

MOL #64295

**Nicotinic acetylcholine receptor transmembrane mutations convert ivermectin
from a positive to a negative allosteric modulator**

Toby Collins and Neil S. Millar

Department of Neuroscience, Physiology & Pharmacology, University College
London, London, WC1E 6BT, UK

MOL #64295

Running Title Page

Running title: Allosteric modulation of $\alpha 7$ nAChRs by ivermectin

Corresponding author:

Professor Neil S. Millar, Research Department of Neuroscience, Physiology & Pharmacology, University College London, Gower Street, London, WC1E 6BT, UK.
Tel. +44-(0)20-7679-7241. Email: n.millar@ucl.ac.uk

Number of text pages: **29**

Number of tables: **2**

Number of figures: **5**

Number of references: **40**

Number of words in the Abstract: **249**

Number of words in the Introduction: **535**

Number of words in the Discussion: **1322**

Abbreviations:

AChBP, acetylcholine binding protein; CPBG, 1-(3-chlorophenyl)biguanide hydrochloride; MLA, methyllycaconitine; nAChR, nicotinic acetylcholine receptor; LY-2087101 [2-(4-fluoro-phenylamino)-4-methyl-thiazol-5-yl]-thiophen-3-yl-methanone; NS1738, 1-(5-chloro-2-hydroxy-phenyl)-3-(2-chloro-5-trifluoromethyl-phenyl)-urea; PNU-120596, 1-(5-chloro-2,4-dimethoxy-phenyl)-3-(5-methyl-isoxazol-3-yl)-urea.

MOL #64295

Abstract

Ivermectin is a macrocyclic lactone that acts as a positive allosteric modulator of $\alpha 7$ nicotinic acetylcholine receptors (nAChRs) but has no modulatory activity on 5-hydroxytryptamine (5-HT) type 3 (5-HT₃) receptors. By examining the influence of ivermectin on subunit chimeras containing domains from the nAChR $\alpha 7$ subunit and 5-HT_{3A} subunit, we have concluded that the transmembrane domains play a critical role in influencing allosteric modulation by ivermectin. A series of mutations located within the α -helical transmembrane domains of the $\alpha 7$ subunit were examined and seven were found to have significant effects on allosteric modulation by ivermectin. Four mutations (A225D, Q272V, T456Y and C459Y) caused a significant reduction in the potency of ivermectin as an allosteric potentiator. Compared to wildtype $\alpha 7$ nAChRs, potentiation by ivermectin was reduced dramatically (by between 89% and 97%) by these mutations. Somewhat unexpectedly, three mutations (S222M, M253L and S276V; located in TM1, TM2 and TM3) converted ivermectin from a positive allosteric modulator into an antagonist. Levels of inhibition of 56%, 84% and 89% were observed on M253L, S276V and S222M, respectively. Antagonism by ivermectin was non-surmountable and had no effect on EC₅₀ of acetylcholine, indicating that it is acting non-competitively. The seven mutations that influence allosteric modulation by ivermectin are located close to a predicted intra-subunit transmembrane cavity. Computer docking simulations provide support for the hypothesis that ivermectin binds in close proximity to this cavity. We conclude that transmembrane mutations in $\alpha 7$ nAChRs are able to convert ivermectin from a positive to a negative allosteric modulator.

MOL #64295

Introduction

Ivermectin is a large macrocyclic lactone (Fig. 1A). It is a synthetic derivative of a naturally-occurring compound, isolated by fermentation of *Streptomyces avermitilis* and is used commercially as an antiparasitic agent in both human and veterinary medicine (Burkhart, 2000). The antiparasitic activity of ivermectin is thought to be due to its action as an agonist of invertebrate glutamate-gated chloride channels (Raymond and Sattelle, 2002; Arena et al., 2004). In addition, ivermectin has been shown to modulate several other ligand-gated ion channels. For example, it is an agonist of invertebrate histamine-gated chloride channels (Zheng et al., 2002) and a positive allosteric modulator of vertebrate $\alpha 7$ nicotinic acetylcholine receptors (nAChRs) (Krause et al., 1998), γ -aminobutyric acid (GABA) type A (GABA_A) receptors (Sigel and Baur, 1987; Krůšek and Zemková, 1994), glycine receptors (Shan et al., 2001) and ATP (P2X) receptors (Khakh et al., 1999). Ivermectin has also been reported to act as an agonist of GABA_A and glycine receptors (Sigel and Baur, 1987; Adelsberger et al., 2000; Shan et al., 2001).

There has been considerable interest in positive allosteric modulators of $\alpha 7$ nAChRs (for recent reviews see Bertrand and Gopalakrishnan, 2007; Moaddel et al., 2007). In part, this interest has been simulated by suggestions that such compounds may have therapeutic potential as cognitive enhancers in connection with disorders such as Alzheimer's disease and schizophrenia (Hurst et al., 2005; Bertrand and Gopalakrishnan, 2007; Moaddel et al., 2007). A wide variety of compounds in addition to ivermectin have been reported to act as positive allosteric modulators of $\alpha 7$ nAChRs. These include proteins such as serum albumins (Conroy et al., 2003)

MOL #64295

and SLURP-1 (Chimienti et al., 2003), as well as small molecules such as galanthamine (Samochocki et al., 2003), 5-hydroxyindole (Zwart et al., 2002), [2-(4-fluoro-phenylamino)-4-methyl-thiazol-5-yl]-thiophen-3-yl-methanone (LY-2087101) (Broad et al., 2006), 1-(5-chloro-2-hydroxy-phenyl)-3-(2-chloro-5-trifluoromethyl-phenyl)-urea (NS1738) (Timmermann et al., 2007) and 1-(5-chloro-2,4-dimethoxy-phenyl)-3-(5-methyl-isoxazol-3-yl)-urea (PNU-120596) (Hurst et al., 2005). All of these compounds potentiate peak agonist-induced responses, but have been classified as being either 'type I' or 'type II' positive allosteric modulators, based on their effect on receptor desensitization (Bertrand and Gopalakrishnan, 2007). Type I compounds (such as ivermectin) have little or no effect on receptor desensitization, whereas type II compounds (such as PNU-120596) reduce levels of desensitization seen during prolonged agonist exposure (Krause et al., 1998; Hurst et al., 2005).

For some of these allosteric potentiators (for example galanthamine), the location of the binding site has been proposed to be on the nAChR extracellular domain (Hansen and Taylor, 2007). In contrast, for allosteric potentiators such as LY-2087101 and PNU-120596, a transmembrane binding site has been proposed (Young et al., 2008). Recently, it has been suggested, on the strength of computer docking simulations, that ivermectin may also interact with nAChRs via a transmembrane binding site (Sattelle et al., 2009). In the present study we have examined the ability of ivermectin to act as an allosteric modulator on a series of mutated and chimeric $\alpha 7$ nAChR subunits. We conclude that the nAChR transmembrane domains play a critical role in influencing allosteric modulation by ivermectin. In addition, we have demonstrated that single point mutations located

MOL #64295

within the $\alpha 7$ nAChR TM1, TM2 and TM3 transmembrane domains are able to convert ivermectin from a positive to a negative allosteric modulator.

