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Assessment of false transmitters as treatments for nerve agent poisoning

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Highlights

- AMECh and ADECh act as false transmitters at human acetylcholine receptors.
- Administration of MECh and DECh led to recovery in a soman-exposed functional neuromuscular preparation.
- Consistent with activity as false transmitters, the functional recovery was maintained after washout.

Abstract

Nerve agents inhibit acetylcholinesterase (AChE), leading to a build-up of acetylcholine (ACh) and overstimulation at cholinergic synapses. Current post-exposure nerve agent treatment includes atropine to treat overstimulation at muscarinic synapses, a benzodiazepine anti-convulsant, and an oxime to restore the function of AChE. Aside from the oxime, the components do not act directly to reduce the overstimulation at nicotinic synapses. The false transmitters acetylmonoethylcholine (AMECh) and acetyldiethylcholine (ADECh) are analogs of ACh, synthesised similarly at synapses. AMECh and ADECh are partial agonists, with reduced activity compared to ACh, so it was hypothesised the false transmitters could reduce overstimulation.

Synthetic routes to AMECh and ADECh, and their precursors, monoethylcholine (MECh) and diethylcholine (DECh), were devised, allowing them to be produced easily on a laboratory-scale. The mechanism of action of the false transmitters was investigated *in vitro*. AMECh acted as a partial agonist at human muscarinic (M_1 and M_3) and muscle-type nicotinic receptors, and ADECh was a partial agonist only at certain muscarinic subtypes. Their precursors acted as antagonists at muscle-type nicotinic, but not muscarinic receptors.

Administration of MECh and DECh improved neuromuscular function in the soman-exposed guinea-pig hemi-diaphragm preparation. False transmitters may therefore help reduce nerve agent induced overstimulation at cholinergic synapses.

1 Introduction

Nerve agents inhibit the enzyme acetylcholinesterase (AChE), which results in an accumulation of acetylcholine (ACh) at cholinergic synapses, including the neuromuscular junction. This build-up of ACh causes the toxic effects of nerve agent poisoning, including but not limited to muscle tremors, fatigue, fasciculations and secretions. There are two types of ACh receptors in the body, muscarinic ACh receptors (mAChR) which activate G-proteins and nicotinic ACh receptors (nAChR) which are ligand-gated ion channels. Current therapeutic approaches to nerve agent poisoning include atropine to reduce the effect of excess ACh at mAChR, an anti-convulsant to reduce or prevent seizures and an oxime to restore AChE activity, so it hydrolyses ACh again and clears excess ACh from the synapse.

These countermeasures do not contain any compounds acting directly at nAChR. Thus, aside from the indirect action of an oxime to reactivate AChE, there is no specific therapy targeting overstimulation of the nAChR. This can be problematic because the ability of the oxime to reactivate nerve agent-inhibited AChE varies depending on the oxime, the nerve agent itself and the time interval between the exposure and the treatment (1). Thus there is an opportunity to improve nerve agent treatment by identifying new therapeutics which can reduce the effects of excess ACh at nAChRs. Such therapies, as they are independent of effects on AChE, would be broad-spectrum, offering the same benefit regardless of the

nerve agent used. Previously it has been shown that administration of a nAChR antagonist, such as the bis-pyridinium compound MB327, is efficacious against nerve poisoning, although there are drawbacks, as MB327 is toxic itself at similar doses to those which result in efficacy (2-4). However, these results suggest that alternative therapeutic strategies which achieve a similar result (reduced effectiveness of nAChR activation) could offer benefits in treating nerve agent poisoning.

ACh is synthesised in the presynaptic nerve terminal by the enzyme choline acetyltransferase (ChAT) from choline and acetyl coenzyme A (acetyl-CoA). Monoethylcholine (MECh) and diethylcholine (DECh) are structural analogs of choline (Figure 1). Like choline, MECh and DECh can be transported into the presynaptic nerve terminal and acetylated by the same enzyme to give acetylmonoethylcholine (AMECh) and acetyldiethylcholine (ADECh) respectively (5-8).

Both AMECh and ADECh are capable of activating postsynaptic AChR if released from the nerve terminal, although as partial agonists they generate reduced receptor activation compared to ACh, approximately 40% lower (9, 10). Thus it is said that AMECh and ADECh act as 'false transmitters' and a schematic of this process is shown in Figure 2. In addition to their use as pharmacological tools for investigating synaptic function and signalling (9), the false transmitters have been investigated for their potential to decrease cholinergic transmission (11). Since they are partial agonists of nAChR, it was hypothesised they might offer therapeutic benefit after nerve agent poisoning by reducing overstimulation of the nAChR and by normalising the kinetics of endplate potentials at synapses without functional AChE (10, 12). Indeed, MECh administration has already been shown to be effective in reducing the mortality caused by the anticholinesterases physostigmine, diisopropyl fluorophosphate (DFP), and diazinon in mice (13).

First, the synthesis routes for the false transmitters and precursors were reviewed, and then modified to make them more amenable to laboratory-scale synthesis. Secondly, the agonist activity of AMECh and ADECh at human AChR was assessed *in vitro*, using an indirect calcium fluorescence assay. Thirdly, as choline is known to act as an antagonist at AChR, the ability of MECh and DECh to antagonise signalling at AChRs was investigated. To date, published work on the false transmitters has described their activity at AChR from various

species including mouse, frog, rat and *Torpedo,* but not human (6, 9, 14). To cover a range of different AChR types and subtypes, three different cell lines have been used – CN21 cells which express both the human muscle-type nAChR (both αβγδ and αβδε) and M_3 mAChR (15-17) and CHOm1 and CHOm3 which express the human M_1 and M_3 mAChR subtypes, respectively. In these fluorescence assays, the addition of an agonist activates the receptor, facilitating an increase of intracellular calcium ion concentration ($[Ca^{2+}]_i$) which is quantified as a fluorescent signal. Addition of a partial agonist, such as a false transmitter, would generate smaller increases than those observed after application of a full agonist like ACh. Similar assays were also used to determine whether MECh and DECh can act as antagonists at AChR. Finally, to assess whether administration of the false transmitters could restore neuromuscular function, MECh and DECh were added to soman-exposed neuromuscular preparations (guinea-pig phrenic nerve/hemi-diaphragm).

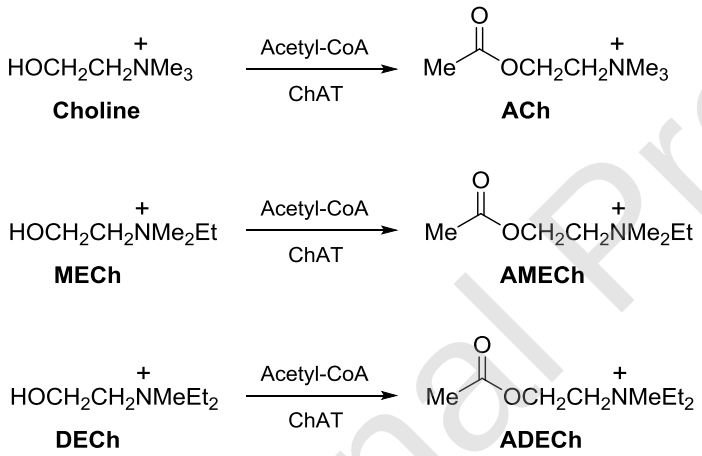

Figure 1. The biological conversion of choline and analogues to their acetyl derivatives. Choline is converted in the nerve terminal to acetylcholine (ACh) by reaction with acetyl coenzyme A (acetyl-CoA), catalysed by choline acetyltransferase (ChAT). The coenzyme A that is generated during this process (not shown) can be re-acetylated in the cell, ready for another cycle. Monoethylcholine (MECh) and diethylcholine (DECh) are acetylated by this system to generate the false transmitters: acetylmonoethylcholine (AMECh) and acetyldiethylcholine (ADECh). Counterions are not shown for ease of structural comparison: the natural counter-ion of choline and ACh is hydroxide. Choline hydroxide is a non-

crystallisable, strongly alkaline syrupy liquid, which deliquesces in air and is soluble in all proportions in water (18). Choline halides crystallise and are easier to handle. The ACh used in the study described herein was the chloride salt. MECh and DECh were iodide salts.

