Published in final edited form as:
Eur J Pharmacol. 2013 November 15; 720(0): 264–275. doi:10.1016/j.ejphar.2013.10.018.

Similar activity of mecamylamine stereoisomers in vitro and in vivo

Roger L. Papke,
Dept. of Pharmacology and Therapeutics, University of Florida, College of Medicine, Gainesville, Florida, USA

Clare Stokes,
Dept. of Pharmacology and Therapeutics, University of Florida, College of Medicine, Gainesville, Florida, USA

Pretal Muldoon, and
Dept. Pharmacology, Virginia Commonwealth University, Richmond, Virginia, USA

M. Imad Damaj
Dept. Pharmacology, Virginia Commonwealth University, Richmond, Virginia, USA

Abstract

A previous characterization of mecamylamine stereoisomers using nicotinic acetylcholine receptors expressed in *Xenopus* oocytes revealed only small differences between the activity of the R and S forms of mecamylamine. However, that work was limited in the breadth of receptor subtypes tested, especially in regard to the discrimination of high and low sensitivity receptors, which differ in the ratios of alpha and beta subunits. We report new data using subunit concatamers, which produce uniform populations of high-sensitivity or low-sensitivity receptors, as well as alpha2, alpha5, and alpha6-containing receptors, which were not studied previously. Consistent with previous studies, we found that beta4-containing receptors were most sensitive to mecamylamine and that the IC_{50} values for the inhibition of net charge were lower than for inhibition of peak currents. No large differences were seen between the activities of the mecamylamine isomers. Additionally, a previously reported potentiation of high-sensitivity α4β2 receptors by S-mecamylamine could not be reproduced in the oocyte system, even with mutants that had greatly reduced sensitivity to mecamylamine inhibition or when the selective agonist TC-2559 was used. In vivo studies suggested that the R-isomer might be somewhat more potent than the S isomer at blocking CNS effects of nicotine. Although the potency difference was no more than a factor of two, it is consistent with lower LD_{50} estimates previously reported for the R isomer. Our results significantly extend knowledge of the nicotinic acetylcholine receptor activity

*To whom correspondence should be addressed: Name: Roger L. Papke, Phone: 352-392-4712, Fax: 352-392-9696, rlpapke@ufl.edu, Address: Department of Pharmacology and Therapeutics, University of Florida, P.O. Box 100267, Gainesville, FL 32610-0267.

profile of mecamylamine and support the hypothesis that these effects are not strongly stereoisomer selective.

Keywords

Tourette's syndrome; depression; nicotine addiction; TC-5214; TC-2559

1 Introduction

There are numerous subtypes of nicotinic acetylcholine receptors (nAChR) expressed in neurons which are pharmacologically distinct from the receptors of the neuromuscular junction (Millar and Gotti, 2009). The ganglionic blocker mecamylamine, a relatively nonselective noncompetitive antagonist for all the neuronal-type nAChR that does not block acetylcholine (ACh) binding, was among the very first drugs developed to target neuronal nAChR (Dennis et al., 1957) but has been long been abandoned for the treatment of hypertension, its original indication.

Normal brain function relies on a balance among neurochemical systems, which can be perturbed by disease, drug abuse, medications, or side effects of medications. It has been hypothesized that hypercholinergic function is an underlying feature of depression. Nicotine self-administration clearly disrupts normal cholinergic function. Certain antipsychotic mediations perturb the balance of dopaminergic and cholinergic function in the basal ganglia, resulting in dyskinesia. In order to treat these imbalances, mecamylamine has recently been considered for adjunct therapy to treat depression, nicotine dependence, and Tourette's syndrome. A prior history of relatively safe use for its original indication has allowed mecamylamine to be clinically tested for these indications, with mixed results (Rose et al., 1998; Silver et al., 2000; Singh et al., 2006).

Mecamylamine is a stereo-active compound, but the vast majority of experimental and therapeutic work has utilized racemic preparations. We previously tested whether the two stereoisomers of mecamylamine had equivalent selectivity and potency for the inhibition of nAChR subtypes expressed in *Xenopus* oocytes (Papke et al., 2001) but failed to find any striking differences in the activity profile of R(-)Mecamylamine (R-Mec) and S(+)Mecamylamine (S-Mec). However, a subsequent study (Fedorov et al., 2009) using nAChR expressed in cell lines reported a novel effect for S-Mec (TC-5214) on a specific α4-containing nAChR subtype with high sensitivity to agonists (HS α4β2 receptors, which have a subunit stoichiometry of two α4 and three β2 subunits). This effect that might have gone unnoticed in the earlier oocyte studies due to a limitation of the original approach.

