPP (EPP$_{10}$) from the corresponding recordings in A–C. D, MPS; E, 20 mM cyclohexanol; F, 10 mM TEA. G, mean EPP$_1$ rise time in control (MPS) solution and after adding either 20 mM cyclohexanol (C6OL) or 10 mM TEA to the same muscles ($N = 3$). Each point is the mean of samples of 2–10 muscle fibres in each muscle. H, box and whisker (95% limits) plots of EPP duration, based on the sum of rise time and time to 90% decay (T90). Open circles are individual data from each muscle fibre in recordings from the same three muscle as in G, mean values from each muscle indicated by filled circles. I, synaptic depression (EPP$_{10}$/EPP$_1$) was 3–5 times greater in TEA than either cyclohexanol or MPS.

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in general is a proximate cause of acute myasthenia and delayed onset ‘intermediate syndrome’ (IMS) that follow self-harming by OP insecticide ingestion, still prevalent among agricultural communities in large areas of rural Asia (Eddleston et al. 2008, 2012; Hulse et al. 2014; Mew et al. 2017; Dissanayake et al. 2021).

**Postsynaptic effects**

Our analysis of potential binding sites for cyclohexanol on AChRs suggests binding to sites that include one identified previously for ethanol, an aliphatic alcohol. However, the predicted binding energies are relatively small, commensurate with the relatively high concentrations required to mediate their pathophysiological effects. In passing, it is perhaps noteworthy that legislation in many countries bans driving of motor vehicles when blood ethanol concentration exceeds 11–17 mM (Otton et al. 2009; World Health Organization, 2018). As implied by our studies, similar concentrations of cyclohexanol have substantial effects on neuromuscular transmission, sufficient to cause potentially fatal neuromuscular paralysis (Eddleston et al. 2012; Dissanayake et al. 2021). Data from previous studies indicate that the effective concentrations of ethanol at NMJs are 10–100 times greater than those shown here for cyclohexanol (Cooper & Dretchen, 1975; Gage et al. 1975; Bradley et al. 1984; Linder et al. 1984; Zuo et al. 2002; Searl & Silinsky, 2010b). Complex effects of aliphatic alcohols have been examined in several studies of nAChR function and transmitter release at NMJs (Gage et al. 1975; Bradley et al. 1984; Zuo et al. 2004; Searl & Silinsky, 2010a,b). Overall, aliphatic alcohols potentiate endplate responses at relatively low concentrations but inhibit them at higher concentrations (Cooper & Dretchen, 1975). However, muscle tension recordings even in high concentrations of ethanol (200 mM) do not show the characteristic fade shown here by cyclohexanol (Gage, 1965).

Prolonged EPPs of reduced amplitude are also a distinctive feature of several known congenital myasthenic syndromes, arising from mutations in AChR subunit proteins or mutations in AChE (Engel et al. 1982; Hutchinson et al. 1993; Zayas et al. 2007; Chevassier et al. 2012; Webster et al. 2012, 2013; Sigoillot et al. 2016). Our preliminary analysis suggests that none of the AChR mutations identified thus far coincides with any of the candidate binding sites for cyclohexanol to AChRα-subunits, including the ethanol binding site, that we identified in our investigation of a nAChR model (see Fig. 8). However, some slow-channel AChRs and ColQ-AChE mutations are associated with myopathy and degeneration at NMJs, which is mitigated by treatment with fluoxetine (Deflorio et al. 2014; Zhu et al. 2015; Vidanagamage et al. 2021). The complexity and diversity of the effects of cyclohexanol on single hnAChR channel activity suggests that it has multiple actions the function of these receptors, possibly including open-channel block, flickery-block, competitive antagonism or some combination. However, if these effects of cyclohexanol induce similar neuromuscular synaptic pathology to that in individuals with slow-channel myasthenic syndromes then perhaps fluoxetine may be an effective prophylactic treatment in patients who have swallowed OP insecticides containing cyclohexanol solvent precursors as well.

Cyclohexanol also modulates GABA A receptor function in the CNS (Hall et al. 2011; Chowdhury et al. 2016) but to our knowledge the present study comprises the first electrophysiological investigation of the actions of a cyclic alcohol on NMJs. Whole-cell currents mediated by neuronal nAChRs, expressed in snail neurones are inhibited by cyclohexanol and by several other cyclic alcohols (McKenzie et al. 1995). The IC 50 for cyclohexanol in that study (about 5.5 mM) was similar to that reported here for the human postsynaptic NMJ form of the receptor (see Fig. 6D). The effects of cyclohexanol merit comparison with its derivatives and with aliphatic alcohols. For instance, the cyclohexanol derivative 2-(4-phenylpiperidino)cyclohexanol (vesamicol) is well established as an inhibitor of synaptic vesicular ACh transport at NMJs (Estrella et al. 1988; Searl et al. 1991). However, the functional effects of vesamicol on amplitudes of MEPPs and EPPs take much longer (hours) to develop, compared with the rapid (seconds to minutes) and rapidly reversible effects we have described here.