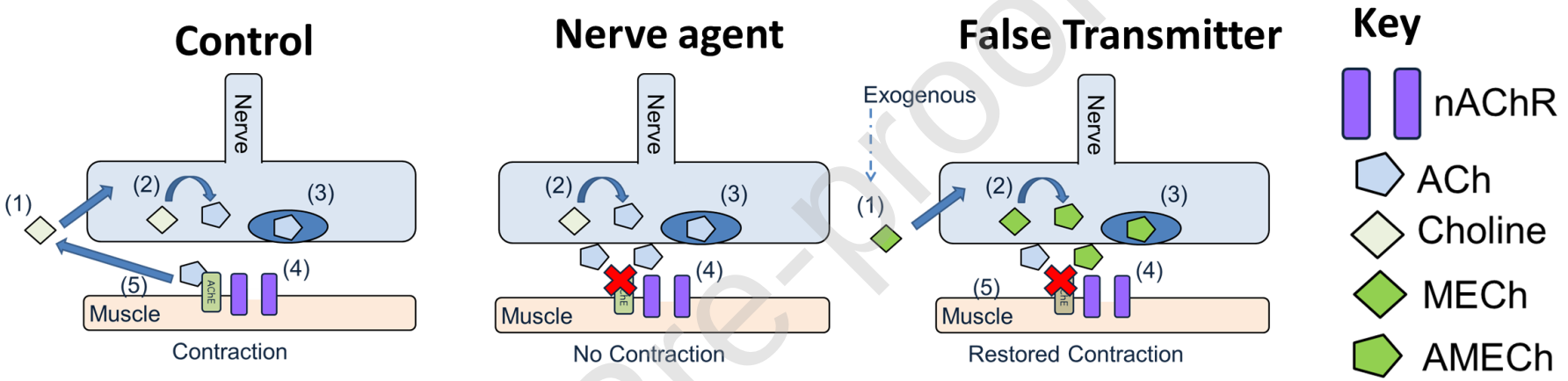

Figure 2. A schematic of the neuromuscular junction (NMJ) showing the cycle of ACh during neuromuscular transmission (control), how this is affected by the presence of nerve agents, and the proposed mechanism for using false transmitters as a nerve agent therapeutic. (*Left image*) During neuromuscular transmission, choline is transported into the pre-synaptic terminal of the NMJ (1) and combines with acetyl CoA to synthesise ACh (2), which is then packaged into vesicles for release (3). In response to stimulation, the vesicles are released, ACh diffuses across the synaptic cleft and activates the nAChR leading to muscle contraction (4). Acetylcholinesterase (AChE) breaks down ACh, terminating its action at the receptor (5) and generating choline, which is taken up into the pre-synaptic terminal (1). (*Middle image*) In the presence of nerve agent, AChE activity is inhibited, resulting in a build-up of ACh at the NMJ and over-stimulation of the muscle (4). (*Right image*) In the absence of choline, exogenous monoethylcholine (MECh) can be transported into the synaptic cleft (1) and combined with acetyl CoA to generate acetylmonoethylcholine (AMECh) (2). Similarly to ACh, AMECh can be packaged into vesicles (3) and released into the synaptic cleft. As a partial agonist, AMECh is predicted to activate the receptor with reduced activity, decreasing the overstimulation at the muscle (4), and to have a therapeutic action.

2 Materials and methods

2.1 Materials

MECh, AMECh, DECh and ADECh were synthesised by the methods described below and solutions at the required concentrations were freshly prepared on the day of the experiment. Soman was synthesised at Dstl Porton Down and supplied at >95% purity diluted in isopropanol. The Molecular Devices FLIPR 5 calcium kit (ThermoFischer Scientific Ltd., UK) was used for the $[Ca^{2+}]_i$ assays. On the day of the assay, the calcium dye was made up according to the manufacturer's instructions. All reagents and solvents were from Sigma-Aldrich Ltd. (Dorset, UK), unless otherwise stated.

2.2 Chemical synthesis

Anhydrous solvents were used for synthesis: tetrahydrofuran (THF) and diethyl ether (hereinafter 'ether'). This was important as the quaternary ammonium salts prepared deliquesced and care had to be taken to exclude water during their production. Melting points were measured using an Electrothermal apparatus and are uncorrected (dec. stands for 'with decomposition'). Nuclear magnetic resonance (NMR) spectroscopic data were collected at 9.4 T using a Bruker Avance III spectrometer equipped with a 5 mm BBFO+ probehead, as solutions in deuterated dimethylsulfoxide (d_6-DMSO) or deuteriochloroform ($CDCl_3$).

Caution! The compounds synthesised have marked physiological action and should be handled cautiously in a fume cupboard. Eye protection and gloves should be worn during all manipulations, and glassware decontaminated in dilute aqueous bleach.

Monoethylcholine iodide (**MECh**). Iodoethane (22.5 ml, 280 mmol) was added dropwise over 25 min to a stirred solution of 2-(dimethylamino)ethanol (17.8 g, 200 mmol) in THF (200 ml). During addition the reaction mixture coloured yellow and a thick white precipitate formed. The mixture was stirred at room temperature overnight. The precipitate was filtered off, rinsed with THF, and quickly transferred to a flask as it deliquesced readily. It was dried to a constant weight (44.2 g) under high vacuum. The solid was recrystallized from hot

isopropanol (about 200 ml). The white crystals were transferred to a flask to avoid deliquescence and dried under vacuum to constant weight (35.9 g, 73%). Mp 272 °C. ^{1}H NMR (600 MHz, d_6-DMSO) 5.25 (1H, dd, *J* = 4.9, 4.9 Hz), 3.84-3.80 (2H, m), 3.41 (2H, q, *J* = 7.3 Hz), 3.38-3.35 (2H, m), 3.05 (6H, s), 1.24 (3H, tt, *J* = 1.7, 7.3 Hz). ^{13}C NMR (151 MHz, d_6-DMSO) 64.5 (t, *J* = 2.7 Hz), 60.1 (t, *J* = 2.7 Hz), 55.4, 50.7 (t, *J* = 3.8 Hz), 8.4 ppm.

Diethylcholine iodide (**DECh**). Iodomethane (8.7 ml, 140 mmol) was added dropwise over 15 min to a stirred solution of 2-(diethylamino)ethanol (11.7 g, 100 mmol) in THF (100 ml). During addition the reaction mixture coloured yellow and a white precipitate formed. The mixture was stirred at room temperature overnight. The white solid was transferred to a flask to avoid deliquescence, and dried under high vacuum to constant weight (25.4 g). The solid was recrystallized from hot isopropanol (about 80 ml). The white crystals were filtered off, rinsed with isopropanol, quickly diverted into a flask, and dried under vacuum to constant weight (22.6 g, 87%). Mp 276 °C (dec.). ^{1}H NMR (600 MHz, d_6-DMSO) 5.24 (1H, t, *J* = 5.0 Hz), 3.82-3.78 (2H, m), 3.36 (4H, q, *J* = 7.3 Hz), 3.33 (2H, dd, *J* = 5.2, 5.2 Hz), 2.98 (3H, s), 1.22 (6H, tm, *J* = 7.3 Hz). ^{13}C NMR (151 MHz, d_6-DMSO) 61.7 (t, *J* = 2.5 Hz), 56.8 (t, *J* = 2.5 Hz), 55.2, 47.7 (t, *J* = 3.7 Hz), 8.1 ppm.