Recently, various nAChR constructs have been developed which allow for the selective expression of receptors with specific subunit composition. We have used several constructs of linked nAChR subunits (concatamers) (Zhou et al., 2003) to extend our oocyte studies to test the activity of the mecamylamine stereoisomers on nAChR subtypes with defined subunit composition that could not previously be studied in isolation. While α4-containing receptors are the most abundant high-affinity nAChR in rodent brain, in primates there are additional high affinity-receptors containing α2 subunits (Han et al., 2000; Han et al., 2003).

The degree to which α2* nAChR have a pharmacological profile similar to α4* receptors has not been well studied. Therefore we have also generated populations of α2 receptors with defined subunit composition and evaluated their responses to agonists, including TC-2559 and the mecamylamine stereoisomers. We also extended these studies to α6-containing receptors formed with a five-subunit concatamer.

We evaluated whether the previously reported selective potentiation of high-sensitivity α4β2 responses by S-Mec could be replicated in the oocyte expression system. Additionally, We have also conducted in vivo experiments to determine whether the mecamylamine stereoisomers given systemically differ in their potency for blocking some of CNS-dependent effects of nicotine in mice.

2 Material and methods

2.1 Chemicals

Fresh acetylcholine (Sigma; St. Louis, MO) stock solutions were made daily in Ringer's solution and diluted. Mecamylamine (N-2,3,3-tetramethylbicyclo[2.2.1]heptan-2-amine) stereoisomers were supplied by Layton Biosciences (Menlo Park CA). TC-2559 was supplied by Targacept (Winston Salem NC) or purchased from Tocris (c/o R&D Systems, Minneapolis MN). All other chemicals for electrophysiology were obtained from Sigma Chemical Co. (St. Louis MO).

2.2 ACh receptor clones

Human nAChR clones, the β2−6−α4 and α4β2α6β2β3 concatamers (Kuryatov and Lindstrom, 2011; Zhou et al., 2003) were obtained from Dr. Jon Lindstrom (University of Pennsylvania, Philadelphia PA). The human β2−6−α2 concatamer and monomeric α2 cDNAs were provided by Dr. Edwin Johnson (Astra Zeneca, Wilmington DE). The identity of the β2−6−α2 concatamer and the human α2 monomer were confirmed by DNA sequencing at the University of Florida. The β4-6-α3 concatamer was constructed as previously described (Stokes and Papke, 2012).

The mutations in the second transmembrane domain of the β2 portion of the β2−6−α4 concatamer were made using the QuikChange kit (Agilent Technologies, Santa Clara CA), as previously described for the double mutation in the β4 monomer (Webster et al., 1999).

2.3 Expression in Xenopus laevis oocytes

Xenopus laevis oocytes were surgically removed from frogs (Nasco, Ft. Atkinson WI) and treated with Type I collagenase (Worthington Biochemical Corporation, Freehold NJ) in calcium-free Barth's solution (88 mM NaCl, 1 mM KCl, 2.38 mM $NaHCO_3$, 0.82 mM $MgS0_4$, 15 mM HEPES (pH 7.6), 12 mg/l tetracycline) in order to remove the follicular layer. Stage-5 oocytes were isolated and injected with 50 nl (3–20 ng) of each cRNA. After linearization and purification of cloned cDNAs, RNA transcripts were prepared in vitro using the appropriate mMessage mMachine kit (Ambion, Austin TX). Recordings were conducted 1–10 days post-injection.

Using concatamers of α4 and β2 (β2−6−α4) (Zhou et al., 2003), α3 and β4 (β4−6−α3) (Stokes et al., 2012), or α2 and β2 (β2−6−α2, provided by Edwin Johnson) subunits, receptors with restricted subunit composition were generated in the following manners:

α4(3)β2(2)	β2−6−α4 co-expressed with α4
α4(2)β2(3)	β2−6−α4 co-expressed with β2
α4(2)β2(2)α5	β2−6−α4 co-expressed with α5
α2(2)β2(3)	β2−6−α2 co-expressed with β2
α2(3)β2(2)	β2−6−α2 co-expressed with α2
α2(2)β2(2)α5	β2−6−α2 co-expressed with α5
α3(2)β4(2)α5	β4−6−α3 co-expressed with five-fold excess α5
α4β2α6β2β3	expressed as a five-subunit concatamer

2.4 Electrophysiology

Experiments were conducted using OpusXpress 6000A (Molecular Devices, Union City CA). OpusXpress is an integrated system that provides automated impalement and voltage clamp of up to eight oocytes in parallel. Both the voltage and current electrodes were filled with 3M KCl. The oocytes were clamped at a holding potential of −60 mV. Data were collected at 50 Hz and filtered at 5 Hz. The oocytes were bath-perfused with Ringer's solution (115 mM NaCl, 10 mM HEPES, 2.5 mM KCl, 1.8 mM $CaCl_2$) containing 1μM atropine to block muscarinic acetylcholine receptors, which may be native in the oocytes. Agonist solutions were delivered from 96-deepwell plates using disposable tips. Flow rates were set at 4 ml/min.