**Presynaptic effects**

The present data also strongly suggest that cyclohexanol increases quantal content by reducing presynaptic K + currents, probably by binding to the same sites as TEA in TEA-sensitive channels, likely to be of the Kv3.3 subtype thought to be selectively expressed in motor nerve terminals (Brooke et al. 2004; Wu et al. 2021). Although inhibition of presynaptic K + currents provides perhaps the simplest explanation for the increase in quantal content (Datynar & Gage, 1980; Molgó et al. 1980; Murray & Newsom-Davis, 1981; Brigant & Mallart, 1982; Penner & Dreyer, 1986) other, plausible alternatives should be considered. Perhaps the most attractive alternative hypothesis would be inhibition of presynaptic ACh receptors (McKenzie et al. 1995). Whether or to what extent presynaptic nicotinic and muscarinic receptors regulate transmitter release at NMJs is a subject of long-standing debate (Bowman et al. 1988, 1990; Minic et al. 2002; Tomàs et al. 2017; Wang et al. 2018). Selective m1 and m2 antagonists appear to have opposing effects on transmitter release (Santafé et al. 2003). Conversely, presynaptic nAChRs have been implicated in regulation of the rate of mobilization of synaptic vesicles to active zones, based on the increase in rate of short-term synaptic...
depression during tetanic stimulation (Bowman et al. 1988, 1990). Reports of the effect of nicotinic antagonists, such as d-tubocurarine or α-bungarotoxin, on quantal content during low-frequency stimulation or at the start of high-frequency trains of stimulation have been conflicting (Auerbach & Betz, 1971; Hubbard & Wilson, 1973; Bowman et al. 1990). However, in a recent study neither atropine, a non-selective muscarinic receptor antagonist, nor α-CtxArIIB, a selective inhibitor of α7-containing nAChRs expressed in motor neurons, had any effect on tubocurarine-induced upregulation of quantal content of EPPs (Wang et al. 2018). Thus, together, while these observations do not rule out the possibility that effects of binding of cyclohexanol to presynaptic AChRs mediate its effect on quantal content, they render these hypotheses less attractive than one based on antagonism of presynaptic K+ channels, supported by the present perineurial recording data.

Nevertheless, the data indicate potential utility for cyclohexanol as a molecular tool that might facilitate investigation of the homeostatic mechanisms that acutely or chronically regulate synaptic strength, in health and disease (Harris & Ribchester, 1979; Wood & Slater, 2001; Davis & Müller, 2015; Jones et al. 2016; Ribchester & Slater, 2018; Frank et al. 2020). Postsynaptic nicotinic antagonists produce acute, reversible up-regulation of evoked ACh release (Wang et al. 2016; 2018). One difference from the findings reported here, albeit preliminary, was that cyclohexanol still increased evoked transmitter release when Ca2+ concentration was reduced and [Mg2+] was increased (see Fig. 9), which was not seen when d-tubocurarine, for example, was used to block postsynaptic nAChRs and to induce upregulation of transmitter release (Wang et al. 2016). By contrast, homeostatic upregulation of release at larval Drosophila NMJs, where glutamate is the excitatory neurotransmitter rather than ACh, occurs over a range of extracellular Ca2+ concentrations (Frank et al. 2006; Newman et al. 2017). The mechanism of feedback upregulation at larval Drosophila NMJs involves a postsynaptic-to-presynaptic signalling cascade involving Ca2+/calmodulin-dependent protein kinase II, semaphorins, BMPs, plexin B, Deg/ENaC channels and regulation of α2-δ subunits of Ca2+ channels (Ribchester & Slater, 2018; Frank et al. 2020). Direct measurements of K+ channel function may help to rule out alternative hypotheses that seek to establish general mechanisms of homeostatic plasticity in representative species of these different phylogenetic classes.

**How does cyclohexanol prolong EPPs?**

We have not been able to resolve unequivocally how cyclohexanol prolongs EPPs and EPCs while apparently curtailing whole-cell current duration during prolonged exposure to ACh. There was no discernible effect of cyclohexanol on membrane time constant or input resistance and cyclohexanol does not significantly inhibit AChE (Dissanayake et al. 2021). A plausible hypothesis is that prolonged EPPs or EPCs – especially when AChE is inhibited – are due to the combined effects of cyclohexanol on binding to AChRs postsynaptically and Kv channels presynaptically. The effect of cyclohexanol on the duration of EPPs was also less than that on EPCs but such discrepancies are to be expected, since membrane capacitance and resistance act together as a low pass filter, retarding polarization in response to transient changes in membrane current. Inspection of computational models of the relationship between EPC and EPP duration (Gage & McBurney, 1973; Ginsborg et al. 1981) suggests that an increase in EPC decay time constant from 1.0 to 3.0 ms would increase EPP half-decay from about 2.5 ms to about 7.5 ms, which is similar to the effect of cyclohexanol on EPCs and EPPs reported here. The overt effects on the pattern of patch current activity (Fig. 7) are consistent with the analysis of whole-cell currents in response to prolonged pulses of ACh and cyclohexanol (Fig. 6).