*2-(Diethylamino)ethyl acetate (*Acetylmonoethylcholine iodide, **AMECh***)*. A solution of acetyl chloride (7.9 g, 100 mmol) in ether (50 ml) was added dropwise over 50 min to a stirred solution of 2-(diethylamino)ethanol (11.7 g, 100 mmol), triethylamine (15.3 ml, 110 mmol) and ether (50 ml) at 0-5 °C. A dense white precipitate formed. Additional ether (50 ml) was added to facilitate stirring and the mixture heated under reflux for 2 h. The mixture was allowed to cool and was stirred at room temperature overnight. The precipitate was filtered off and thoroughly rinsed with ether. The solvent was removed from the filtrate to leave a yellow liquid (16.1 g). This was distilled using a one-piece distillation rig to leave a brown oily residue and provide a colourless distillate (9.02 g; bp 58-62 °C/10 mmHg). Analysis by ^{1}H NMR spectroscopy showed this to contain about 85% of the desired compound. The crude product was therefore dissolved in ether (75 ml) and washed with saturated aqueous sodium bicarbonate (2 × 75 ml). The organic phase was dried over anhydrous magnesium sulfate. The drying agent was filtered off and the solvent removed from the filtrate to leave a colourless liquid (4.8 g, 30%). Bp 60-61 °C/10 mmHg. Analysis by ^{1}H NMR spectroscopy indicated that this material was around 90% pure; this was used for the next step without

further purification. ^{1}H NMR (600 MHz, $CDCl_3$) 4.13 (2H, t, *J* = 6.2 Hz), 2.70 (2H, t, *J* = 6.1 Hz), 2.58 (4H, q, *J* = 7.2 Hz), 2.04 (3H, s), 1.03 (6H, t, *J* = 7.2 Hz). ^{13}C NMR (151 MHz, $CDCl_3$) 171.1, 62.5, 50.9, 47.6, 21.1, 11.6 ppm.

Acetyldiethylcholine iodide (**ADECh**). Iodomethane (2.18 ml, 35 mmol) was added in one portion to a stirred solution of 2-(diethylamino)ethyl acetate (3.98 g, 25 mmol) in THF (25 ml). The mixture was left to stir at ambient temperature overnight. The solution went milky and an orange oil separated. The solvent was removed by rotary evaporation to leave a liquid. This was dried to constant weight (7.9 g) under high vacuum. Recrystallization from isopropanol was attempted but no crystals formed: signs of solid formation were evident if the mixture was chilled, but no recrystallization occurred upon warming slowly to room temperature. The solvent was removed and the liquid dried under vacuum to a constant weight (7.6 g, 100%). ^{1}H NMR (400 MHz, $CDCl_3$) 4.57 (2H, dd, *J* = 4.9, 4.9 Hz), 3.94 (2H, tt, *J* = 2.3, 2.5 Hz), 3.67 (4H, q, *J* = 7.3 Hz), 3.34 (3H, s), 2.12 (3H, s), 1.44 (6H, t, *J* = 7.2 Hz). ^{13}C NMR (101 MHz, $CDCl_3$) 170.1, 59.5, 57.9, 57.6, 48.8, 21.2, 8.6 ppm.

2.3 Cell lines

Three different cell lines were used: CHO-CHRM1 (ECACC 10110203), T02J-7/10 (CHO-M3 (CHRM3)) (ECACC 10031603) and CN21. The first two are Chinese Hamster Ovary (CHO) cells stably transfected with the AChR M1 and M3 receptor cDNA sequences respectively. Hereinafter, these cell lines are abbreviated to CHOm1 and CHOm3. They were obtained from the European Collection of Authenticated Cell Cultures (ECACC) maintained by Public Health England. CN21 cells were obtained from Dr. David Beeson (Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, UK) (15). These were derived from the TE671 rhabdomyosarcoma cell line by a stable transfection of the ε-subunit to express both the foetal and adult human muscle-type nicotinic receptor.

CHOm1 and CHOm3 were grown in Ham's F12 media supplemented with 10% FBS (foetal bovine serum), 50 U/mL penicillin, 50 µg/mL streptomycin and either 2 mM L-glutamine or GlutaMAX™ (Life Technologies, California, USA). CN21 cells were grown in DMEM

(Dulbecco's Modified Eagle Medium) supplemented with 10% FBS, 50 U/mL penicillin, 50 µg/mL streptomycin, 2 mM L-glutamine, 0.5 mg/mL geneticin.

All cells were grown in 150 cm^2 flasks at 37 °C, 5% CO_2 with the appropriate media until approximately 70-80% confluent. Cells were then washed with PBS (phosphate-buffered saline) and harvested with either Trypsin-EDTA or TrypLE Select (Fischer Scientific Ltd.). The resulting cell suspension was either re-plated into tissue culture flasks at split ratios of 1:6-1:10 for continued proliferation, or onto Corning clear-bottomed, black-walled, 96 well half-well plates for use in the assay. Cells were plated out in 50 µL of media 24-48 h before the experiment at densities of 20,000 or 10,000 cells per well for CN21 and 30,000 or 15,000 cells per well for CHOm1 and CHOm3.

2.4 Flexstation assays

On the day of the assay, the growth medium was removed and 50 µL of calcium dye was added to the cells. The calcium dye, made according to the manufacturer's instructions, was further diluted (50:50) in a 50:50 mixture of HEPES-buffered (20 mM HEPES (Fischer Scientific Ltd.) at pH 7.4) Hanks Buffered Saline Solution (HBSS) and DMEM/Ham's F12 as appropriate before use. For experiments with CHOm1 or CHOm3 cells, probenecid (Life Technologies) at a final concentration of 2.5 mM was added to the calcium dye. For experiments with CN21 cells, the calcium dye contained either atropine (at a final concentration of 40 µM) to block the muscarinic response, or tubocurarine (at a final concentration of 50 µM to block the nicotinic response), ensuring only stimulation at the nAChR or mAChR respectively was measured.

AMECh, DECh and MECh were made up as stock solutions in HEPES-buffered HBSS on the day of the experiment, while stock solutions of 250 mM ADECh were prepared in advance, stored at -20 °C and thawed on the day of the experiment. These were then serially diluted in compound buffer across a 96 well plate in half-log_{10} steps to provide the required range of concentrations.

Following 30 min incubation with the calcium dye, the cells were analysed using the FlexStation 3 (Molecular Devices Ltd., Berkshire, UK), a multi-mode plate reader, which was programmed to measure baseline fluorescence for 15 s before adding the agonist (ACh, AMECh or ADECh) and measuring the fluorescence for another 45 s. For experiments assessing the antagonist activity of the false transmitter precursors, MECh and DECh were added to the cells after 25 min of dye incubation, and ACh diluted in HEPES-buffered HBSS to give a final concentration of 30 µM (for the CN21 and CHOm1 cells) and 0.3 µM (for the CHOm3 cells) was used as the agonist.

2.5 Data analysis

Raw fluorescence values, corrected for background fluorescence, were exported from SoftMax Pro software (Molecular Devices Ltd.) to GraphPad Prism version 6.00 for Windows. The concentrations were converted to $\log_{10}$ values and non-linear regression analysis was performed using the sigmoidal concentration dose response (variable slope function). Interpolation of this curve generated the EC_{50} values for ACh, AMECh and ADECh. A statistical sum-of-squares F test was performed on the output of the linear regression to compare the IC_{50} values and the maximal response.

For analysis of MECh and DECh as antagonists, the non-linear regression analysis provided best-fit values for the top and bottom of the curve, which were used to normalise the data for presentation. Where a concentration-response curve could not be fitted, values were normalised to the mean of the maximal response.

Once values were normalised, they were analysed statistically using non-linear regression analysis, specifically a dose-response curve (log (inhibitor) versus normalised response) on GraphPad Prism version 6.02 for Windows, which gave the IC_{50}. IC_{50} values were generated when the inhibition was greater than 50% at the highest concentration tested.

If IC_{50} values could not be determined from the curve, the degree of antagonism was estimated by taking the mean of the normalised fluorescence intensity values at the highest concentration tested. This will also be provided in tables where appropriate.

2.6 Hemi-diaphragm preparations

Male Dunkin-Hartley guinea pigs (250-300 g, Harlan UK) were killed by concussion, a UK Home Office Schedule 1 method. Guinea pig hemi-diaphragm tissues isolated by dissection were set up according to a method (19) based on that of Bulbring (20). As both left and right hemi-diaphragms were prepared, experiments were organised such that each experimental group had a mixture of left and right preparations. Tissues were suspended in modified Tyrode's solution (137 mM NaCl (Sigma-Aldrich/BDH), 5 mM KCl, 12 mM $NaHCO_3$, 1 mM $NaH_2PO_4 \cdot 2H_2O$ (Fischer Scientific/Sigma-Aldrich), 25 mM glucose, 2 mM $CaCl_2$ and 1 mM $MgCl_2$), continually gassed with 95% O_2/5% CO_2 and maintained at 37 °C.