2.5 Experimental protocols and data analysis

Responses were calculated as both net charge and peak currents. Each oocyte received initial control applications of ACh, then experimental drug applications, and follow-up control applications of ACh. Responses to experimental drug applications were calculated relative to the preceding ACh control responses in order to normalize the data, compensating for the varying levels of channel expression among the oocytes. Then responses were divided by average ACh maxima for each receptor type in order to show the efficacy of the test drug relative to the fully active reference agonist. Average values and standard errors (S.E.M.) were calculated from the normalized responses of at least four oocytes for each experimental condition. Cells were tested with progressively higher concentrations of mecamylamine co-applied with control ACh (with alternating applications of ACh alone). If control ACh responses obtained after the co-applications of ACh and mecamylamine were less than 75% of the preceding ACh controls, then new sets of cells were used to test any higher concentrations of mecamylamine with similar internal controls. Control ACh concentrations were 100 μM for all subtypes tested except the high sensitivity (HS) forms of α4β2 (α4(2)β2(3)) and α2β2 (α2(2)β2(3)), for which the controls were 10 μM, and for α4β2α6β2β3 receptors, for which the ACh controls were 30 μM.

For concentration-response relations, data were plotted using Kaleidagraph 3.0.2 (Abelbeck Software; Reading, PA), and EC_{50} curves were generated from the Hill equation:

$$\text{Response} = [\text{agonist}]^n/([\text{agonist}]^n + (EC_{50})^n)$$

I_{max} denotes the maximal response relative to the maximal ACh-evoked currents, and *n* represents the Hill coefficient. I_{max} was fixed at 1 for the ACh concentration response curves. For studies of nicotine and TC-2559, I_{max}, *n*, and the EC_{50} were all unconstrained for the fitting procedures. For the calculation of IC_{50} values, the maximal responses relative to control were fixed at 1, and the Hill coefficient *n* was assigned a negative value.

2.6 Evaluation of mecamylamine stereoisomers in vivo

2.6.1 Animals—Experimentally naïve male ICR mice were obtained from Harlan Laboratories (Indianapolis, IN). Animals were 8–10 weeks of age at the start of the experiments and were group-housed in a 21°C humidity-controlled Association for Assessment and Accreditation of Laboratory Animal Care-approved animal care facility with ad libitum access to food and water. Experiments were performed during the light cycle and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. Different groups of mice (n= 6–8 per group) were injected with mecamylamine stereoisomers and nicotine and tested at different time points after injection. Three responses to nicotine were measured: antinociception using the tail-flick and hot-plate tests, and changes in body temperature. Separate groups of mice were used for each test. (-)-Nicotine and mecamylamine stereoisomers were dissolved in physiological saline (0.9% sodium chloride) and injected subcutaneously (s.c.) at a volume of 10 ml/kg body weight. All doses are expressed as the free base of the drug.

2.6.2 Antinociception—Antinociception was assessed by the tail-flick method of D'Amour and Smith (D'Amour and Smith, 1941) and the hot plate test. For the tail-flick test, mice were lightly restrained while a radiant heat source was directed onto the upper portion of the tail. A control response (2–4 s) was determined for each mouse before treatment, and test latency was determined 5 min after nicotine administration. The apparatus has an automatic cut-off of 10 s to minimize tissue damage. In the hot plate test, mice were placed into a 10-cm wide glass cylinder on a hot-plate (Thermojust Apparatus, Columbus, OH). The hot plate is a rectangular heated surface surrounded by plexiglass and maintained at 55°C. The device is connected to a manually operated timer that records the amount of time the mouse spends on the heated surface before showing signs of nociception (e.g. jumping, paw licks). A control response (8–12 s) was determined for each mouse before treatment, and test latency was determined 5 min after nicotine administration. The timer has an automatic cut-off of 40 s to avoid tissue damage. Antinociceptive response for the both tests was calculated as percentage of maximum possible effect (%MPE), where %MPE = [(test - control)/(10 (40 for the hot-plate) - control)]×100. Increased latency in either test is indicative of antinociception. Mice were pretreated s.c. with saline, R-Mec or S-Mec 10 min before nicotine. Nicotine was administered at a dose of 2.5 mg/kg s.c., and mice were tested 5 min later.