In addition, protracted episodes of reduced amplitude, flickery-block would be expected, on summation, to both attenuate and prolong overall endplate current and depolarization. More detailed analysis of single-channel currents during rapid transient application of ACh ± cyclohexanol (see for example, Wyllie et al. 1998) would perhaps allow a critical test of this hypothesis.

Finally, we may speculate on whether cyclohexanol might impair function of other ion channels or receptors in axons, skeletal muscle or NMJs. Preliminary in silico analysis suggests a weak interaction with the gating region of voltage-sensitive Nav 1.4 channels, the μCTX-sensitive isoform expressed in skeletal muscle (F. Margetiny, unpublished observations). However, our intracellular and perineurial recordings imply that significant impairment of voltage-sensitive Na+ channel function is unlikely, since nerve stimulation had no effect on perineurally recorded Na+ currents or action potential generation in muscle fibres. Nevertheless, our previous study showed that cyclohexanol can also inhibit muscle contraction directly (Dissanayake et al. 2021). Further investigations are required to establish whether cyclohexanol additionally uncouples muscle excitation from contraction, for example by detubulation (Gage & McBurney, 1973; Kurihara & Brooks, 1975), or by direct interference with either sarcoplasmic reticulum or contractile protein function, or some other mechanism.

**References**

Auerbach A & Akk G (1998). Desensitization of mouse nicotinic acetylcholine receptor channels. A two-gate mechanism. J Gen Physiol 112, 181–197.
Auerbach A & Betz W (1971). Does curare affect transmitter release? *J Physiol* **213**, 691–705.

Bekoff A & Betz WJ (1977). Physiological properties of dissociated muscle fibres obtained from innervated and denervated adult rat muscle. *J Physiol* **271**, 25–40.

Beman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN & Bourne PE (2000). The protein data bank. *Nucleic Acids Res* **28**, 235–242.

Bird SB, Krajacic P, Sawamoto K, Bunya N, Loro E & Khurana TS (2016). Pharmacotherapy to protect the neuromuscular junction after acute organophosphorus pesticide poisoning. *Ann N Y Acad Sci* **1374**, 86–93.

Bowman WC, Marshall IG, Gibb AJ & Harborne AJ (1988). Feedback control of transmitter release at the neuromuscular junction. *Trends Pharmacol Sci* **9**, 16–20.

Bowman WC, Prior C & Marshall IG (1990). Presynaptic receptors in the neuromuscular junction. *Ann N Y Acad Sci* **604**, 69–81.

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

Braga MF, Anderson AJ, Harvey AL & Rowan EG (1992). Apparent block of K⁺ currents in mouse motor nerve terminals by tetrodotoxin, mu-conotoxin and reduced external sodium. *Br J Pharmacol* **106**, 91–94.

Braga MF, Harvey AL & Rowan EG (1991). Effects of tacrine, valnacrine (HP029), suronacrine (HP128), and 3,4-diaminopyridine on skeletal neuromuscular transmission in vitro. *Br J Pharmacol* **102**, 909–915.

Brigant JL & Mallart A (1982). Presynaptic currents in mouse motor endings. *J Physiol* **333**, 619–636.

Brooke RE, Moores TS, Morris NP, Parson SH & Deuchars J (2004). Kv3 voltage-gated potassium channels regulate neurotransmitter release from mouse motor nerve terminals. *Eur J Neurosci* **20**, 3313–3321.

Buratti FM & Testai E (2007). Evidences for CYP3A4 auto-activation in the desulfuration of dimethoate by the human liver. *Toxicology* **241**, 31–46.

Burd PF & Ferry CB (1987). A prolonged contraction at the end-plate region of the diaphragm of rats and mice after anticholinesterase in vitro. *J Physiol* **391**, 429–440.

Cetin H, Liu W, Cheung J, Cossins J, Vanhaesebrouck A, Maxwell S, Vincent A, Beeson D & Webber R (2019). Rapsyn facilitates recovery from desensitization in fetal and adult acetylcholine receptors expressed in a muscle cell line. *J Physiol* **597**, 3713–3725.

Chang CC & Hong SJ (1986). A regenerating release of acetylcholine from mouse motor nerve terminals treated with anticholinesterase agents. *Neurosci Lett* **69**, 203–207.

Chevassier F, Peter C, Mersdorf U, Girard E, Kreeci E, Mc Ardle JJ & Witzemann V (2012). A new mouse model for the slow-channel congenital myasthenic syndrome induced by the AChR eL221F mutation. *Neurobiol Dis* **45**, 851–861.