Muscle contractions were recorded from a baseline tension of 4 g using a 100 g isometric force transducer (TSD 125D, Buxco Research Systems, Wilmington NC, USA) using Buxco recording software (Biosystems for Windows version 2.7.5). Diaphragms were stimulated via the phrenic nerve using a Grass S88 stimulator with 0.2 ms pulses delivered at supramaximal voltage (1.5 times the maximum response, usually between 2 V and 15 V).

Figure 3 shows the experimental protocol used to test the efficacy of the false transmitters. Tetanic stimuli were delivered at a frequency of 50 Hz for 3 s every 15 min, with twitch stimuli delivered at a frequency of 1 Hz. After establishing stable baseline contractions for 30 min, contractions were decreased to approximately 5% of control values abolished by adding an irreversible cholinesterase inhibitor, the nerve agent soman at a concentration of 150 nM. After 30 min (a period of 2 tetanic contractions), the soman was washed out and diaphragm preparations were incubated with MECh and DECh. For control diaphragms, an equivalent volume of modified Tyrode's solution was added. Finally, two further tetanic contractions were assessed following a washout.

2.7 Data analysis

Force analysis was performed automatically by the Buxco recording system for all tetanic contractions, except those where soman was present. These were analysed manually, as the

software did not analyse them accurately. To analyse diaphragm force, the area under the curve (AUC) of the 50 Hz tetanic contractions was measured, normalised to the control value (mean of 2 initial control responses) using Microsoft Excel 2010 and analysed using GraphPad Prism version 6.0 for Windows. A 2-way ANOVA, with a post-hoc Tukey's multiple comparisons test, was performed on the maximal functional recovery achieved with MECh or DECh using GraphPad Prism version 6.0 for Windows.

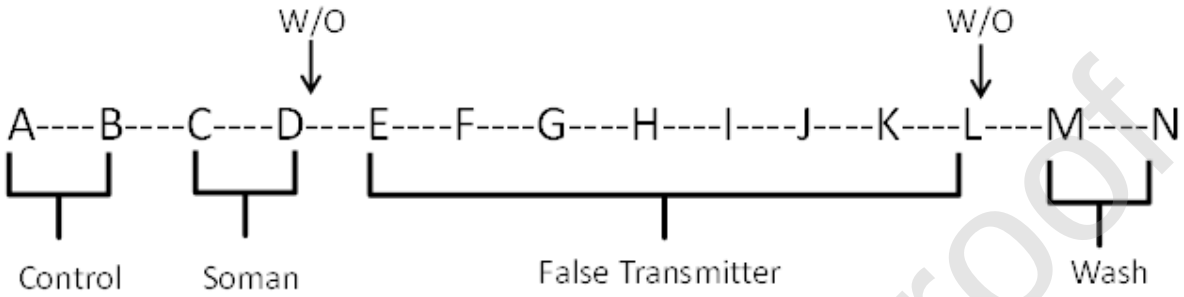

Figure 3. Protocol for analysis of false transmitters in hemi-diaphragms used to evaluate the actions of MECh and DECh on guinea pig phrenic nerve/hemi-diaphragm preparations. Each letter (A to N) represents a tetanic stimulus (delivered at a frequency of 50 Hz), with twitch stimuli delivered at a frequency of 1 Hz in the 15 min between tetanic stimuli. The labels under the schematic indicate the experimental conditions, with the washouts (W/O) shown by vertical arrows.

3 Results

3.1 False transmitter synthesis

To avoid the use of gaseous or hazardous reagents, we devised alternative routes for the synthesis of AMECh, MECh, DECh and ADECh. Rather than use the expensive unsymmetrical amines - dimethylethylamine (Me_2NEt) or diethylmethylamine ($MeNEt_2$) - as precursors, we started from the readily-available and comparatively non-toxic 2-(dialkylamino)ethanol compounds.

Quaternization of 2-(dimethylamino)ethanol and 2-(diethylamino)ethanol in THF with ethyl iodide and methyl iodide, respectively, gave MECh and DECh iodide salts in acceptable yields (Figure 4A). These quaternary ammonium salts had the physical properties expected of ionic salts: they were crystalline solids of defined melting points (272 °C and 276 °C with decomposition, respectively), dissolved in water or other polar solvents, and were insoluble in ethers (diethyl ether or THF). The decomposition observed at the melting point is characteristic of such salts; quaternary ammonium halides generally decompose on heating to give the tertiary amine and alkyl halide (21). Treatment of 2-(diethylamino)ethanol with acetyl chloride in diethyl ether in the presence of triethylamine gave 2-(diethylamino)ethyl acetate (Figure 4B). Quaternization of this colourless liquid with methyl iodide in THF gave ADECh as a liquid that failed to crystallise; it was used in the biological experiments as a liquid. Note that all quaternary ammonium salts prepared in this study, whether solid or liquid, were deliquescent, absorbing quickly moisture from the air. They were isolated in an anhydrous state upon prolonged drying under high vacuum. The dry material was best weighed as rapidly as possible for biological experiments to reduce the potential for stoichiometry errors. This was the approach taken here. Such handling challenges are posed by many quaternary ammonium salts, not just those reported in this publication, as they are notoriously hygroscopic as a class: some form stable hydrates and are difficult to prepare in a completely anhydrous state (21).

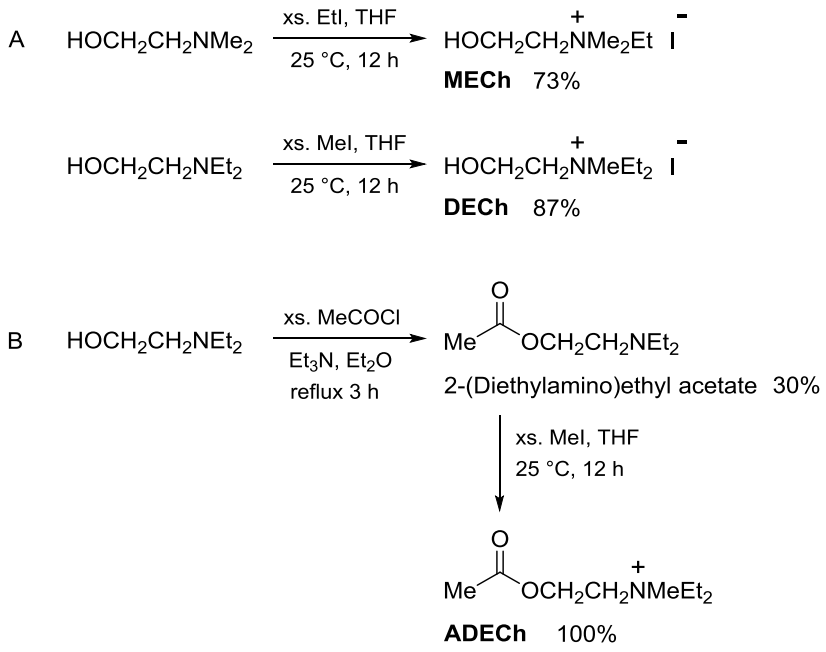

Figure 4. Synthetic routes to make the precursors and false transmitters. (A) Quaternization chemistry used to produce MECh and DECh. (B) Two step route to ADECh. All routes use straightforward economical procedures suitable for research laboratories, avoiding toxic reagents used by industry to effect similar transformations.

3.2 Agonist action of the false transmitters

First, a calcium/fluorescence assay was used to test whether AMECh and ADECh can activate the ACh receptors expressed in CHOm1, CHOm3 and CN21 cells (Table 1 and Figure 5). If AMECh and ADECh were partial agonists, they would be expected to produce a smaller maximal response in comparison to ACh. Additionally, the EC_{50} (concentration which generated 50% of the maximal response) may be shifted to the right, indicative of a lower potency.