2.6.3 Body temperature—Rectal temperature was measured by a thermistor probe (inserted 24 mm) and digital thermometer (YSI Inc., Yellow Springs, OH). Mice were pretreated s.c. with saline, R-Mec or S-Mec 10 min before nicotine. Nicotine was administered at a dose of 2.5 mg/kg s.c., and mice were tested 5 min later. The maximum effect of nicotine-induced hypothermia in the mouse was found in many of our studies to be between 30 and 60 min after nicotine injection (Damaj et al., 1993; 1995; 1999). Therefore readings were taken just before and at 30 min after nicotine injection. The difference in rectal temperature before and after treatment was calculated for each mouse. The ambient temperature of the laboratory varied from 21–24°C from day to day.

2.6.4 Statistical analysis—AD50 values with 95% confidence limits (CL) were also calculated for the antinociception and body temperature studies by unweighted least-squares linear regression as described by Tallarida and Murray (1987). If confidence limit values did not overlap, the shift in the dose-response curve was considered significant.

3 Results

3.1 Characterization of HS and LS α2β2 receptors

Additional experimental tools have become available in recent years that make it possible to study various nAChR subtypes with defined subunit composition. Receptors containing α4 and β2 subunits at a ratio of 2:3 (α4(2)β2(3)) are pharmacologically distinct from receptors with the subunit ratio 3:2 (α4(3)β2(2)). Receptors with the (α4(2)β2(3)) composition respond to relative low concentrations of ACh or nicotine and therefore have been identified as high sensitivity (HS) receptors, while α4(3)β2(2) receptors are identified as low sensitivity (LS). Although under carefully controlled conditions, monomers can be valuable tools when studying HS and LS versions of nicotinic receptors (Harpsoe et al., 2011), some earlier studies that relied on the co-expression of subunit monomers produced mixed populations of HS and LS receptors. The use of a α4−β2 concatamer with either monomeric α4 or β2 allows for pure populations of LS or HS α4β2 receptors to be generated, respectively (Zhou et al., 2003). Alpha5 co-expressed with the α4−β2 concatamer also generates receptors with high sensitivity to agonist but in other ways pharmacologically distinct from α4(2)β2(3) HS receptors.

Receptors containing α2 and β2 subunits are believed to be in low abundance in rodent brain (Wada et al., 1989) but more widely expressed in the brains of primates (Han et al., 2000; Han et al., 2003) and therefore perhaps also in humans. These receptors also manifest HS and LS forms (Timmermann et al., 2012), the expression of which can be regulated with a β2−6−α2 concatamer similar to the β2−6−α4 construct (Gurley et al., 2008). A characterization of the ACh and nicotine sensitivity of these α2β2 receptor subtypes is shown in Figure 1 (curve fit values provided in Table 1), along with data for α2β2 receptors incorporating α5 as a structural subunit. In general, the data for the α2-containing receptors align well with those previously reported for the α4* receptors (Papke et al., 2010).

Note that the slope of the nicotine response curves for the HS receptors, α2(2)β3(3) and α2(2)β2(2)α5 are rather shallow. It is difficult to interpret Hill slopes of macroscopic concentration-response curves since many factors impact the dynamics of the responses

(Papke, 2010), and this is especially true for data which may be impacted by factors such as noncompetitive inhibition associated with channel block by nicotine (Papke et al., 2007) or mecamylamine. While such shallow slopes are sometimes seen when there are mixed populations of receptors, this is unlikely to be the case for these receptors which were formed with monomers and the α2−β2 concatamer, since the concatamer show negligible function when expressed alone.

3.2 Characterization of TC-2559 as a tool to identify receptors with defined subunit composition

TC-2559 was identified as a more efficacious agonist of α4β2 receptors formed when the ratio of α4 and β2 RNAs were skewed 1:5 in favor of β2 than when the ratio was reversed (Zwart et al., 2006). Although the use of an RNA ratio skewed toward β2 probably does still result in a mixed population of receptors, the population was highly enriched in the HS subtype. To confirm the selectivity of TC-2559 for HS receptors in our system, we compared its activity to that of ACh for receptors formed when the β2−6−α4 concatamer was co-expressed with either β2 or α4 monomers. Consistent with results obtained with the RNA ratio approach, we confirmed that TC-2559 was more efficacious than ACh for HS α4β2 receptors but only a relatively weak partial agonist for LS α4β2 (Figure 2A). Maximal TC-2559-evoked currents were approximately 20-fold greater in HS than LS receptors when each were normalized to their respective ACh maximum responses (Table 2). TC-2559 was also relatively selective for HS α2β2 receptors compared to LS α2β2 (Figure 2A). Although the efficacy of TC-2559 compared to ACh was only about half as great for the HS α2β2 receptors as for HS α4β2, the relative efficacy between the HS and LS forms of the two receptors was similar.