Chowdhury L, Croft CJ, Goel S, Zaman N, Tai AC-S, Walch EM, Smith K, Page A, Shea KM, Hall CD, Jishkariani D, Pillai GG & Hall AC (2016). Differential potency of 2,6-dimethylcyclohexanol isomers for positive modulation of GABA_A receptor currents. *J Pharmacol Exp Ther* **357**, 570–579.

Cooper SA & Dretchen KL (1975). Biphasic action of ethanol on contraction of skeletal muscle. *Eur J Pharmacol* **31**, 232–236.

Costanzo EM, Barry JA & Ribchester RR (1999). Co-regulation of synaptic efficacy at stable polynervously innervated neuromuscular junctions in reinnervated rat muscle. *J Physiol* **521**, 365–374.

Dawson G & Gage PW (1980). Phasic secretion of acetylcholine at a mammalian neuromuscular junction. *J Physiol* **303**, 299–314.

Davis GW & Müller M (2015). Homeostatic control of presynaptic neurotransmitter release. *Ann Rev Physiol* **77**, 251–270.

Deflorio C, Catalano M, Fucile S, Limatola C & Grassi F (2014). Fluoxetine prevents acetylcholine-induced excitotoxicity blocking human endplate acetylcholine receptor. *Muscle Nerve* **49**, 90–97.

Ding J, Xu TH & Shi YL (2001). Different effects of toosendanin on perineurally recorded Ca²⁺ currents in mouse and frog motor nerve terminals. *Neurosci Res* **41**, 243–249.

Dissanayake KN, Chou RC-C, Thompson A, Margetiny F, Davie C, McKinnon S, Patel V, Sultatos L, McArdle JJ, Clutton RE, Eddleston M & Ribchester RR (2021). Impaired neuromuscular function by conjoint actions of organophosphorus insecticide metabolites omethoate and cyclohexanol with implications for treatment of respiratory failure. *Clin Toxicol (in press)* doi: 10.1080/15563650.2021.1916519.

Eddleston M (2000). Patterns and problems of deliberate self-poisoning in the developing world. *QJM* **93**, 715–731.

Eddleston M, Buckley NA, Eyer P & Dawson AH (2008). Management of acute organophosphorus pesticide poisoning. *Lancet* **371**, 597–607.

Eddleston M, Street JM, Self I, Thompson A, King T, Williams N, Naredo G, Dissanayake K, Yu L-M, Worek F, John H, Smith S, Thiernmann H, Harris JB & Clutton RE (2012). A role for solvents in the toxicity of agricultural organophosphorus pesticides. *Toxicology* **294**, 94–103.

Engel AG, Lambert EH, Mulder DM, Torres CF, Sahashi K, Bertorini TE & Whitaker JN (1982). A newly recognized congenital myasthenic syndrome attributed to a prolonged open time of the acetylcholine-induced ion channel. *Ann Neurol* **11**, 553–569.

Estrella D, Green KL, Prior C, Dempster J, Halliwell RF, Jacobs RS, Parsons SM, Parsons RL & Marshall IG (1988). A further study of the neuromuscular effects of vesamicol (AH5183) and of its enantiomer specificity. *Br J Pharmacol* **93**, 759–768.

Frank CA, James TD & Müller M (2020). Homeostatic control of *Drosophila* neuromuscular junction function. *Synapse* **74**, e22133.

Frank CA, Kennedy MJ, Goold CP, Marek KW & Davis GW (2006). Mechanisms underlying the rapid induction and sustained expression of synaptic homeostasis. *Neuron* **52**, 663–677.

Fucile S, Sucapane A, Grassi F, Eusebi F & Engel AG (2006). The human adult subtype ACh receptor channel has high Ca²⁺ permeability and predisposes to endplate Ca²⁺ over-loading. *J Physiol* **573**, 35–43.
Gage PW (1965). The effect of methyl, ethyl and n-propyl alcohol on neuromuscular transmission in the rat. J Pharmacol Exp Ther 150, 236–243.

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 Membr 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.

Gilley J, Ribchester RR & Coleman MP (2017). Sarm1 deletion, but not WldF, confers lifelong rescue in a mouse model of severe axonopathy. Cell Rep 21, 10–16.

Gillingwater TH, Thomson D, Mack TGA, Soffin EM, Mattison RJ, Coleman MP & Ribchester RR (2002). Age-dependent synapse withdrawal at axotomised neuromuscular junctions in Wld^mutant and Ube4b/Nmnat transgenic mice. J Physiol 543, 739–755.

Ginsborg BL, McLachlan EM, Martin AR & Searl JW (1981). Improved patch-clamp techniques for studying GABA_A receptor currents and act as general anaesthetics. Eur J Pharmacol 66, 175–181.