These experiments showed that both AMECh and ADECh acted as partial agonists at both the M_1 subtype of mAChR expressed in CHOm1 cells and the M_3 subtype expressed in CHOm3 cells. An extra sum-of-squares F test performed on the curve fits indicated that both the maximal fluorescent responses and EC_{50} values for AMECH and ADECh were significantly different from ACh and each other (Table 1 and Figure 5A-B).

AMECh also acted as a partial agonist at the mAChR expressed in CN21 cells, as although the EC_{50} value was slightly higher (Table 1), the maximal fluorescent response generated by AMECh was lower than that generated by ACh (Figure 5C). In contrast to its effects in the CHO cells, ADECh at concentrations up to 6 mM did not have any agonist activity at the mAChR expressed in CN21 cells.

AMECh acted as a partial agonist at the human muscle-type nAChR in CN21 cells, as AMECh generated much reduced maximal raw fluorescent values when compared to ACh and has a lower EC_{50} value than ACh (Figure 5D). Contrastingly, ADECh did not act as an agonist at the human muscle-type nAChR, displaying only slight activity at 6 mM, the highest concentration tested.

In summary, AMECh acted as a partial agonist at both human muscle-type nAChR and mAChR (M_1 and M_3 subtypes), whilst ADECh acted as an agonist at certain mAChR subtypes, but not human muscle-type nAChR. ADECh has reduced binding affinity in comparison to both ACh and AMECh. As AMECh generated higher maximal responses, had lower EC_{50} values at mAChR, and also acted at human muscle-type nAChR, it should be considered a more efficient partial agonist than ADECh (Table 1 and Figure 5).

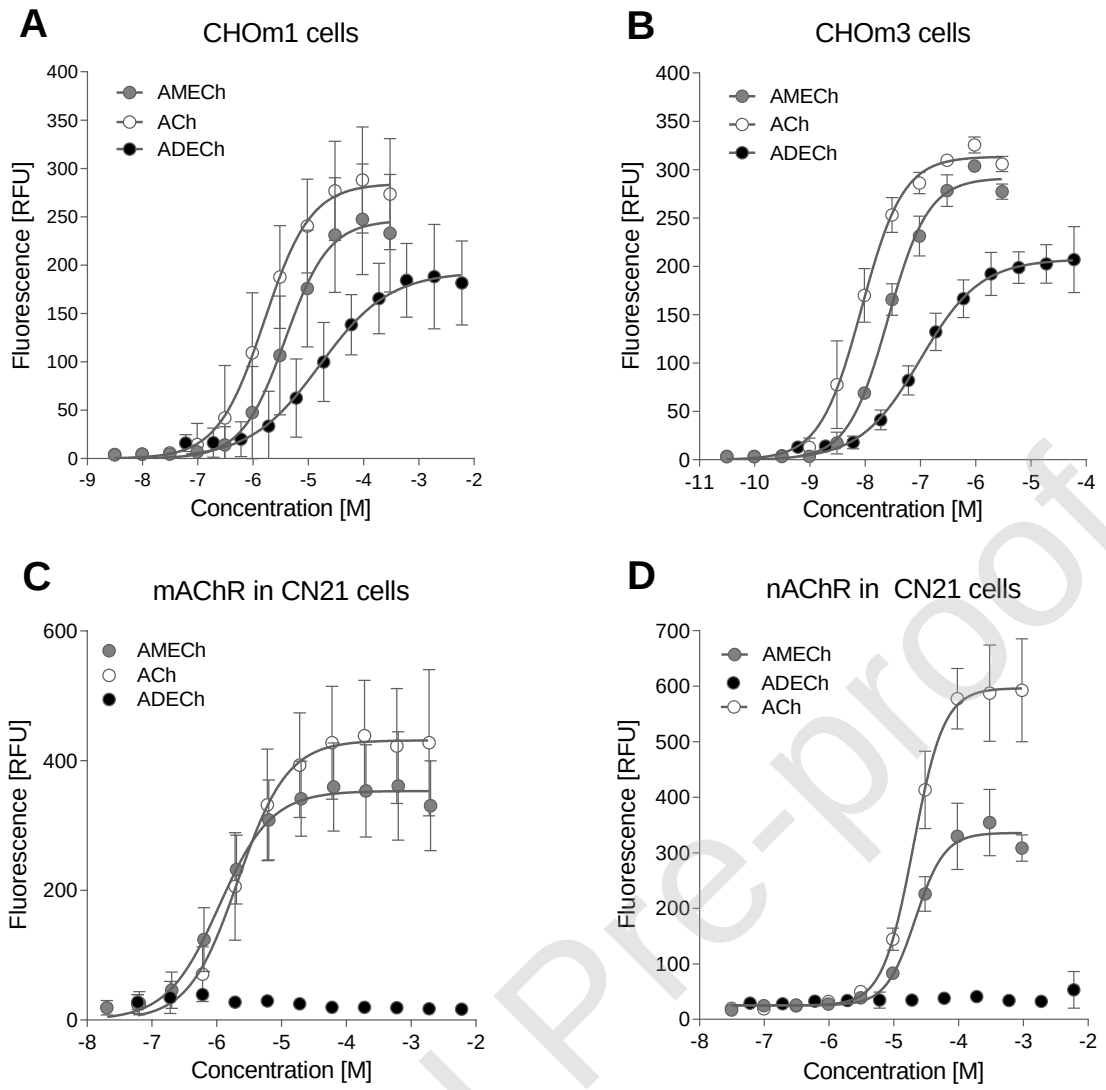

Figure 5. Agonist actions of false transmitters at ACh receptors. The graphs show the agonist actions of ACh, AMECh and ADECh at mAChR, in (A) CHOm1, (B) CHOm3, and (C) both muscle-type nAChR, and (D) mAChR in CN21 cells. Data show the raw fluorescence units recorded on the Flexstation following stimulation with the agonist, with the error bars showing the standard deviation. In CHOm1 cells, n = 6 for all three agonists; in CHOm3 cells, n = 3 for AMECh and ACh, n = 5 for ADECh; in CN21 cells, n = 3 for AMECh and ACh and n = 4 for ADECh. Where appropriate, a dose response curve was fitted using GraphPad Prism version 6.0 for Windows, where the bottom was constrained to 0 as the baseline values had already been subtracted.

Cell line and receptor	EC_{50} with (95% Confidence Interval) (nM)			Relative efficacy	
	ACh	AMECh	ADECh	AMECh	ADECh
CHOm1 AChR M1	1591 (1074 – 2358)	3823 (2436 – 5999)	16060 (8173 – 31580)	86.5%*	67.9%*
CHOm3 AChR M3	8.6 (7.16 – 10.3)	25.8 (22.4 – 29.8)	99.4 (74.9 - 131.9)	93.0%*	66.2%*
CN21 nAChR	3845 (2421 – 6107)	14060 (7746 – 25520)	NA	56.6%*	NA
CN21 mAChR	2136 (1345 – 3392)	1096 (744 – 1615)	NA	81.9%*	NA

Table 1 Agonist EC_{50} values and relative efficacy for the false transmitters.

This table shows the calculated EC_{50} values for ACh and AMECh in CHOm1 and CHOm3 cells and at muscle-type nAChR in CN21 cells. EC_{50} values were calculated from a log-dose response curve generated using a Sigmoidal dose-response (variable slope) function in GraphPad Prism version 6.02 for Windows, with the bottom constrained to 0 as the basal response had already been subtracted. For mAChR and nAChR in CN21 cells, there was no meaningful agonist response with ADECh (indicated by NA and shading in the table). The 95% confidence interval is shown. The relative efficacy was calculated from the maximal value generated in the non-linear regression analysis, with ACh set at 100%. * Denotes where an extra sum-of-squares F test, performed using GraphPad Prism, indicated that the top value of the fit was different for each data set.