It has been reported that α5 subunits are expressed in numerous cell lines of similar origin (Chini et al., 1992) to the SH-EP1 cells used by Fedorov et al., although it is unclear if the RNA detected in these cells is processed appropriately to form functional α5 protein (Ron Lukas, personal communication). Receptors formed with α4β2 dimers and α5 as a structural subunit also have relatively high sensitivity to ACh and nicotine (Kuryatov et al., 2008); therefore we tested the sensitivity of α4(2)β2(2)α5 and α2(2)β2(2)α5 receptors to TC-2559 (Figure 2B). We determined that the potency of TC-2559 was relatively high (EC_{50} = 1.5 ± 0.3 μM, Table 2) and that while the efficacy of TC-2559 for α5-containing receptors was 3-fold greater than for LS α4β2 and α2β2 receptors, it was still 5-fold less than for HS receptors (relative to ACh). Likewise, the efficacy of TC-2559 for α6-containing receptors formed by the expression of a pentameric concatamer containing α4β2α6β2α3 (Kuryatov and Lindstrom, 2011) was greater than for LS α4β2 receptors, but significantly less than for HS receptors (Table 2).

Since the activity of α4* and α2* receptors was highly dependent on the presence or absence of β2 as the fifth (structural) subunit, we tested whether this could be generalized to α3-containing receptors. Since α3−β2 concatamers were not available, we took the alternative approach of injecting α3 and β2 RNA at either 6:1 or 1:6 ratios. Although receptors formed with the overexpression of β2 relative to α3 responded more potently to

TC-2559 than receptors formed with the reverse subunit ratio (Figure 2C), the efficacy of TC-2559 was comparable for both populations of receptors (Table 2).

3.3 Effects of mecamylamine stereoisomers on receptors with defined subunit composition expressed in Xenopus oocytes

We have previously studied the effects of mecamylamine stereoisomers on receptors formed from the co-expression of RNA at equal ratios (Papke et al., 2001). Since mixed populations of receptors were represented in those experiments, we redid them with defined populations of α4β2 nAChR having subunit stoichiometry of HS (α4(2)β2(3)) or LS (α4(3)β2(2)) by co-expressing the β2−6−α4 concatamer (Zhou et al., 2003) with either monomeric β2 or α4, respectively. After obtaining ACh control responses for purposes of normalization, co-applications of ACh and progressively increasing concentrations of either R-Mec or S-Mec were made, with alternating application of ACh alone to evaluate the stability of the ACh controls (see Methods). The data for the inhibition of peak current and net charge are shown in Figure 3, and the IC_{50} values are given in Table 3. Mecamylamine appeared more potent for inhibition of LS α4β2 receptors than for HS α4β2 receptors, and for both subtypes IC_{50} values for inhibition of net charge were lower than for inhibition of peak currents. In these experiments the mecamylamine isomers appeared to have equivalent activity for the respective subtypes and the two measurements of receptor function, peak current and net charge. There was no indication of potentiating effects for either isomer on either subtype in these experiments.

The two stereoisomers of mecamylamine were also tested on other nAChR with defined subunit composition, including α2β2* receptors, α3β4 and α4β2 receptors with α5 as the fifth subunit, and α4β2α6β2β3 receptors. Note, receptors containing α5 in combination with α3 and β4 subunits are likely to be found in the medial habenula and have recently been implicated as important for the aversive effects of nicotine (Fowler et al., 2011; Frahm et al., 2011). The co-assembly of α5 with α3 and β4 subunits in the oocyte expression system has been sometimes problematic (Kuryatov et al., 2008); however, we have previously shown that the use of the α3β4 concatamer 1:5 with α5 (Stokes and Papke, 2012) gives reliable incorporation of the α5 subunit. The potency of mecamylamine for inhibition of these nAChR subtypes varied significantly (Table 3), although there were no striking differences in the effects of the stereoisomers (Figure 4).

3.4 Inhibition of TC-2559-evoked responses by mecamylamine stereoisomers

In order to test whether the potentiating effect of S-Mec on HS receptors reported in Fedorov et al. ((Fedorov et al., 2009), Figure 5) might be due to a specific interaction between allosteric effects of S-Mec and the activation mechanism of TC-2559, we tried to emulate the protocol used in the Fedorov paper. That protocol involved repeated co-applications of 0.5 μM TC-2559 combined with 1 μM S-Mec (TC-5214) or R-Mec (TC-5213). They reported a potentiation of the TC-2559-evoked responses after the second co-application of TC-2559 and S-Mec, while there was inhibition of the TC-2559 responses when R-Mec was co-applied.

Our protocol involved sequential application to HS or LS α4β2-expressing oocytes, initially measuring ACh control responses and then applying, in alternation, either 0.6 μM TC-2559 or ACh, followed by three co-applications of TC-2559 plus 1 μM of either S-Mec or R-Mec. As expected, the applications of 0.6 μM TC-2559 were more effective at activating α4(2)β2(3) receptors than α4(3)β2(2) receptors (Figure 5). However, with this protocol the mecamylamine co-applications produced only inhibition of the TC-2559-evoked responses, regardless of which isomer was used and which subtype was tested.