MOL #64295

Materials and Methods

Materials. All chemicals were obtained from Sigma (Poole, UK) with the exception of 1-(3-chlorophenyl)biguanide hydrochloride (CPBG) and methyllycaconitine (MLA), which were obtained from Tocris Bioscience (Bristol, UK).

Subunit cDNAs, subunit chimeras and site-directed mutagenesis. Rat nAChR $\alpha 7$ subunit cDNA was obtained from Jim Patrick, Baylor College of Medicine. Mouse 5-HT3A subunit cDNA was obtained from David Julius, University of California, San Francisco. Subunit chimeras $\alpha 7^{4TM-5HT3A}$ and $\alpha 7^{V201-5HT3A}$, containing domains derived from the rat $\alpha 7$ subunit and mouse 5-HT3A subunit in plasmid pZeoSV2 have been described previously (Cooper and Millar, 1998; Gee et al., 2007), as has a plasmid expression construct (pcDNA3-hRIC-3) containing the human RIC-3 cDNA (Lansdell et al., 2005). Construction of $\alpha 7$ nAChRs containing site-directed mutations has also been described previously (Young et al., 2008).

Xenopus oocyte electrophysiology. *Xenopus laevis* oocytes were isolated and defolliculated as described previously (Young et al., 2007). Heterologous expression was achieved by injection of plasmid cDNA constructs (10-30 ng) into oocyte nuclei. In the case of the $\alpha 7$ cDNA containing the A225D mutation (Young et al., 2008), expression of functional receptors was inefficient. For this reason, the A225 mutation was co-expressed with the molecular chaperone RIC-3, which has been found previously to enhance nAChR maturation (Lansdell et al., 2005; Millar, 2008). Oocytes were injected in a volume of 18.4nl using a Drummond variable volume microinjector. Two electrode voltage-clamp recordings were performed essentially as

MOL #64295

described previously (Young et al., 2007) using an Axon Geneclamp 500B amplifier, Axon Digidata 1200 and pClamp software (Molecular Devices, Sunnyvale, CA). Rapid solution exchange was achieved using a computer-controlled eight-way solenoid valve perfusion system (BPS-8; ALA Scientific Instruments, Westbury, NY). Data were analyzed using GraphPad Prism software using the Hill equation. In all cases, data were best fit with a single-site model. Statistical significance was determined by analysis of variance (ANOVA).

Computer docking simulations. Computational molecular docking was performed with AUTODOCK 4 (Morris et al., 1998) using a homology model of the human $\alpha 7$ nAChR transmembrane region (Cheng et al., 2006), as described previously (Young et al., 2008). To avoid bias, a 'blind docking' approach was used in which no assumptions were made concerning where within the transmembrane region ivermectin might be expected to bind. Flexibility of rotatable bonds in ivermectin was permitted during the docking simulation. Predicted Gibbs free energy of binding (ΔG) was calculated as described (Morris et al., 1998), as was the predicted equilibrium constant for binding (K_{eq}), using the equation $K_{eq} = e^{-\Delta G/RT}$ (where R = gas constant, T = absolute temperature).

MOL #64295

Results

Ivermectin displayed no agonist activity on rat $\alpha 7$ nAChRs expressed in *Xenopus* oocytes and caused little or no potentiation of agonist-induced responses when it was co-applied with acetylcholine (in the absence of pre-application). Strong potentiation was, however, observed when ivermectin was pre-applied (for 60 seconds) in addition to it being co-applied with acetylcholine (Fig. 1B and 1C). Consequently, this application protocol (a combination of pre- and co-application of ivermectin) was used for all experiments described in the present study. These findings, conducted with rat $\alpha 7$ nAChRs, are in agreement with previous studies conducted with human and chick $\alpha 7$ nAChRs (Krause et al., 1998; Raymond et al., 2000) and are consistent with the classification of ivermectin as a positive allosteric modulator of $\alpha 7$ nAChRs.

Initial studies, performed with rat $\alpha 7$ nAChRs, were aimed at determining the influence of ivermectin on responses evoked by an EC_{50} concentration of acetylcholine (100 μ M). Ivermectin potentiated acetylcholine responses (with an EC_{50} of 6.8 ± 1.2 μ M) and maximal levels of potentiation were obtained with 30 μ M ivermectin (Fig. 1B). The effect of this maximal concentration of ivermectin was then examined on a range of concentrations of acetylcholine. In the absence of ivermectin, acetylcholine activated $\alpha 7$ nAChRs with an EC_{50} of 141 ± 23 μ M and a Hill coefficient of 1.2 ± 0.1 . In the presence of ivermectin the acetylcholine dose-response curve was shifted to the left ($EC_{50} = 50 \pm 3$ μ M) and had a steeper slope (Hill coefficient = 1.6 ± 0.4). Both of these are effects that have been observed consistently with nAChR potentiators and are consistent with an allosteric mechanism of action

MOL #64295

(Bertrand and Gopalakrishnan, 2007). Responses to a maximal concentration of acetylcholine (3 mM) were potentiated 2.0 ± 0.3 fold ($n=4$) by ivermectin, whereas higher levels of potentiation were observed with sub-maximal concentrations of acetylcholine (Fig. 1C). Responses to an EC_{50} concentration of acetylcholine (100 μ M) were potentiated 3.6 ± 0.2 fold ($n=4$) and responses to an EC_{20} concentration (50 μ M) were potentiated 4.4 ± 0.6 fold ($n=8$). In contrast, 30 μ M ivermectin caused no significant potentiation of agonist-evoked responses with the 5-HT3A subunit expressed in *Xenopus* oocytes (Fig. 2). The influence of ivermectin was also examined on two artificial subunit chimeras ($\alpha 7^{4TM-5HT3A}$ and $\alpha 7^{V201-5HT3A}$), both of which contain the $\alpha 7$ nAChR extracellular domain fused to the 5-HT3A subunit transmembrane domain (Fig. 2). In contrast to what was observed with the $\alpha 7$ and 5-HT3A, ivermectin caused a significant inhibition of both of the chimeras (Fig. 2 and Table 1). Ivermectin (30 μ M) caused responses evoked by an EC_{50} concentration of acetylcholine (50 μ M) to be inhibited by $44.9 \pm 8.8\%$ ($n=5$) with $\alpha 7^{4TM-5HT3A}$ and by $26.6 \pm 3.4\%$ with $\alpha 7^{V201-5HT3A}$.