3.3 Antagonist actions of the false transmitter precursors

Over the concentration range tested, neither MECh nor DECh caused appreciable inhibition of the mAChR-mediated responses in any of the three cell lines (CHOm1, CHOm3 and CN21) (Table 2 and Figure 6). In contrast, both MECh and DECh acted as antagonists at human muscle-type nAChR, albeit at low potency, reducing signalling to 42% (± 9.4%) and 41% (± 5.6%) respectively, at the highest concentration tested, with estimated $\log_{10}$ IC_{50} values of -2.6 and -2.8 M, respectively. As shown in Table 2, these IC_{50} values were markedly higher than that achieved with the known muscle-type nAChR antagonist tubocurarine, which caused a near 100% inhibition of the fluorescent response induced by ACh, with a log IC_{50} of -6.1 M.

In summary, the false transmitter precursors MECh and DECh did not act as antagonists at the mAChR expressed in CHOm1, CHOm3 and CN21 cells. MECh and DECh acted as weak antagonists at the human muscle-type nAChR and were approximately 1000 fold less potent than tubocurarine.

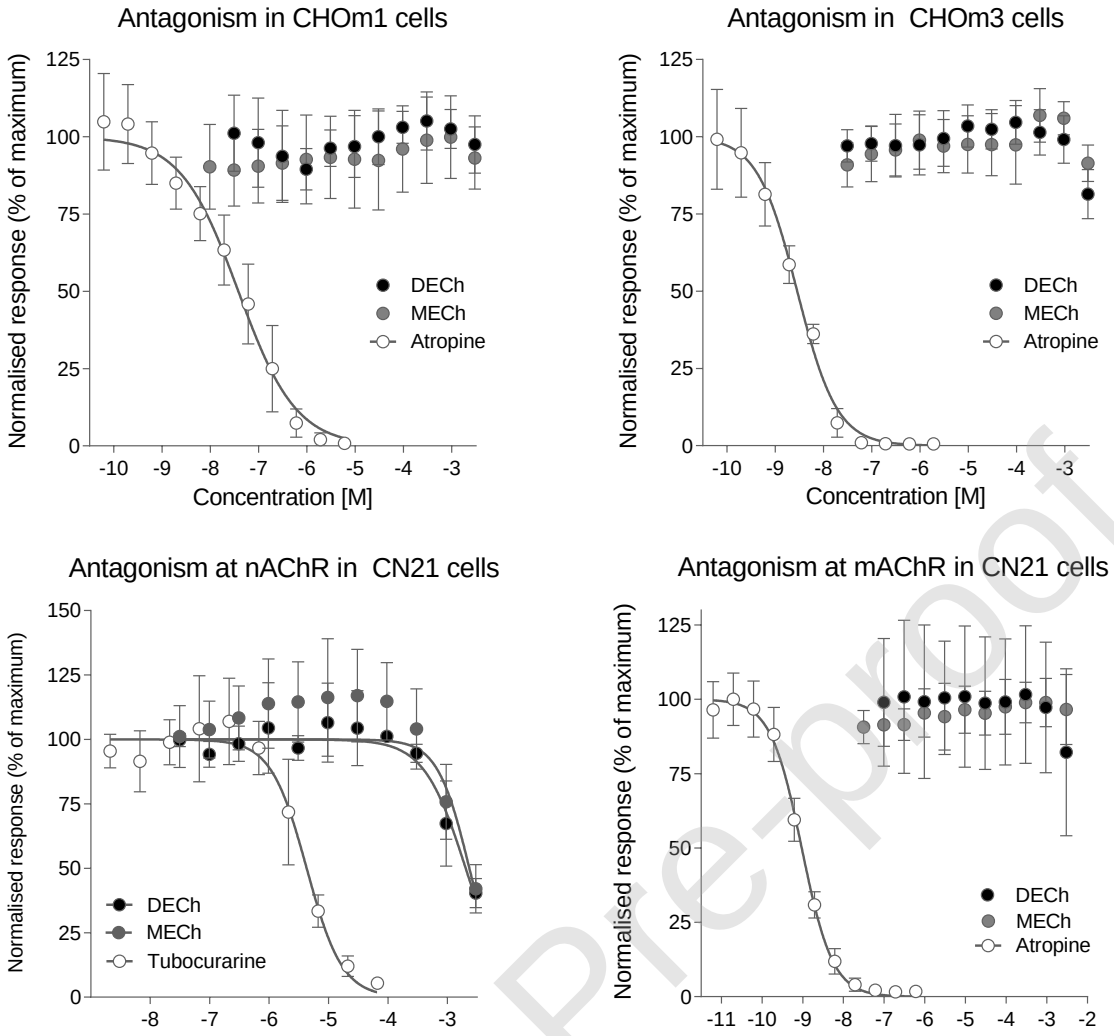

Figure 6. Antagonism actions of the false transmitter precursors. These graphs show the antagonist actions of DECh and MECh against ACh stimulation at both human muscle-type nAChR (CN21 cells) and mAChR (CHOm1, CHOm3 and CN21 cells). Values have been normalised to baseline fluorescence responses and show mean ± SEM. For comparative purposes, the dose-response curves for known antagonists at mAChR (atropine) and nAChR (tubocurarine) are shown. In CHOm1 cells, n = 5 for all three treatments groups; in CHOm3 cells, n = 5 for MECh and atropine, n = 6 for DECh; in CN21 cells at mAChR, n = 4 for MECh and atropine, n = 5 for DECh; in CN21 cells at nAChR, n = 4 for DECh and tubocurarine, and n = 5 for MECh.

Cell Line	IC_{50} with (95% Confidence Interval) (μM)			
	MECh	DECh	Atropine	Tubocurarine
CHOm1			0.038 (0.029 – 0.051)	
CHOm3			0.0029 (0.0024 – 0.0034)	
CN21 - nAChR	2341 (1645 – 3332)	2030 (1549 – 2661)		4.29 (3.28 – 5.6)
CN21 - mAChR			0.00094 (0.00082 – 0.0011)	

Table 2. Antagonist actions of the false transmitter precursors. Antagonist IC_{50} values were determined for MECh and DECh at human muscle-type nAChR, shown as the IC_{50} value, with the 95% confidence interval. At mAChR, MECh and DECh were insufficiently active as antagonists in the concentration range tested to allow the determination of IC_{50} values. Instead, to indicate their antagonist actions, the normalised relative fluorescence intensity (RFI) values at the highest concentration tested ± SD are shown. SD is the standard deviation. SEM is the standard error of measurement.

3.4 Effect of the false transmitters on neuromuscular function

A functional neuromuscular preparation, the guinea-pig phrenic nerve/hemi-diaphragm preparation, was used to assess whether the false transmitters would improve neuromuscular function after nerve agent exposure. The build-up of ACh which arises as a consequence of nerve agent exposure has a pronounced effect on skeletal muscle function, with muscle fibres no longer able to sustain a tetanic contraction (22). Addition of soman caused the expected rapid and sustained fade of the tetanic contraction that was maintained after washout of the nerve agent and is reflected in the marked decrease in the tetanic AUC measured (Figure 7). Addition of 1 mM MECh in the absence of soman had no effect on neuromuscular function, with contraction force similar to that seen with addition of saline (data not shown). Addition of 0.3 mM MECh or DECh to the tissue bath after the washout of soman led to a continuous improvement in neuromuscular function, with the maximal improvement in neuromuscular force seen at the end of the 2 h incubation period (Figure 7A-B). At a concentration of 1 mM, the improvement shown by both MECh and DECh peaked earlier then plateaued or declined slightly respectively.

When MECh or DECh were applied at the highest concentration tested (3 mM), the tetanic response initially improved more rapidly than at lower concentrations. This was then followed by a decline in tetanic contraction to levels lower than those exhibited by the soman control (Figure 7A-B). Statistical analysis (2-way ANOVA with a post-hoc Tukey's multiple comparisons test) of the maximal recovery achieved at each concentration studied showed that the presence of MECh and DECh significantly improved diaphragm function over and above saline alone.

Interestingly, for all treatment groups, there was a rapid and marked increase in tetanic contraction following washout of both MECh and DECh, in comparison to values during the incubation period (Figure 7A-C). For all groups except the DECh 3.0 mM group, these values were significantly increased in comparison to saline.