3.5 Shifting the inhibitory potency of mecamylamine for HS and LS α4β2 nAChR

In order to test the hypothesis that the antagonism of the HS receptors by S-Mec might have obscured our ability to detect potentiation previously reported (Fedorov et al., 2009), we incorporated mutations in the second transmembrane domain (TM2) that we have previously shown to have reduced sensitivity to mecamylamine inhibition when introduced into β4 subunits (Webster et al., 1999). The mutant is designated β2†. The mutations substitute the sequence of β1 subunit from muscle-type receptor, which is relatively insensitive to mecamylamine, at the 6' and 10' positions in the TM2 (numbering from (Miller, 1989)) as indicated below (the 6' and 10' residues are in bold and underlined):

β2	MTLCI**S**VLL**A**LTVFLLLISK
β1	MGLSI**F**ALL**T**LTVFLLLLAD
β2†	MTLCI**F**VLL**T**LTVFLLLISK

The β2† sequence with the 6' and 10' mutations was engineered into both the β2 monomer and the β2 segment of the β2−6−α4 concatamer. As shown in Figure 6, the mutations were very effective at decreasing the potency of the mecamylamine stereoisomers for the inhibition of both mutant HS and LS forms of the receptor (Table 3). The mutations nullified the apparent difference in potency for the inhibition of net charge relative to peak current, and the greatest effect of the mutations was a more than 1000-fold decrease in IC_{50} for the net charge inhibition of the HS subtype, which incorporated the double mutations into three of five subunits. In general, the mutations had parallel effects on the inhibition by both isomers. There was a small trend toward potentiation of the HS mutant responses by low concentrations of R-Mec, but these effects were not statistically significant, and in any case would not relate to the effects reported by Fedorov et al., since they saw putative potentiation of wild-type HS α4β2 receptors with S-Mec.

3.6 Evaluation of in vivo activity of mecamylamine stereoisomers

While our in vitro data would suggest that the two stereoisomers of mecamylamine have very similar activity profiles across a large range of nAChR subtypes, there might nonetheless be enantioselectivity in blocking nicotine's effects in vivo, based on differences in blood-brain barrier permeation or other pharmacokinetic factors. We therefore evaluated their pharmacological potency after systemic administration in mice.

Nicotine-induced antinociception in the hot-plate (Figure 7A) and tail-flick (Figure 7B) tests after systemic administration in mice (2.5 mg/kg) was blocked by either R-Mec or S-Mec in

a dose-dependent manner. Similarly, both stereoisomers blocked nicotine-induced hypothermia in a dose-related manner. The relative potencies of mecamylamine stereoisomers were calculated from their dose-response data. Calculation of the AD_{50} values (Table 4) showed that R-Mec was 2.4 times more potent in blocking the antinociceptive effect of nicotine in the tail flick test than S-Mec (0.03 versus 0.08 mg/kg). A similar trend was seen in the hot-plate test and hypothermia, but the difference in potencies was not significant since the confidence limits of the curves overlapped.

It should be noted that these behavioral tests were not intended to specifically address the potential efficacy of the isomers for the indications mentioned per se, but rather, to test whether the two isomers were both able to penetrate the blood-brain barrier with similar efficiency. This extension of the in vitro data suggests that blood-brain barrier penetration will not be a significant factor for distinguishing the in vivo efficacy of the mecamylamine stereoisomers.

4 Discussion

The principal high-affinity nAChR subtype of mammalian brain is composed of five subunits of usually of two different types, α4 or α2 plus β2, in some cases co-assembled with alternative subunits such as α6, α5, or β3 (Gotti et al., 2009). While subunit composition may be differentially regulated in specific cell types or tissues in vivo, heterologous expression systems are permissive for forming mixed populations of receptors which require both α and β subunits. Since the original characterization of the mecamylamine stereoisomers was based on the expression of RNA for α and β subunit monomers and therefore resulted in a mixed population of receptors, any effects that were specific for either HS or LS receptors may have been missed. While the population of α4β2 in the cell line that was used to study the mecamylamine stereoisomers was also mixed, those researchers were able to pharmacologically isolate the response of HS α4β2 nAChR with an agonist (TC-2559) that selectively activates that subtype. They observed that repeated applications of relatively low concentrations of S-Mec, but not R-Mec, were able to produce apparent potentiation of TC-2559-evoked responses.