Hamill OP, Marty A, Neher E, Sakmann B & Sigworth FJ (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Arch 391, 85–100.

Harris JB & Ribchester RR (1979). The relationship between end-plate size and transmitter release in normal and dystrophic muscles of the mouse. J Physiol 296, 245–265.

He F, Xu H, Qin F, Xu L, Huang J & He X (1998). Intermediate myasthenia syndrome following acute organophosphates poisoning—an analysis of 21 cases. Hum Exp Toxicol 17, 40–45.

Hubbard JI (1963). Repetitive stimulation at the mammalian neuromuscular junction, and the mobilization of transmitter. J Physiol 169, 641–662.

Hubbard JI & Wilson DF (1973). Neuromuscular transmission in a mammalian preparation in the absence of blocking drugs and the effect of D-tubocurarine. J Physiol 228, 307–325.

Hulse EJ, Davies JOJ, Simpson AJ, Scuito AM & Eddleston MJ (2014). Respiratory complications of organophosphorus nerve agent and insecticide poisoning. Implications for respiratory and critical care. Am J Respir Crit Care Med 190, 1342–1354.

Hutchinson DO, Walls TJ, Nakano S, Camp S, Taylor P, Harper CM, Groover RV, Peterson HA, Jamieson DG & Engel AG (1993). Congenital endplate acetylcholinesterase deficiency. Brain 116, 633–653.

Jaghoori MM, Bleijlevens B & Olabarriaga SD (2016). 1001 ways to run AutoDock vina for virtual screening. J Comput Aided Mol Des 30, 237–249.

Jones RA, Reich CD, Dissanayake KN, Kristmundsdottir F, Findlater GS, Ribchester RR, Simmen MW & Gillingwater TH (2016). NMJ-morph reveals principal components of synaptic morphology influencing structure-function relationships at the neuromuscular junction. Open Biol 6, 160240.

Karalliedde LD, Edwards P & Marrs TC (2003). Variables influencing the toxic response to organophosphates in humans. Food Chem Toxicol 41, 1–13.

Karunaratne A, Gunnell D, Konradsen F & Eddleston M (2019). How many premature deaths from pesticide suicide have occurred since the agricultural Green Revolution? Clin Toxicol 58, 227–232.

Katz B & Milêdi R (1973). The binding of acetylcholine to receptors and its removal from the synaptic cleft. J Physiol 231, 549–574.

Kuriraha T & Brooks JE (1975). Excitation-contraction uncoupling. The effect of hyperosmolar glycerol solution and antelone sodium on mammalian muscle in vitro. Arch Neurol 32, 92–97.

Laskowski MB, Olson WH & Dettbarn WD (1975). Ultrastructural changes at the motor end-plant produced by an irreversible cholinesterase inhibitor. Exp Neurol 47, 290–306.

Leonard JP & Salpeter MM (1979). Agonist-induced myopathy at the neuromuscular junction is mediated by calcium. J Cell Biol 82, 811–819.

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.

Luzhkov VB, Osterberg F & Aqvist J (2003). Structure-activity relationship for extracellular block of K^+ channels by tetraalkylammonium ions. FEBS Lett 554, 159–164.

Matthies D, Bae C, Toombe ES, Fox T, Bartesaghi A, Subramanian S & Swartz KJ (2018). Single-particle cryo-EM structure of a voltage-activated potassium channel in lipid nanodiscs. Elife 7, e37558.

McArdele JJ, Anguat-Petit D, Mallart A, Bournaud R, Faille L & Brignet JL (1981). Advantages of the triangularis sterni muscle of the mouse for investigations of synaptic phenomena. J Neurosci Methods 4, 109–115.

McArdele JJ, Sellin LC, Coakley KM, Potian JG, Quinones-Lopez MC, Rosenfeld CA, Sultatos LG & Hoganson K (2005). Melphelone inhibits cholinesterases at the mouse neuromuscular junction. Neuropharmacology 49, 1132–1139.

McGrath CD & Hunter JM (2006). Monitoring of neuromuscular block. Contin Educ Anaesth Crit Care Pain 6, 7–12.

McKenzie D, Franks NP & Lieb WR (1995). Actions of general anaesthetics on a neuronal nicotinic acetylcholine receptor in isolated identified neurones of Lymnaea stagnalis. Br J Pharmacol 115, 275–282.

McLachlan EM & Martin AR (1981). Non-linear summation of end-plate potentials in the frog and mouse. J Physiol 311, 307–324.
Moshul CK, Boyne AF, Deshpande SS & Albuquerque EX (1985). Comparison of the ultrastructural myopathy induced by anticholinesterase agents at the end plates of rat soleus and extensor muscles. Exp Neurol 89, 96–114.

Mew EJ, Padmanathan P, Konradsen F, Eddleston M, Chang S-S, Phillips MR & Gunnell D (2017). The global burden of fatal self-poisoning with pesticides 2006–15: Systematic review. J Affect Disord 219, 93–104.