The increase in the AUC after treatment with MECh or DECh appeared to be associated with the ability of the diaphragm to maintain the tetanic contraction. Whilst the peak contraction

did not approach that achieved prior to soman addition, the contraction in treated diaphragms did not show the marked fade normally associated with anticholinesterase poisoning in this preparation (Figure 7C).

In summary, the experiments with these functional neuromuscular preparations showed that administration of MECh and DECh improved neuromuscular function in hemi-diaphragm preparations exposed to soman, and this improvement was maintained, or even enhanced, upon washout of MECh and DECh.

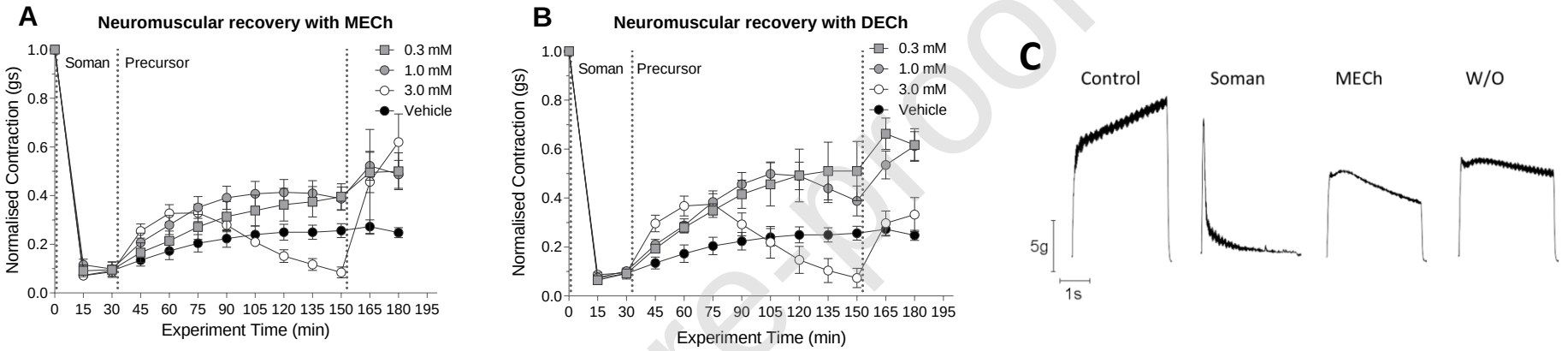

Figure 7. Normalised contraction following false transmitter administration. A and B show normalised diaphragm contraction (gs) following exposure to 150 nM soman and treatment with either MECh or DECh. After 2 baseline tetanic contractions, diaphragms were incubated with soman for 30 min, before washing out (W/O) the soman and incubation of 120 min with either vehicle, DECh or MECh at 3 concentrations: 0.3, 1.0 and 3.0 mM. Contractions were normalised to the average of the 2 baseline tetanic contractions. Data show mean ± SEM, with n = 4 for all dose groups. C shows example muscle contractions from an individual hemi-diaphragm preparation exposed to soman and treated with 1 mM MECh, followed by a trace 30 minutes after washout of MECh or DECh.

4 Discussion

This paper has shown that alternative synthesis routes avoiding the use of toxic chemicals can be used successfully to generate AMECh, ADECh, MECh and DECh on a laboratory scale. AMECh was shown to be a partial agonist at human mAChR and muscle-type nAChR, whilst ADECh was shown to be a partial agonist at mAChR only, with no appreciable activity at nAChR. The precursors MECh and DECh acted as antagonists at muscle-type nAChR, but not at mAChR. Finally, it was demonstrated that administration of MECh and DECh to nerve agent exposed hemi-diaphragm preparations significantly improved neuromuscular function and this improvement persisted upon washout of the compound.

4.1 Chemistry

Synthetic procedures that have been used to produce choline include the action of aqueous trimethylamine on ethylene oxide to yield choline hydroxide, and the reaction between trimethylamine and 2-chloroethanol to yield choline chloride (mp 302-305 °C dec.) (23, 24). Similarly, ACh bromide or ACh chloride has been prepared from trimethylamine and 2-bromoethyl or 2-chloroethyl acetate (24). These procedures, suitable for industrial manufacture, are possible due to the availability and cheapness of trimethylamine (a gas, bp 3 °C). However, they use as the other reagent, chemicals of an unpleasant nature: ethylene oxide (a gas, bp 12 °C; carcinogenic), 2-chloroethanol (a liquid, bp 128 °C; toxic) (24), or 2-haloethyl acetates (volatile liquids, whose vapours are irritating and cause lung oedema) (25, 26).

To avoid the use of gaseous or hazardous reagents, we devised alternative routes for the synthesis of AMECh, MECh, DECh and ADECh. Rather than use the expensive unsymmetrical amines - dimethylethylamine (Me_2NEt) or diethylmethylamine ($MeNEt_2$) - as precursors, we started from the readily-available and comparatively non-toxic 2-(dialkylamino)ethanol compounds. It was envisaged that their quaternization reactions – Menshutkin reactions – with an appropriate alkyl halide would furnish the required ethyl-bearing choline derivatives as halide salts. The reaction is a replacement of the halogen atom by the nucleophilic

tertiary amine; its rate depends on the nature of the alkyl halide. Methyl halides are more reactive than other alkyl halides, and among the halides having the same alkyl group, the iodide is the most reactive (27, 28). The solvent also influences the rate of reaction; since the transition states and products have considerable separation of charge whereas the reactants do not, solution in solvents of high dielectric constant, such as acetonitrile or tetrahydrofuran (THF), facilitates quaternization by lowering the activation energy (27, 28).

The acetylated false transmitters from an electronic perspective are expected to be similarly susceptible towards enzymatic or uncatalysed hydrolysis; the acetyl group being activated towards nucleophilic attack similarly by the distant electron-withdrawing trialkylammonium entity. The degree of positive charge on the nitrogen atom is expected to decline in the order $–N^+Me_3$ > $–N^+Me_2Et$ > $–N^+MeEt_2$ (an ethyl group is more electron-donating than a methyl group) and the size of this grouping increases the more ethyl groups that are present (the ethyl group is larger than a methyl group). These factors might influence the interaction of the acetylated transmitters with AChE and other binding sites involved in neurotransmission, and this might account for the differences in potency at nAChR observed for ACh, AMECh and ADECh (6, 9, 14, 29).

4.2 Agonism at acetylcholine receptors

To date, the activity of AMECh and ADECh has been determined at muscarinic receptors in the rat (6, 11, 14) and muscle-type nicotinic receptors in the rat (6), mouse (9, 29), frog (9) and *Torpedo* electric organ (14). For the false transmitters to be effective in reducing overstimulation at the NMJ after nerve agent poisoning, they would need to act at human ACh receptors. Consistent with the results previously reported in the rat (11), here it has been shown that AMECh activated human mAChR M1 and M3 receptors, and achieved a comparable maximal response to ACh, albeit with a higher EC_{50}. In contrast, ADECh acted as a lower potency partial agonist, producing lower maximal responses than both ACh and AMECh.

Although AMECh acted as an agonist at the mAChR known to be expressed in CN21 cells, ADECh did not. The CN21 cells are derived from the TE671 cell line (16), which is known to

express muscarinic receptors (30), specifically the M3 subtype (17). In this study a difference in agonist activity was identified between the CHOm3 cells and the CN21 cells – the EC_{50} of ACh was markedly different between these cell types and additionally ADECh did not activate the mAChR in CN21 cells. This could be related to differences between the cell types expressing the receptor, Chinese Hamster Ovary versus human rhabdomyosarcoma, and differences in coupling to the Ca^{2+} response.

Consistent with previous reports on AMECh and ADECh activity at rat and *Torpedo* nAChR (9, 11), AMECh acted as a partial agonist at human muscle-type nAChR, generating lower maximal responses compared to ACh. Although ACh and AMECh generate unitary current events of the same amplitude when bound to the receptor, in comparison to ACh, AMECh has a reduced potency for the nAChR and a reduced channel opening time, approximately 40% shorter (6, 9, 10).