Our data support the usefulness of TC-2559 as an experimental tool to discriminate HS from LS α4* and α2* receptor subtypes and, to a lesser degree, receptors that contain α5 from those that do not. The observation that TC-2559 has similar profiles for α4* and α2* receptors will limit its utility for discriminating between these receptor subtypes, but may ultimately guide structure-function studies to define the basis for the HS selectivity of the drug. Studies of α3* receptors indicate that the efficacy of TC-2559 is not specifically determined by the presence of a β2 "structural" subunit, since the drug does not strongly activate α3(2)β4(2)β2, α3(3)β2(2), or α3(2)β2(3) receptors.

Our data support the usage of mecamylamine as a broad-spectrum antagonist of nAChR found in the CNS and fail to identify large differences in the activity of the stereoisomers. Mecamylamine inhibition of neuronal nAChR is noncompetitive and voltage dependent (Webster et al., 1999). In acute co-application experiments, the ganglionic blocker mecamylamine appears most potent for blocking α3β4 receptors, least potent for α7, and

roughly equipotent for the muscle receptors and the β2-containing nAChR (Papke et al., 2008; Papke et al., 2012; Papke et al., 2006). However, the block of both β4- and β2-containing receptors is slowly reversible, consistent with effective targeting of these receptors in vivo. Although mecamylamine has some selectivity for β4-containing ganglionic receptors, hypothetically it may used to target receptors in the CNS without serious side effects. This may be due to a large receptor reserve in autonomic ganglia, so that mecamylamine treatments result in small changes in the dominant tone of the peripheral targets. However, it should also be noted that there is a growing appreciation for the potential importance of α3β4 receptors in brain, where they are most highly concentrated in the medial habenula. Some α3β4 receptors in brain are co-assembled with α5, and these α5-containing receptors have been implicated in establishing aversive effects of high nicotine doses (Frahm et al., 2011), and independent α5, α3β4 receptors have also been implicated in nicotine reward and withdrawal (Jackson et al., 2013). Our data are the first to report the high sensitivity of these receptors to both mecamylamine stereoisomers.

Based on its broad activity profile, mecamylamine has been very commonly used as a pharmacological tool when the specific blockade of particular receptor subtypes was not required. It has also been used for several human studies and was, most notably, tested as a therapy for the management of Tourette's syndrome (Sanberg et al., 1998). The preliminary studies of mecamylamine (Inversine®) for Tourette's syndrome provided the initial impetus to the study of functional profiles of the mecamylamine stereoisomers (Papke et al., 2001).

An early characterization of mecamylamine and related compounds (Suchocki et al., 1991) evaluated the in vivo effects of the mecamylamine isomers, and, consistent with our findings, they found no enantioselectivity for nicotine-induced antinociception in the acute thermal tail-flick pain test. The initial in vitro studies of the mecamylamine stereoisomers on heterologously expressed receptors suggested that there was relatively little difference between S-Mec and R-Mec in terms of the IC_{50} values for given receptor subtypes. Accounting for error estimates, the IC_{50}s overlapped or differed by no more than 22%. There did appear to be significant differences in the off-rates of the mecamylamine isomers. Specifically, S-Mec appeared to dissociate more slowly from α4β2 and α3β4 receptors than did R-Mec. However, this difference was not large and was similar for both α4β2 and α3β4 receptors.

A relatively large-scale trial for mecamylamine as a monotherapy for Tourette's was unsuccessful (Silver et al., 2001). Since, in the promising early small-scale studies of Tourette's patients, the subjects were using the drug in conjunction with standard antipsychotics, in retrospect, it may be that the positive effects of mecamylamine were due to more effective management of side effects rather than the disease itself. Although mecamylamine was subsequently abandoned as therapy for Tourette's, interest in its possible therapeutic development remained when post-hoc analysis on the human data and subsequent animal studies suggested that it might be useful for the treatment of depression (Rabenstein et al., 2006; Shytle et al., 2011). This motivated interest in the further characterization of the stereoisomers, and in 2008 a team of scientists from Targacept reported that S-Mec (TC-5214) had the effect of selectively potentiating TC-2559 responses of HS α4β2 receptors over a narrow range of concentration (Fedorov et al., 2009). This

effect was not reproducible in the present study using nAChR expressed in *Xenopus* oocytes, and we are at a loss to explain this discrepancy. It is possible, but unlikely, that S-Mec might have selectively affected the trafficking of the TC-2559-sensitive (putative HS α4β2) receptors in the host cells. It is also possible, but unprecedented, that differences in post-translational modification of the HS receptors in the mammalian cells, compared to those expressed in oocytes, were responsible for allowing the HS receptors to be sensitive to a unique effect of S-Mec. It would be of interest to determine if the effect reported by Fedorov et al. could be observed in some other preparation of native α4β2 receptors, perhaps utilizing TC-2559 to selectively activate HS receptors, as was done with the SH-EP1 cells.