To investigate in greater detail the influence of the $\alpha 7$ transmembrane domain upon allosteric modulation by ivermectin, a series of mutations within the four α -helical transmembrane regions (TM1-TM4) were examined (Fig. 3). As in a previous study that examined allosteric modulation of $\alpha 7$ nAChRs (Young et al., 2008), the rationale that we have used for mutagenesis is to alter amino acids in the $\alpha 7$ subunit to the corresponding amino acid in 5-HT3A. In all cases, the influence of 30 μ M ivermectin was examined on an EC_{20} concentration of acetylcholine. Of eleven transmembrane mutations examined, four (G221V, L230Y, M260L and F455A) had no significant effect on allosteric modulation by ivermectin, whereas seven mutations had

MOL #64295

significant effects (Fig. 3 and Table 2). Four mutations caused a significant reduction in potentiation by ivermectin (A225D, Q272V, T456V and C459Y). These mutations are located in TM1 (A225D), TM3 (Q272V) and TM4 (T456V and C259Y). Compared to the level of potentiation observed in wild-type $\alpha 7$ nAChRs in response to an EC_{20} concentration of acetylcholine (4.6 ± 0.6 fold), ivermectin caused potentiation of only 1.1 to 1.4-fold ($n=3-7$) in $\alpha 7$ receptors containing one of these four mutations (Table 2). Three mutations (S222M, M253L and S276V, located in TM1, TM2 and TM3, respectively) had a particularly dramatic and somewhat unexpected effect. All of these mutations caused ivermectin to act as an inhibitor, rather than as a potentiator. Ivermectin caused agonist-evoked responses to be inhibited by $89.1 \pm 6.6\%$ ($n=4$) in S222M, $55.6 \pm 8.8\%$ ($n=3$) in M253L and $84.2 \pm 3.5\%$ ($n=4$) in S276V (Fig. 3 and Table 2).

Of the three mutations that caused ivermectin to act as an inhibitor, one (M253L) was selected for more detailed investigation (because it was found to generate functional receptors most reliably). The influence of a range of ivermectin concentrations was examined on responses evoked by $100 \mu\text{M}$ acetylcholine (an EC_{50} concentration) on $\alpha 7$ nAChRs containing the M253L mutation (Fig. 4A and 4B). The IC_{50} value for inhibition by ivermectin on receptors containing the M253L mutation was $1.4 \pm 0.2 \mu\text{M}$ ($n=3$; Fig. 4C), similar to the EC_{50} value for potentiation by ivermectin of agonist ($100 \mu\text{M}$ acetylcholine) evoked responses on wildtype $\alpha 7$ nAChRs ($6.7 \pm 1.2 \mu\text{M}$; $n=3$; Fig. 1B). The influence of ivermectin on $\alpha 7$ nAChRs containing the M253L mutation was also examined on a range of acetylcholine concentrations (Fig. 4C). As would be expected of a non-competitive antagonist, ivermectin ($3 \mu\text{M}$) caused an insurmountable reduction in the maximal agonist response with no significant effect

MOL #64295

on the EC₅₀ value for acetylcholine. In the absence of ivermectin, the EC₅₀ value for acetylcholine was 110±27 μM (with a Hill coefficient of 1.4±0.2; n=3) and in the presence of ivermectin (3μM) was 115±24 μM (with a Hill coefficient of 1.6±0.3; n=3). However, the maximum agonist-evoked response in the presence of 3μM ivermectin was only 42±3% (n=3) of that detected in the absence of ivermectin (Fig. 4.C). In contrast, inhibition of nAChRs M253L by the competitive antagonist methylcaconitine (MLA) was typical of what would be expected of a competitive antagonist. MLA (3nM) caused a significant (P<0.001) rightward shift of the agonist dose-response curve (the EC₅₀ for acetylcholine was 570±86 μM; with a Hill coefficient of 1.2±0.2; n=3 in the presence of MLA) and the antagonism was completely surmountable by high concentrations of acetylcholine (Fig. 4C).

Computational docking simulations (Morris et al., 1998) were performed with a nAChR α7 subunit homology model (Cheng et al., 2006) based on the 4Å structure of the *Torpedo* nAChR (Unwin, 2005), as described previously for docking studies with PNU-120596 (Young et al., 2008). Our aim was to examine whether the α7 transmembrane region was a plausible binding site for ivermectin. The most favourable docked position of ivermectin (that of lowest predicted binding free energy and, hence, highest predicted affinity) was towards the top of a cavity located between the four α-helical transmembrane domains (Fig. 5). Two equally favourable conformations of ivermectin were identified in these studies (illustrated in yellow and green in Fig. 5). For both conformations the predicted binding free energy (ΔG) of ivermectin within the α7 homology model was -8.39 kcal/mol, equivalent to a predicted binding affinity of 0.7 μM. Of the seven amino acids identified as influencing allosteric modulation in this study, both of the lowest energy docked positions of

MOL #64295

ivermectin were in closest proximity to S276V, a mutation that had no significant effect on the type II allosteric modulator PNU-120596 (Young et al., 2008). PNU-120596 is a much smaller molecule than ivermectin and is predicted to bind at a lower position within the intrasubunit cavity (Young et al., 2008).

MOL #64295

Discussion

There has been a considerable amount of interest in understanding the mechanism of action of allosteric modulators of nAChRs. In part, this has been prompted by evidence that such modulators may have useful therapeutic applications as cognitive enhancers (Hurst et al., 2005; Bertrand and Gopalakrishnan, 2007; Moaddel et al., 2007). We have previously identified an intrasubunit transmembrane cavity as a plausible binding for small positive allosteric modulators of $\alpha 7$ nAChRs such as PNU-120596 (Young et al., 2008). The work presented here extends these studies and provides evidence that mutations located in the transmembrane region can influence allosteric modulation of agonist-evoked responses by much larger compounds such as ivermectin.

Mutagenesis of amino acids located within the four transmembrane helices of the nAChR $\alpha 7$ subunit lead us to conclude that the transmembrane region is critical in determining the allosteric modulatory activity of ivermectin. Interestingly, studies conducted with ivermectin on P2X receptors have suggested that it acts as a positive allosteric modulator of the P2X receptors via interaction with transmembrane regions (Silberberg et al., 2007; Jelínková et al., 2008). This may, therefore, be a common mechanism by which ivermectin is able to modulate a variety of diverse ion channels. The finding that ivermectin acts as an inhibitor of $\alpha 7/5$ -HT3A subunit chimeras was surprising, given that it acts as a potentiator of $\alpha 7$ nAChRs and has no significant effect on 5-HT₃ receptors. This finding is, however, consistent with previous studies conducted with other $\alpha 7/5$ -HT3A chimeras (Bertrand et al., 2008), although the inhibitory action of ivermectin described previously was less pronounced than in the

MOL #64295

present study. Given the inhibitory action of ivermectin on $\alpha 7/5$ -HT3A chimeras, the lack of any significant effect of ivermectin on wild-type 5-HT₃ receptors requires some explanation. It could be argued that, in constructing the $\alpha 7/5$ -HT3A chimeras, a novel ivermectin binding site has been created. A more plausible explanation may be that the conformation of the extracellular domain of the 5-HT3A subunit blocks access of ivermectin to a potential transmembrane binding site and that access by ivermectin is possible only when replaced with the extracellular domain of the nAChR $\alpha 7$ subunit (for example in the two $\alpha 7/5$ -HT3A chimeras). This possibility would be consistent with our findings that mutagenesis of transmembrane amino acids in the nAChR $\alpha 7$ subunit into the analogous amino acids in 5-HT3A converts ivermectin into an negative allosteric modulator.