Minic J, Molgó J, Karlsson E & Krejci E (2002). Regulation of acetylcholine release by muscarinic receptors at the mouse neuromuscular junction depends on the activity of acetylcholinesterase. Eur J Neurosci 15, 439–448.

Molgó J, Lundh H & Thesleff S (1980). Potency of 3,4-diaminoypyridine and 4-aminopyridine on mammalian neuromuscular transmission and the effect of pH changes. Eur J Pharmacol 61, 25–34.

Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS & Olson AJ (2009). AutoDock4 and AutoDockTools4: automated docking with selective receptor flexibility. J Comput Chem 30, 2785–2791.

Mráz J, Gálová E, Nohová H & Vítková D (1994). Uptake, metabolism and elimination of cyclohexanone in humans. Int Arch Occup Environ Health 66, 203–208.

Murray NM & Newsom-Davis J (1981). Treatment with oral anaesthetics. NEnglJMed 304, 1388–1404.

Neher E (1983). The charge carried by single-channel currents of rat cultured muscle cells in the presence of local anaesthetics. J Physiol 339, 663–678.

Newman ZL, Hoagland A, Aghi K, Worden K, Levy SL, Son JH, Lee LP & Isacoff EY (2017). Input-specific plasticity and homeostasis at the Drosophila larval neuromuscular junction. Neuron 93, 1388–1404.

Noori HR, Mücksch C, Vengeliene V, Schöning K, Takahashi TT, Mukhtasimova N, Bagher Oskouei M, Mosqueira M, Bartsch D, Fink R, Urbaske HM, Spanagel R & Sine SM (2018). Alcohol reduces muscle fatigue through atomistic interactions with nicotinic receptors. Commun Biol 1, 159.

Otton HJ, Janssen A, O’Leary T, Chen PE & Wyllie DJA (2009). Inhibition of rat recombinant GluN1/GluN2A and GluN1/GluN2B NMDA receptors by ethanol at concentrations based on the US/UK drink-drive limit. Eur J Pharmacol 614, 14–21.

Penner R & Dreyer F (1986). Two different presynaptic calcium currents in mouse motor nerve terminals. Pflugers Arch 406, 190–197.

Ribecheer RR, Mao F & Betz WJ (1994). Optical measurements of activity-dependent membrane recycling in motor nerve terminals of mammalian skeletal muscle. Proc Biol Sci 255, 61–66.

Ribecheer RR & Slater CR (2018). Rapid retrograde regulation of transmitter release at the NMJ. Carr Opin Physiol 4, 82–87.

Ribecheer RR, Thomson D, Wood NI, Hinks T, Gillingwater TH, Wishart TM, Court FA & Morton AJ (2004). Progressive abnormalities in skeletal muscle and neuromuscular junctions of transgenic mice expressing the Huntington’s disease mutation. Eur J Neurosci 20, 3092–3114.

Ring A, Strom BO, Turner SR, Timperley CM, Bird M, Green AC, Chad JE, Worek F & Tattersall JEH (2015). Bispyridinium compounds inhibit both muscle and neuronal nicotinic acetylcholine receptors in human cell lines. PLoS One 10, e0135811.

Ruiz R, Cano R, Casañas JJ, Gaffield MA, Betz WF & Tabares L (2011). Active zones and the readily releasable pool of synaptic vesicles at the neuromuscular junction of the mouse. J Neurosci 31, 2000–2008.

Saint DA, Quastel DM & Guan YY (1987). Multiple potassium conductances at the mammalian motor nerve terminal. Pflugers Arch 410, 408–412.

Santafe MM, Salon I, Garcia N, Lanuza MA, Uchitel OD & Tomás J (2003). Modulation of ACh release by presynaptic muscarinic autoreceptors in the neuromuscular junction of the newborn and adult rat. Eur J Neurosci 17, 119–127.

Searl T, Prior C & Marshall IG (1991). Acetylcholine recycling and release at rat motor nerve terminals studied using (−)-vesamicol and troxpyrrolium. J Physiol 444, 99–116.

Searl TJ & Silinsky EM (2010a). The mechanism for pre-junctional enhancement of neuromuscular transmission by ethanol in the mouse. J Pharmacol Exp Ther 335, 465–471.

Searl TJ & Silinsky EM (2010b). Post-junctional interactions between neuromuscular blocking agents and ethanol at the mouse neuromuscular junction. Br J Pharmacol 161, 659–667.

Seeger T, Eichhorn M, Lindner M, Niessen KV, Tattersall JEH, Timperley CM, Bird M, Green AC, Thiermann H & Worek F (2012). Restoration of soman-blocked neuromuscular transmission in human and rat muscle by the bispyridinium non-oxime MB327 in vitro. Toxicology 294, 80–84.