In addition, here it was identified that ADECh does not activate human muscle-type nAChR in CN21 cells, although previous studies showed that ADECh activated nAChR from the *Torpedo* electric organ and rat muscle, albeit with much reduced potency compared to ACh and AMECh (9, 11). Thus, given that ADECh is a weak partial agonist, the difference in activity is likely due to species differences in the structure of the nAChR.

4.3 Antagonism at nAChR

One of the hydrolysis products of ACh, choline, can antagonise signalling through the nAChR, since it blocks the nicotinic ion channel and may act as a partial agonist at human muscle-type nAChRs (31). Given this dual action of choline, the potential for the false transmitters to act as antagonists of nAChR was investigated. Here, it was identified that, in high concentrations, MECh and DECh are antagonists of the human muscle-type nAChR. Although their potency was much reduced in comparison to the known nAChR antagonist tubocurarine, their potency is comparable to the parent compound choline, which has an equilibrium constant for channel block at the muscle-type nAChR of 6.9 mM (31).

Densitisation of nAChR depends on the subunit composition of the receptor and whilst some nAChR such as α7 desensitise rapidly, others such as the muscle-type nAChR

investigated here desensitise more slowly (32). As reviewed in Giniatullin et al., 2005 different ligands have different effects on desensitisation of the nAChRs (32), so it is possible that the false transmitters could alter these kinetics. However, activation of the nAChR by the false transmitters results in a reduced channel opening time (6, 9, 10), and it is considered more likely that this, plus the reduced potency of the false transmitters for the receptor, lead to the partial agonism (and subsequent functional recovery in the neuromuscular preparation).

4.4 False transmitter precursors as treatments for nerve agent poisoning.

A previous study showed that administration of MECh and a related false transmitter precursor, N-aminodeanol (NAD), protected mice from the lethal effects of AChE inhibitors, when administered at the same time as the inhibitor (13). In this same publication, the authors also showed *in vitro* that, at these efficacious doses, MECh and NAD both inhibited AChE activity and acted as antagonists at both nAChR and mAChR, specifically M_1 and M_2 (13). Thus, the authors' proposed mechanism of action for the protection seen with MECh and NAD was through either interaction with AChE or competition with ACh for receptor occupancy (13). The authors made no mention of the false transmitter effect. Here, an alternative mechanism of action has been investigated, which could contribute to the protection seen in the mice (13). This is the manipulation of neuromuscular transmission to reduce the effects of ACh overstimulation at the NMJ, countering the build-up ACh caused by AChE inhibitors.

It was predicted that introducing the false transmitter precursor would improve NMJ function after nerve agent poisoning by (a) reducing the amount of ACh released, (b) increasing the contribution of the lower efficacy false transmitter to the released pool of ACh and (c) changing the kinetics of the human muscle-type nAChR channel opening, which would at least partially correct the kinetics of synaptic potentials in the presence of AChE inhibitors. Moreover, it is known that incorporation of the false transmitter into releasable pools occurs more rapidly when AChE is inhibited, as choline is not being generated from the breakdown of ACh (9).

The improvement in muscle contraction seen here after administration of MECh and DECh to the soman exposed neuromuscular preparations is consistent with this hypothesis. The recovery in muscle contraction develops over time and is concentration-dependent; administering a larger dose of the precursor results in a quicker recovery time. The most effective concentration of MECh and DECh restored approximately 40-50% of control muscle function. This degree of recovery is comparable to that achieved with a muscle-type nAChR antagonist, like MB327 (33, 34), and greater than that achieved with oximes including HI-6 (22). Although high concentrations of MECh and DECh have been used and show this benefit *in vitro,* potential toxicities *in vivo* may impact on their utility. Although this does not impact on their ability to protect against anti-cholinesterases *in vivo* (13), an assessment of potential toxicity would be a key aspect of any further exploration of this approach.

The maintained and enhanced effect seen on washout is also consistent with this mechanism of action, as the acetylated transmitter will continue to be released from the pre-synaptic terminal even after the precursor has been washed out. It shows there must be a persistent change and one not due to a pharmacological action that would reverse on washout. In contrast, the inhibitory effect of the highest concentration does washout, hence the rebound in function upon washout of the 3 mM dose. Additionally, the presence of MECh or DECh improved and restored the tetanic shape of the muscle contraction, even during the washout period. This is important and suggests the presence of the false transmitter has corrected some of the fundamental aspects of neuromuscular transmission.

Additionally, it is interesting that MECh and DECh led to comparable improvements in muscle contraction, given that ADECh did not activate human muscle-type nAChR here. This positive result suggests that reducing the amount of ACh released is key. Alternatively, the functional recovery could be a result of the competition between ACh and the false transmitter, which would reduce ACh stimulation, regardless of whether the false transmitter activated the receptor.

It should be noted that the *in vitro* assessment of receptor agonism and antagonism was performed in cell lines expressing the human ACh receptors, whilst the functional

neuromuscular preparation used guinea-pig tissue. It is possible these species differences could affect some of these parameters, such as competitive binding or precursor processing rates. Indeed species differences between the human and guinea-pig nAChR may contribute to the discrepancy between the beneficial effects seen with DECh and the lack of activity at human muscle-type nAChR.

Returning to the study which demonstrated the positive benefits of MECh and NAD in countering the toxic effects of AChE inhibitors (13), one explanation for the positive benefits seen with MECh and NAD was that they might interact with AChE directly (13). That is unlikely to be a contributing factor in these studies, as with this experimental design it is likely that all the AChE in these neuromuscular preparations was already inhibited by soman before the precursors were added. The second explanation was that MECh and NAD might have some direct interactions at the AChRs (13). Here, it was shown by us that the MECh and DECh have little appreciable antagonist activity at mAChR, but at high concentrations they do antagonise ACh signalling at nAChR. This antagonist activity at the nAChR is unlikely to be solely responsible for the protection seen in mice, given that no additional anti-muscarinic was administered (13). However, it should be noted that Patterson and colleagues assessed activity at both M_1 and M_2 receptors, but here activity at M_2 receptors was not assessed (13). In addition, their binding experiment showed antagonist activity for MECh and DECh at both these receptor subtypes, but our functional assays showed no antagonist activity at M_1 and M_3 receptors. Again this could be attributed to the coupling between binding to the receptor and the movement of $[Ca^{2+}]_i$, alongside the degree of block needed to achieve an effect on the functional response.

5 Summary

Chemical synthesis routes were devised for MECh, DECh, AMECh and ADECh. All of these routes used straightforward economical procedures suitable for research laboratories and avoided the toxic reagents used by industry to effect similar transformations.

These studies showed that both AMECh and ADECh act as agonists at human M1 and M3 receptors, but only AMECh activated the nAChR and mAChR expressed in CN21 cells. It was

also shown that administration of MECh and DECh improved neuromuscular function in isolated neuromuscular preparations exposed to soman. This recovery of function was maintained after washout of MECh and DECh, suggesting it can be attributed to release of the acetylated false transmitter from the pre-synaptic terminal. Incorporation of the false transmitters into releasable pools of ACh may modulate neuromuscular transmission to restore function after nerve agent poisoning. Indeed this mechanism may contribute to the protection seen in mice after administration of the false transmitters (13).

This work suggests that ways of manipulating ACh release could be beneficial in the treatment of nerve agent poisoning, so consideration should be given to investigating other compounds, including adenosine, and other false trasmitter precursors, such as selenonium choline and pyrrolcholine, which become acetylselenonium choline and acetylpyrrolcholine respectively (35, 36).

Given the promising results presented here, and the previous work demonstrating that administration of MECh protected mice from exposure to other compounds that inhibit AChE (13), it is recommended that the efficacy of MECh in the treatment of nerve agent poisoning should be investigated *in vivo*. However, as MECh and DECh demonstrate a lack of appreciable mAChR antagonism, at least for the M_1 and M_3 subtypes, more effective *in vivo* results might be expected if the precursors were used as an adjunct to therapy containing an effective anti-muscarinic.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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