Several previous studies have demonstrated that ivermectin acts as a positive allosteric modulator of $\alpha 7$ nAChRs (Krause et al., 1998; Raymond et al., 2000; Bertrand et al., 2008; Sattelle et al., 2009). Of some significance to the findings reported in the present study is that, in addition to acting as an allosteric potentiator of $\alpha 7$ nAChRs, ivermectin has been reported to cause a slight attenuation of agonist-evoked responses in ACR-16, a nAChR from the nematode *C. elegans* (Raymond et al., 2000; Sattelle et al., 2009). Our findings would suggest that the inhibitory effect of ivermectin on ACR-16 might be a consequence of amino acid residues located within the transmembrane domain. Interestingly, of the three mutations that we have identified in this study that convert ivermectin from a potentiator into an inhibitor (S222M, M253L and S276V), none are conserved between $\alpha 7$ and ACR-16 (the corresponding amino acids in ACR-16 are threonine, leucine and cysteine, respectively). Indeed, the M253L mutation corresponds exactly to a change into the

MOL #64295

analogous amino acid in ACR-16. Of the four mutations that had no effect on allosteric modulation by ivermectin in $\alpha 7$ (G211V, L230Y, M260L and F455A), three of these amino acids (G211, L230 and F455) are conserved between $\alpha 7$ and ACR-16. It seems plausible that amino acid differences at one or more of the positions identified in the present study may explain the different modulatory effect of ivermectin on these two nAChRs.

Inspection of the 4Å resolution structure of the *Torpedo* nAChR reveals an intrasubunit cavity located between the four transmembrane α -helices (Miyazawa et al., 2003; Unwin, 2005). The cavity is open at the extracellular side of the helices but closes approximately half way into the lipid bilayer and it has been suggested as being water-accessible (Miyazawa et al., 2003; Lobo et al., 2006). In a recent study involving site-directed mutagenesis of amino acids located in the $\alpha 7$ transmembrane regions we have obtained evidence to suggest that low molecular weight allosteric potentiators such as PNU-120596 binds close to the bottom of this transmembrane cavity (Young et al., 2008). Other studies have suggested that allosteric modulators of GABA_A and glycine receptors, such as neurosteroids and volatile anaesthetics, may also bind near to or within this cavity (Mihic et al., 1997; Ye et al., 1998; Hosie et al., 2006). Thus, it appears that this region may correspond to a common site for allosteric modulation of Cys-loop ligand-gated ion channels.

In a previous study of mutations affecting the $\alpha 7$ nAChR positive allosteric modulator PNU-120596, all of the amino acids that were found to influence PNU-120596 were predicted to line the base and sides of the putative intrasubunit transmembrane cavity and had side chains pointing towards its interior (Young et al., 2008). Also,

MOL #64295

those amino acids that had no significant effect upon allosteric modulation by PNU-120596 had side chains that point away from the interior of the cavity or are located towards the ends of the α -helices – either below the cavity or above the central part of it (Young et al., 2008). These findings are consistent with computer docking simulations that predict that the lowest energy docked conformation of PNU-120596 is situated close to the bottom of the cavity. Four of the mutations that we have identified in the present study as influencing allosteric modulation by ivermectin (S222M, A225D, M253L and C459Y) also reduce potentiation by PNU-120596. In contrast, three mutations (Q272V, S276V and T456V) have a significant effect on ivermectin but have no effect on PNU-120596. The side chain of S276 is predicted to point towards the interior of the cavity (as are all of those found to influence PNU-120596) but it is located much closer to the top of the transmembrane cavity than are the mutations that effect PNU-120596 (Fig. 5). In contrast to all of the above mutations, the side chains of Q272 and T456V are not predicted point towards the centre of the cavity (Fig. 5). Whereas the predicted binding site for PNU-120596 is in very close proximity to all amino acids that, when mutated, exert a significant effect on potentiation (Young et al., 2008), this is not the case for ivermectin. The two lowest energy docked positions of ivermectin lie in closest proximity to Q272 which, interestingly, is one of the three amino acids which, when mutated, affect ivermectin but have no significant effect on potentiation by PNU-120596 (Fig. 5). It is tempting to speculate that the positive and negative modulatory activity of ivermectin might be a consequence of its ability to interact with the receptor in different orientations but we do not wish to over-interpret these computer-docking studies, particularly since they are conducted with a homology model (based on the *Torpedo* nAChR). Nevertheless, these docking studies provides support for the possibility that ivermectin binds in the

MOL #64295

nAChR transmembrane domain. At present, we cannot conclude whether the side chains of these mutated amino acids interact directly with bound ivermectin or whether these mutations have a longer-range effect on ivermectin due to induced conformational changes.

We conclude that point mutations located within the transmembrane domain of the $\alpha 7$ nAChR can convert a positive allosteric modulator into a negative allosteric modulator. These findings are consistent with previous studies that have demonstrated that nAChR mutations can convert agonists binding to the conventional (orthosteric) binding site into competitive antagonists (Hussy et al., 1994; Young et al., 2007). It seems plausible that ivermectin binds to a common site in wildtype and in mutated $\alpha 7$ nAChRs and exerts its effect by either stabilising an open conformation (resulting in positive allosteric modulation) or a closed conformation (resulting in non-competitive antagonism or 'negative allosteric modulation').