Senanayake N & Karalliedde L (1987). Neurotoxic effects of organophosphorus insecticides. An intermediate syndrome. N Engl J Med 316, 761–763.

Sigoillot SM, Bourgeois F, Karmouch J, Molgó J, Dobbertin M, Nacional R, Sandoval V & Thiermann H (2010). Active zones and the readily releasable pool of synaptic vesicles at the neuromuscular junction of the newborn and adult rat. Eur J Neurosci 31, 3092–3114.

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Tomáš J, García N, Lanuza MA, Santafé MM, Tomáš M, Nadal L, Hurtado E, Simó A & Cilleros V (2017). 
Pre-synaptic membrane receptors modulate ACh release, axonal competition and synapse elimination during neuromuscular junction development. *Front Mol Neurosci* 10, 132.

Trott O & Olson AJ (2010). AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J Comput Chem* 35, 455–461.

Turner SR, Chad JE, Price M, Timperley CM, Bird M, Green AC & Tattersall JEH (2011). Protection against nerve agent poisoning by a noncompetitive nicotinic antagonist. *Toxicol Lett* 206, 105–111.

Unwin N & Fujiyoshi Y (2012). Gating movement of acetylcholine receptor caught by plunge-freezing. *J Mol Biol* 422, 617–634.

Vale A & Lotti M (2015). Organophosphorus and carbamate insecticide poisoning. *Handb Clin Neurol* 131, 149–168.

Vidanagamage A, Gooneratne IK, Nandasiri S, Gunaratne K, Fernando A, Maxwell S, Cossins J, Beeson D & Chang T (2021). A rare mutation in the *COLQ* gene causing congenital myasthenic syndrome with remarkable improvement to fluoxetine: a case report. *Neuromuscular Disord* 31, 246–248.

Wacker SJ, Jurkowski W, Simmons KJ, Fishwick CWG, Johnson AP, Madge D, Lindahi E, Rolland J-F & de Groot BL (2012). Identification of selective inhibitors of the potassium channel Kv1.1-1.2(a) by high-throughput virtual screening and automated patch clamp. *ChemMedChem* 7, 1775–1783.

Wang X, McIntosh JM & Rich MM (2018). Muscle nicotinic acetylcholine receptors may mediate trans-synaptic signaling at the mouse neuromuscular junction. *J Neurosci* 38, 1725–1736.

Wang X, Pinter MJ & Rich MM (2016). Reversible recruitment of a homeostatic reserve pool of synaptic vesicles underlies rapid homeostatic plasticity of quantal content. *J Neurosci* 36, 828–836.

Webster R, Maxwell S, Spearman H, Tai K, Beckstein O, Sansom M & Beeson D (2012). A novel congenital myasthenic syndrome due to decreased acetylcholine receptor ion-channel conductance. *Brain* 135, 1070–1080.

Webster RG, Cossins J, Lashley D, Maxwell S, Liu WW, Wickens JR, Martinez-Martinez P, de Baets M & Beeson D (2013). A mouse model of the slow channel myasthenic syndrome: neuromuscular physiology and effects of ephedrine treatment. *Exp Neurol* 248, 286–298.

Wood SJ & Slater CR (1995). Action potential generation in rat slow- and fast-twitch muscles. *J Physiol* 486, 401–410.

Wood SJ & Slater CR (1997). The contribution of postsynaptic folds to the safety factor for neuromuscular transmission in rat fast- and slow-twitch muscles. *J Physiol* 500, 165–176.

Wood SJ & Slater CR (2001). Safety factor at the neuromuscular junction. *Prog Neurobiol* 64, 393–429.

World Health Organization (2018, June 17). *Global status report on road safety*, World Health Organisation. https://www.who.int/publications/i/item/9789241565684

Wu X-S, Subramanian S, Zhang Y, Shi B, Xia J, Li T, Guo X, El-Hassar I, Szegeti-Buck K, Henao-Mejia J, Flavell RA, Horvath TL, Jonas EA, Kaczmarek LK & Wu L-G (2021). Presynaptic Kv3 channels are required for fast and slow endocytosis of synaptic vesicles. *Neuron* 109, 938–946.e5.

Wyllie DJ, Béhé P & Colquhoun D (1998). Single-channel activations and concentration jumps: comparison of recombinant NR1a/NR2A and NR1a/NR2D NMDA receptors. *J Physiol* 510, 1–18.

Zayas R, Groshong JS & Gomez CM (2007). Inositol-1,4,5-triphosphate receptors mediate activity-induced synaptic Ca\(^{2+}\) signals in muscle fibers and Ca\(^{2+}\) overload in slow-channel syndrome. *Cell Calcium* 41, 343–352.

Zhu H, Grajales-Reyes GE, Alicea-Vázquez V, Grajales-Reyes JG, Robinson K, Pytel P, Báez-Pagán CA, Lasalde-Dominici JA & Gomez CM (2015). Fluoxetine is neuroprotective in slow-channel congenital myasthenic syndrome. *Exp Neurol* 270, 88–94.