MOL #64295

References

- Adelsberger H, Lepier A and Dudel J (2000) Activation of rat recombinant $\alpha_1\beta_2\gamma_{2S}$ GABA_A receptor by the insecticide ivermectin. *Eur J Pharmacol* 394:163-170.
- Arena JP, Liu KK, Paress PS, Frazier EG, Cully DF, Mrozik H and Schaeffer JM (2004) The mechanism of action of avermectins in *Caenorhabditis elegans*: correlation between activation of glutamate-sensitive chloride current, membrane binding and activity. *J Parasitol* 81:286-294.
- Bertrand D, Bertrand S, Casser S, Gubbins E, Li J and Gopalakrishnan M (2008) Positive allosteric modulation of the α_7 nicotinic acetylcholine receptor: ligand interactions with distinct binding sites and evidence for a prominent role of the M2-M3 segment. *Mol Pharmacol* 74:1407-1416.
- Bertrand D and Gopalakrishnan M (2007) Allosteric modulation of nicotinic acetylcholine receptors. *Biochem Pharmacol* 74:115-1163.
- Broad LM, Zwart R, Pearson KH, Lee M, Wallace L, McPhie GI, Emkey R, Hollinshead SP, Dell CP, Baker SR and Sher E (2006) Identification and pharmacological profile of a new class of selective nicotinic acetylcholine receptor potentiators. *J Pharmacol Exp Ther* 318:1108-1117.
- Burkhart CN (2000) Ivermectin: an assessment of its pharmacology, microbiology and safety. *Vet Hum Toxicol* 42:30-35.
- Cheng X, Benzhuo L, Grant B, Law RJ and McCammon JA (2006) Channel opening motion of α_7 nicotinic acetylcholine receptor as suggested by normal mode analysis. *J Mol Biol* 355:310-234.
- Chimienti F, Hogg RC, Plantard L, Lehmann C, Brakch N, Fischer J, Huber M, Bertrand D and Hohl D (2003) Identification of SLURP-1 as epidermal

MOL #64295

neuromodulator explains the clinical phenotype of Mal de Meleda. *Hum Mol Genet* 12:3017-3024.

Conroy WG, Liu Q-S, Nai Q, Margiotta JF and Berg DK (2003) Potentiation of $\alpha 7$ -containing nicotinic acetylcholine receptors by select albumins. *Mol Pharmacol* 63:419-428.

Cooper ST and Millar NS (1998) Host cell-specific folding of the neuronal nicotinic receptor $\alpha 8$ subunit. *J Neurochem* 70:2585-2593.

Gee VJ, Kracun S, Cooper ST, Gibb AJ and Millar NS (2007) Identification of domains influencing assembly and ion channel properties in $\alpha 7$ nicotinic receptor and 5-HT₃ receptor subunit chimaeras. *Br J Pharmacol* 152:501-512.

Hansen SB and Taylor P (2007) Galanthamine and non-competitive inhibitor binding to ACh-binding protein: evidence for a binding site on non- α -subunit interfaces of heteromeric neuronal nicotinic receptors. *J Mol Biol* 369:895-901.

Hosie AM, Wilkins ME, da Silva HMA and Smart TG (2006) Endogenous neurosteroids regulate GABA_A receptors through two different discrete transmembrane sites. *Nature* 444:486-489.

Hurst RS, Hajós M, Raggenbass M, Wall TM, Higdon NR, Lawson JA, Rutherford-Root KL, Berkenpas MB, Hoffmann WE, Piotrowski DW, Groppi VE, Allaman G, Ogier R, Bertrand S, Bertrand D and Arneric SP (2005) A novel positive allosteric modulator of the $\alpha 7$ neuronal nicotinic acetylcholine receptor: *in vitro* and *in vivo* characterization. *J Neurosci* 25:4396-4405.

Hussy N, Ballivet M and Bertrand D (1994) Agonist and antagonist effects of nicotine on chick neuronal nicotinic receptors are defined by α and β subunits. *J Neurophysiol* 72:1317-1326.

MOL #64295

- Jelínková I, Vávra V, Jindrichova M, Obsil T, Zemkova HW, Zemkova H and Stojilkovic SS (2008) Identification of P2X₄ receptor transmembrane residues contributing to channel gating and interaction with ivermectin. *Pflugers Arch Eur J Physiol* 456:939-950.
- Khakh BS, Proctor WR, Dunwiddie TV, Labarca C and Lester HA (1999) Allosteric control of gating and kinetics at P2X₄ receptor channels. *J Neurosci* 19:7289-7299.
- Krause RM, Buisson B, Bertrand S, Corringer P-J, Galzi J-L, Changeux J-P and Bertrand D (1998) Ivermectin: a positive allosteric effector of the $\alpha 7$ neuronal nicotinic acetylcholine receptor. *Mol Pharmacol* 53:283-294.
- Krůšek J and Zemková H (1994) Effect of ivermectin on γ -aminobutyric acid-induced chloride currents in mouse hippocampal embryonic neurones. *Eur J Pharmacol* 259:121-128.
- Lansdell SJ, Gee VJ, Harkness PC, Doward AI, Baker ER, Gibb AJ and Millar NS (2005) RIC-3 enhances functional expression of multiple nicotinic acetylcholine receptor subtypes in mammalian cells. *Mol Pharmacol* 68:1431-1438.
- Lobo IA, Trudell JR and Harris RA (2006) Accessibility to residues in transmembrane segment four of the glycine receptor. *Neuropharmacol* 50:174-181.
- Mihic SJ, Ye Q, Wick MJ, Koltchine VV, Krasowski MD, Finn SE, Mascia MP, Valenzuela CF, Hanson KK, Greenblatt EP, Harris RA and Harrison NL (1997) Sites of alcohol and volatile anaesthetic action on GABA_A and glycine receptors. *Nature* 389:385-389.
- Millar NS (2008) RIC-3: a nicotinic acetylcholine receptor chaperone. *Br J Pharmacol* 153:S177-S183.

MOL #64295

Miyazawa A, Fujiyoshi Y and Unwin N (2003) Structure and gating mechanism of the acetylcholine receptor pore. *Nature* 423:949-955.

Moaddel R, Jozwiak K and Wainer IW (2007) Allosteric modifiers of neuronal nicotinic acetylcholine receptors: new methods, new opportunities. *Med Res Rev* 27:723-753.

Morris GM, Goodsell DS, Halliday RS, Huey R, Hart WE, Belew RK and Olson AJ (1998) Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. *J Comput Chem* 19:1639-1662.

Raymond V, Mongan NP and Sattelle DB (2000) Anthelmintic actions on homomer-forming nicotinic acetylcholine receptor subunits: chicken $\alpha 7$ and ACR-16 from the nematode *Caenorhabditis elegans*. *Neurosci* 101:785-791.

Raymond V and Sattelle DB (2002) Novel animal-health drug targets from ligand-gated chloride channels. *Nature Rev Drug Discovery* 1:427-436.

Samochocki M, Höffle A, Fehrenbacher A, Jostock R, Ludwig J, Vhrstner C, Radina M, Zerlin M, Ullmer C, Pereira EFR, Lübbert H, Albuquerque EX and Maelicke A (2003) Galantamine is an allosterically potentiating ligand of neuronal nicotinic but not of muscarinic acetylcholine receptors. *J Pharmacol Exp Ther* 305:1024-1036.

Sattelle DB, Buckingham SD, Akamatsu M, Matsuda K, Pienarr I, Jones AK, Sattelle BM, Almond A and Blundell CD (2009) Comparative pharmacology and computational modelling yields insights into allosteric modulation of human $\alpha 7$ nicotinic acetylcholine receptors. *Biochem Pharmacol* 78:836-843.

Shan Q, Haddrill JL and Lynch JW (2001) Ivermectin, an unconventional agonist of the glycine receptor chloride channel. *J Biol Chem* 276:12556-12564.

MOL #64295

- Sigel E and Baur R (1987) Effect of avermectins B_{1a} on chick neuronal γ -aminobutyrate receptor channels expressed in *Xenopus* oocytes. *Mol Pharmacol* 32:749-752.
- Silberberg SD, Li M and Swartz KJ (2007) Ivermectin interaction with transmembrane helices reveals widespread rearrangements during opening of P2X receptor channels. *Neuron* 54:263-274.
- Timmermann DB, Grønlien JH, Kohlhaas KL, Nielsen EØ, Dam E, Jørgensen TD, Ahring PK, Peters D, Holst D, Chrsitensen JK, Malysz J, Briggs CA, Gopalakrishnan M and Olsen GM (2007) An allosteric modulator of the $\alpha 7$ nicotinic acetylcholine receptor possessing cognition-enhancing properties in vivo. *J Pharmacol Exp Ther* 323:294-307.
- Unwin N (2005) Refined structure of the nicotinic acetylcholine receptor at 4Å resolution. *J Mol Biol* 346:967-989.
- Ye Q, Koltchine VV, Mihic SJ, Mascia MP, Wick MJ, Finn SE, Harrison NL and Harris RA (1998) Enhancement of glycine receptor function by ethanol is inversely correlated with molecular volume at position $\alpha 267$. *J Biol Chem* 273:3314-3319.
- Young GT, Broad LM, Zwart R, Astles PC, Bodkin M, Sher E and Millar NS (2007) Species selectivity of a nicotinic acetylcholine receptor agonist is conferred by two adjacent extracellular $\beta 4$ amino acids that are implicated in the coupling of binding to channel gating. *Mol Pharmacol* 71:389-397.
- Young GT, Zwart R, Walker AS, Sher E and Millar NS (2008) Potentiation of $\alpha 7$ nicotinic acetylcholine receptors via an allosteric transmembrane site. *Proc Natl Acad Sci USA* 105:14686-14691.

MOL #64295

Zheng Y, Hirschberg B, Yuan J, Wang AP, Hunt DC, Ludnerer SW, Schmatz DM and Cully DF (2002) Identification of two novel *Drosophila melanogaster* histamine-gated chloride channel subunits expressed in the eye. *J Biol Chem* 277:2000-2005.

Zwart R, De Filippi G, Broad LM, McPhie GI, Pearson KH, Baldwinson T and Sher E (2002) 5-Hydroxyindole potentiates human $\alpha 7$ nicotinic receptor-mediated responses and enhances acetylcholine-induced glutamate release in cerebellar slices. *Neuropharmacol* 43:374-384.

MOL #64295

Footnotes

This work was supported by a Doctoral Training Grant from the Biotechnology and Biological Sciences Research Council (BBSRC) [Grant BB/D526961/1].

MOL #64295

Figure Legends

Fig. 1. Positive allosteric modulation of $\alpha 7$ nAChRs by ivermectin. A, the chemical structure of ivermectin. The preparation of ivermectin used in this study (Sigma I8898) is composed primarily of ivermectin B_{1a} (where R=CH₂CH₃) with ivermectin B_{1b} (where R=CH₃) being a minor component. B, Dose-response curve for ivermectin on rat $\alpha 7$ nAChR expressed in *Xenopus* oocytes illustrating potentiation of responses to an EC₅₀ concentration of acetylcholine (100 μ M) by ivermectin. Data points are means of 3-4 responses and are normalised to the responses in the absence of ivermectin. C, Acetylcholine dose-response curve obtained in the presence (open circles) and absence (closed circles) of 30 μ M ivermectin. Data points are means of 4-5 responses and are normalised to the maximum response in the absence of ivermectin.

Fig. 2. Representative two-electrode voltage-clamp recordings from *Xenopus* oocytes illustrating the effects of ivermectin on agonist-evoked responses. Agonist-evoked responses are shown for rat $\alpha 7$ subunit, $\alpha 7^{4\text{TM-5HT3A}}$ chimera, $\alpha 7^{\text{V201-5HT3A}}$ chimera and mouse 5-HT3A subunit. Control EC₅₀ agonist responses elicited with either acetylcholine (in the case of $\alpha 7$, $\alpha 7^{4\text{TM-5HT3A}}$ and $\alpha 7^{\text{V201-5HT3A}}$) or CPBG (5-HT3A) are shown (black dashed lines). Also shown are responses evoked by EC₅₀ concentration of agonists after co-application and 60 sec pre-application of 30 μ M ivermectin (solid lines).

Fig. 3. The influence of $\alpha 7$ transmembrane mutations on allosteric modulation by ivermectin. A bar graph illustrates the influence of single point mutations on allosteric

MOL #64295

modulation by ivermectin. The effect of ivermectin is expressed either as potentiation (fold effect; right hand axis) or inhibition (% change; left hand axis), compared to the magnitude of responses obtained with an EC₂₀ concentration of acetylcholine in the absence of ivermectin. Levels of potentiation and inhibition are summarized in Table 2. Data are means \pm SEM (n=3-8).

Fig. 4. Ivermectin acts as a non-competitive antagonist (negative allosteric modulator) of $\alpha 7$ nAChRs containing the M253L mutation. A, Dose-dependent inhibition by ivermectin of agonist-evoked responses with $\alpha 7^{M253L}$. Data (n=3) are normalised to responses obtained with an EC₅₀ concentration of acetylcholine (100 μ M) in the absence of ivermectin. B, Representative traces illustrating responses to a range of concentrations acetylcholine in the absence (upper panel) and presence (lower panel) of ivermectin (3 μ M). C, Acetylcholine dose-response curve for $\alpha 7^{M253L}$ nAChRs (filled circles). Also shown are acetylcholine dose-response curve for $\alpha 7^{M253L}$ nAChRs determined in the presence of 3 μ M ivermectin (open triangles) and 3nM methyllycaconitine (open squares). Data (n=3) are normalised to responses obtained with a maximum concentration of acetylcholine in the absence of antagonist.