Zuo Y, Kuryatov A, Lindstrom JM, Yeh JZ & Narahashi T (2002). Alcohol modulation of neuronal nicotinic acetylcholine receptors is \(\alpha\) subunit dependent. Octanol modulation of neuronal nicotinic acetylcholine receptor single channels. *Alcohol Clin Exp Res* 26, 779–784.

Zuo Y, Yeh JZ & Narahashi T (2004). Octanol modulation of neuronal nicotinic acetylcholine receptor single channels. *Alcohol Clin Exp Res* 28, 1648–1656.

**Additional information**

**Data availability statement**

Raw data from electrophysiological recordings and summary data included in the Figures will be provided on reasonable requests directed to the corresponding author. Figure 6A–E is © Crown copyright (2019), Dstl and this material is licensed under the terms of the Open Government Licence.

**Competing interests**

None of the authors has any competing or conflicting interests to declare.

**Funding**

This research was supported by the Medical Research Council under grant MR/M024075; the Wellcome Trust under grant GR104972; and a Lister Research Prize Fellowship to M.E. C.R. was supported by the Motor Neurone Disease Association, under grant 838–791. K.N.D. was initially supported by a Charles Darwin Scholarship of the University of Edinburgh. F.M. is an MRC student on the Precision Medicine PhD Programme at The University of Edinburgh.
Author contributions

K.N.D. – developed methodology, performed experiments (Edinburgh, New Jersey, Oxford), analysed data, helped write the paper. F.M. – developed methodology, performed experiments (Edinburgh), wrote software, analysed data, helped write the paper. C.M. – developed methodology, performed experiments (Porton Down), analysed data. C.R. – developed methodology, performed experiments (Edinburgh). V.P. – developed methodology, assisted with experiments (New Jersey). J.J.M. – supervised research (New Jersey), helped write the paper. R.W. – developed methodology, supervised experiments (Oxford), helped write the paper. D.B. – developed methodology, supervised research (Oxford), provided cell lines and reagents. J.E.H.T. – developed methodology, supervised experiments (Porton Down), provided cell lines and reagents. D.J.A.W. – developed methodology, supervised experiments (Edinburgh), helped analyse data, helped write the paper. M.E. – instigated the research, acquired funding, helped design the study, helped write the paper (Edinburgh). R.R.R. – designed and directed the study, acquired funding, developed methodology, supervised and performed experiments, analysed data, wrote the paper (Edinburgh). All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Acknowledgements

The authors thank Professors Eddie Clutton, John Harris, Clarke Slater, Steve Traynelis, Angela Vincent for advice and comments on earlier drafts of the manuscript; Dr Matthew Livesey for technical advice on outside-out patch recording from AChR; Dr David Sterratt for valuable discussions of data analysis; and Charlotte Davie and Scott McKinnon for complementary research findings made during their respective BSc and MSc research projects.

Keywords

alcohol, neuromuscular junction, synaptic transmission

Supporting information

Additional supporting information can be found online in the Supporting Information section at the end of the HTML view of the article. Supporting information files available:

Peer Review History
Statistical Summary Document

Translational perspective

Pesticide toxicity is a global health issue, especially in rural Asia, where tens of thousands of people die each year as a delayed consequence of ingesting agricultural formulations of organophosphorus insecticides. One cause is neuromuscular weakness (‘myasthenia’) leading to lethal paralysis, previously poorly understood. Recent studies identified links to cyclohexanol, a circulating metabolite produced by one common insecticide solvent. Here we tested the hypothesis that cyclohexanol causes paralysis by interfering with protein molecules responsible for release and action of neurotransmitters at nerve–muscle junctions. We used a combination of electrophysiological recording and modelling techniques to identify likely molecular targets on nerve endings and their muscle contact points (endplates). Cyclohexanol attenuated and prolonged responses to neurotransmitter, probably as a consequence of binding to several sites, including an alcohol binding site identified previously on endplate acetylcholine receptors. Simultaneously, cyclohexanol enhanced initial release of acetylcholine by nerve impulses, which compensated for reduced endplate sensitivity to this neurotransmitter. This effect was associated with binding of cyclohexanol to potassium ion channels on nerve endings. However, transmitter release decreased with repeated activation of neuromuscular junctions. This, together with the reduced endplate sensitivity, reduced the ‘safety margin’ for nerve-to-muscle transmission, significantly impairing overall neuromuscular function. The data thus revealed a compelling mechanism for neuromuscular paralysis that follows self-poisoning with those organophosphorus insecticides that produce cyclohexanol in circulating blood. This better understanding of paralysis in pesticide poisoning should allow advances in antidote development and guide better formulation of pesticides that are less toxic to exposed humans.