Fig. 5. Computer docking simulation. A homology model of the $\alpha 7$ transmembrane domain, illustrating the location of mutated amino acids. The backbone of the four transmembrane α -helices (TM1-TM4) are coloured grey. The model is shown from a side-on view (right panel) and as viewed from above, looking down from the extracellular face of the lipid membrane (left panel). Also illustrated are the two lowest energy (highest predicted binding affinity) docked positions of ivermectin

MOL #64295

within the $\alpha 7$ homology model (represented as a stick model in yellow and green). Side chains of amino acids which, when mutated, had a significant effect on potentiation by ivermectin are indicated by colored spheres. Different colors have been used to indicate differing effects of mutations on allosteric modulation by ivermectin (this study) and PNU-120596 (Young et al., 2008). A225D (TM1) and C459Y (TM4), which caused reduced potentiation of both ivermectin and PNU-120596 are shown in blue. Q272V (TM3) and T456V (TM4), which caused reduced potentiation of ivermectin but had no effect on PNU-120596 are shown in purple. S222M (TM1) and M253L (TM2), which caused ivermectin to act as an inhibitor and caused reduced potentiation by PNU-120596 are shown in red. S276V (TM3), which caused ivermectin to act as an inhibitor but had no effect on PNU-120596 is shown in orange.

MOL #64295

TABLE 1

The influence of ivermectin on agonist-evoked responses was examined on the $\alpha 7$ nAChR, the 5-HT₃R 3A subunit (5-HT3A) and two chimeras containing domains from the $\alpha 7$ and 5-HT3A subunits ($\alpha 7^{4TM-5HT3A}$ and $\alpha 7^{V201-5HT3A}$). In each case, the influence of a maximal concentration of ivermectin (30 μ M) was examined on an EC₅₀ concentration of acetylcholine. The effect of ivermectin is expressed either as potentiation (fold effect) or inhibition (%), compared to the magnitude of responses obtained with an EC₅₀ concentration of acetylcholine in the absence of ivermectin. Significant differences to the $\alpha 7$ subunit are indicated (***) P < 0.001).

Subunit	EC ₅₀ agonist (μ M)	Potentiation (fold)	Inhibition (%)	n
$\alpha 7$	100 (ACh)	3.6±0.2	-	4
$\alpha 7^{4TM-5HT3A}$	100 (ACh)	-	44.9±8.8 ***	5
$\alpha 7^{V201-5HT3A}$	100 (ACh)	-	26.6±3.4 ***	5
5-HT3A	1 (CPBG)	1.0±0.02 ***	-	4

MOL #64295

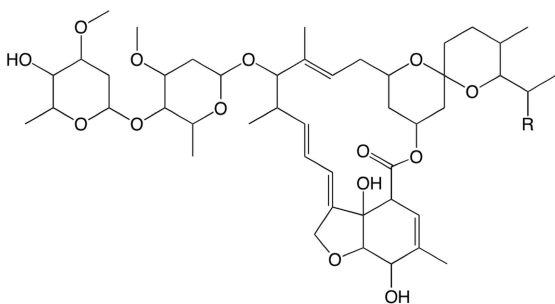
TABLE 2

The influence of ivermectin on agonist-evoked responses was examined on wildtype $\alpha 7$ nAChRs and a series of $\alpha 7$ nAChRs containing single point mutations. In each case, the influence of a maximal concentration of ivermectin (30 μ M) was examined on an EC₂₀ concentration of acetylcholine. The effect of ivermectin is expressed either as potentiation (fold effect) or inhibition (%), compared to the magnitude of responses obtained with an EC₂₀ concentration of acetylcholine in the absence of ivermectin. Significant differences to wildtype $\alpha 7$ nAChRs, determined by ANOVA and Tukeys multiple comparison test, are indicated (***) P < 0.001).

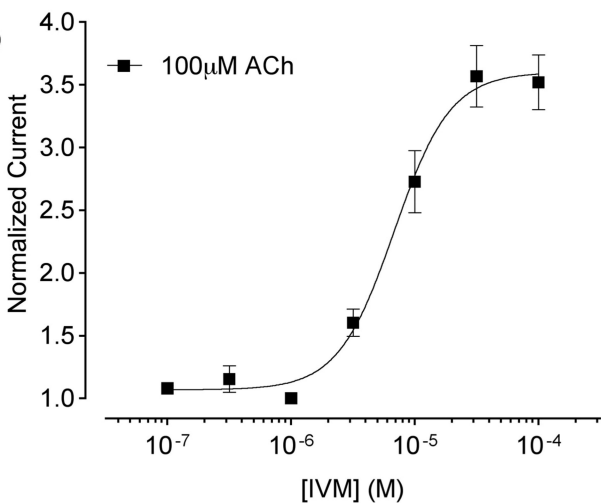
Subunit	Location	EC ₂₀ ACh (μ M)	Potentiation (fold)	Inhibition (%)	n
$\alpha 7$		50	4.6 \pm 0.6	-	8
G211V	TM1	50	4.7 \pm 0.5	-	6
S222M	TM1	50	-	89.1 \pm 6.6 ***	4
A225D	TM1	50	1.1 \pm 0.1**	-	4
L230Y	TM1	50	4.7 \pm 0.5	-	5
M253L	TM2	50	-	55.6 \pm 8.8 ***	3
M260L	TM2	25	4.4 \pm 0.6	-	5
Q272V	TM3	60	1.2 \pm 0.1*	-	3
S276V	TM3	50	-	84.2 \pm 3.5 ***	4
F445A	TM4	50	4.6 \pm 1.0	-	6
T456V	TM4	50	1.2 \pm 0.2 ***	-	7
C459Y	TM4	50	1.4 \pm 0.2 ***	-	7

FIGURE 1

A



B



C

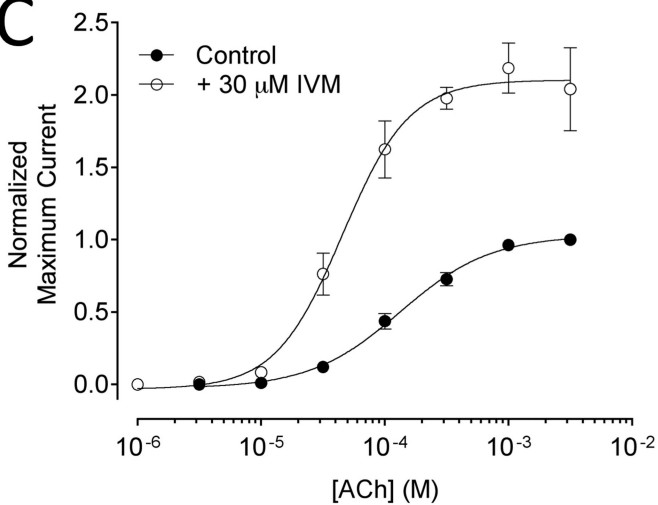
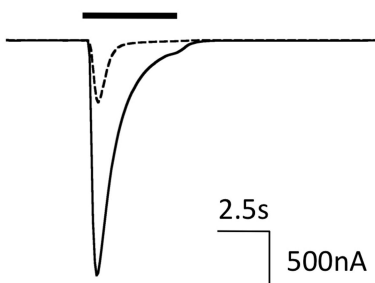


FIGURE 2



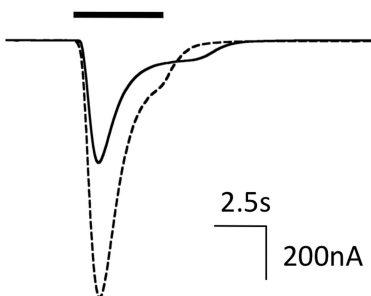
$\alpha 7$

ACh



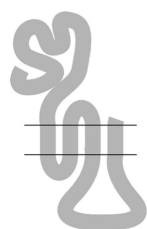
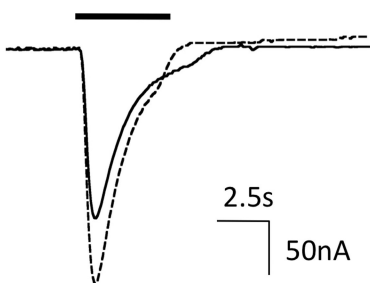
$\alpha 7^{4TM-5HT3A}$

ACh



$\alpha 7^{V201-5HT3A}$

ACh



5-HT^{3A}

CPBG

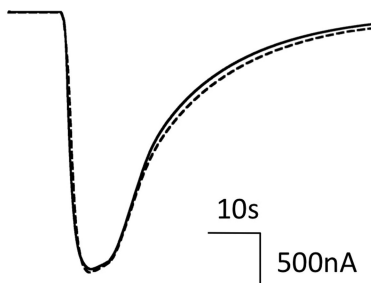


FIGURE 3

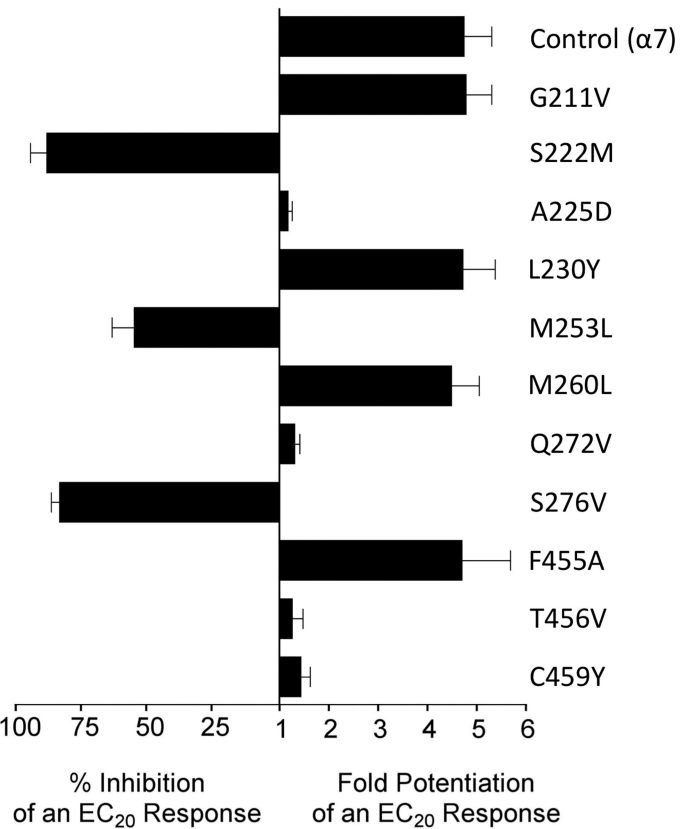


FIGURE 4

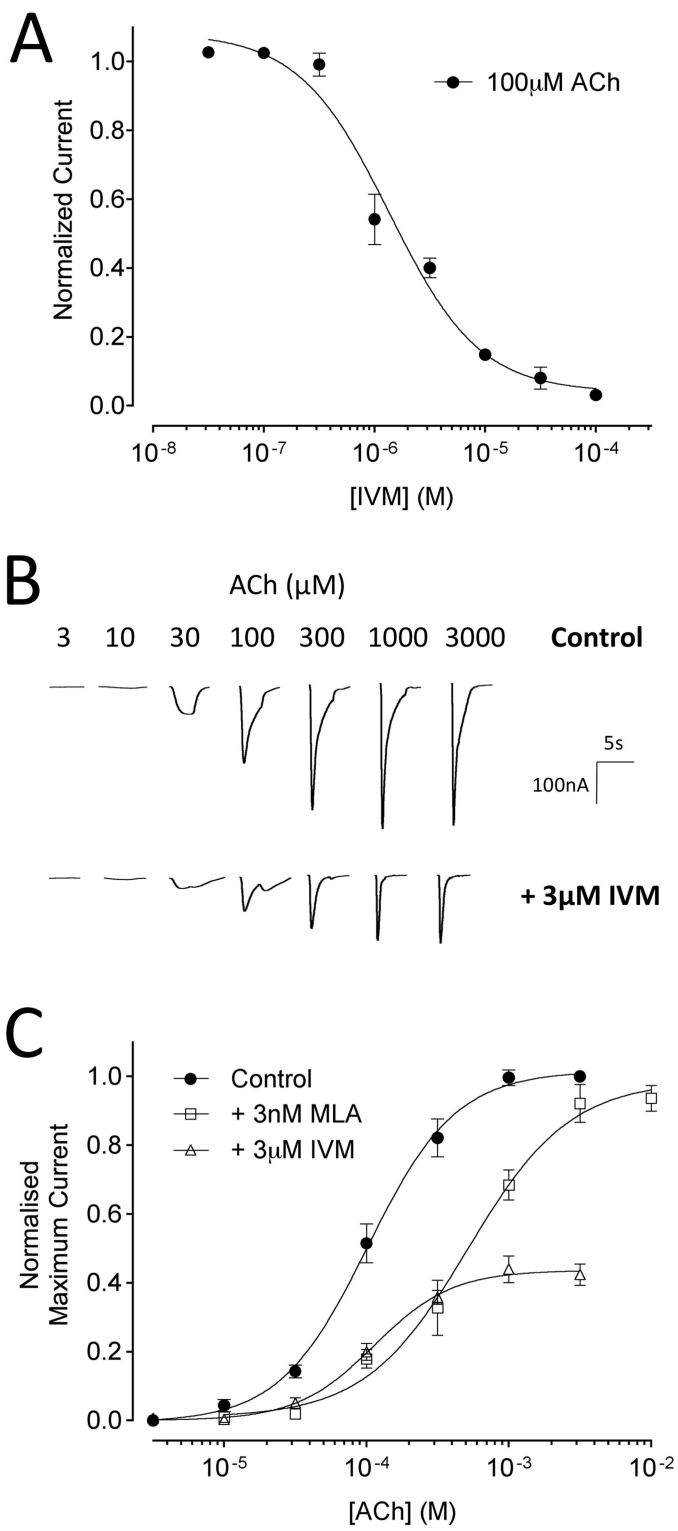


FIGURE 5