5 Conclusions

5.1

In the present study, we have extended our knowledge of the in vitro activity (selectivity/potency) of mecamylamine for previously unstudied receptors. Our data suggest that mecamylamine will more effectively produce acute inhibition of LS forms of α4* and α2* receptors than the HS forms, which have been suggested to increase in abundance with chronic nicotine use. However, our analyses of net charge inhibition suggest that under chronic conditions there will be roughly equivalent inhibition of all common nAChR subtypes in brain, aside from α7.

5.2

Our current in vitro data also suggest that there is very little to differentiate the pharmacological profiles of the mecamylamine stereoisomers, although there may be a potency difference for in vivo applications. The potency difference suggested by our data would be consistent with the previous report of lower LD_{50}s for R-Mec relative to S-Mec. However, a significant and modest difference of potency was only found in the tail-flick test. While the nicotinic responses we measured in our in vivo testing are in part mediated by α4β2* subtypes, non-α4β2* subtypes play a more important role in the tail-flick test compared to hot-plate and body temperature effects (Jackson et al., 2010; Marubio et al., 1999). The reason for this potency difference is unclear. However, it is interesting to speculate that if there is any facilitated transport of the drug into the brain, the mediator of that transport may provide a more stereo-selective site than the receptor ion channels.

5.3

The future of mecamylamine in therapeutics will doubtless be impacted by the failure of the recent trials (Lindsley, 2010) that utilized the S-isomer of mecamylamine (TC-5214). TC-5214 was negative in four trials which were run in two different ways. Whether those were failures of the drug itself or of the trial designs has been discussed in detail elsewhere (Papke and Picciotto, 2012). Perhaps the mecamylamine isomer might have had efficacy as a mono-therapy or would have been better paired with a different primary therapy with cholinergic side effects. Perhaps, because of its putative potentiating activity, S-Mec was the wrong choice for a drug intended to decrease hypercholinergic function. In any case, it seems unlikely that mecamylamine will encourage further investments in large-scale trials unless future studies uncover other novel unique properties of one or the other of the

stereoisomers. The challenge therefore now exists to look at the properties of mecamylamine, as described in this work and by others, and ask whether the drug's profile is really optimized for the desired CNS targets. Our work suggests that the trend toward β4-containing receptor selectivity is something that might be best engineered out of an optimized drug. Not only are β4-containing nAChR in the autonomic nervous system likely to be off-target receptors, but recent work has indicated that the activity of β4-containing receptors in the medial habenula is important for limiting nicotine selfadministration and modulation of mood (Frahm et al., 2011; Shytle et al., 2011).

5.4

It might be possible to optimize a new family of drugs, beginning with the scaffold of an alternative non-competitive antagonist, as is being done with bupropion analogs (Lukas et al., 2010). Alternatively, many novel partial agonists are being developed which show a high degree of selectivity for specific brain nAChR subtypes, making it seem more likely that the future of nicotinic cholinergic therapeutics will come from designing well-tuned partial agonists that improve on current drugs such as varenicline (Benowitz, 2009; Coe et al., 2005; Rollema et al., 2010) or other cytisine derivatives (Mineur et al., 2009).

Acknowledgments

We thank Targacept for supplying TC-2559 and Drs. R. Douglas Shytle and Ron Lukas for their comments. We also thank Drs. Jon Lindstrom and Edwin Johnson for supplying nAChR subunit DNA clones and concatamers. We thank Shehd A. A. Al Rubaiy, Lynda Cortes, Sara Copeland, Matthew R. Kimbrell, Matthew Isaacson, Lu W. Corrie, and Thomas F. Pack for conducting the OpusXpress experiments. We thank Dr. Lynn Wecker (University of South Florida) for the use of an additional OpusXpress 6000 and Tie-Shan Han for his technical support on the in vivo experiments. This work was supported by grants from the National Institute of Health [GM57481, DA12001 and DA019377] and James and Esther King Biomedical Research foundation [KG12].

References

Benowitz NL. Pharmacology of nicotine: addiction, smoking-induced disease, and therapeutics. Annu Rev Pharmacol Toxicol. 2009; 49:57–71. [PubMed: 18834313]

Chini B, Clementi F, Hukovic N, Sher E. Neuronal-type alpha-bungarotoxin receptors and the alpha 5-nicotinic receptor subunit gene are expressed in neuronal and nonneuronal human cell lines. Proc. Natl. Acad. Sci. USA. 1992; 89:1572–1576. [PubMed: 1542648]

Coe JW, Brooks PR, Vetelino MG, Wirtz MC, Arnold EP, Huang J, Sands SB, Davis TI, Lebel LA, Fox CB, Shrikhande A, Heym JH, Schaeffer E, Rollema H, Lu Y, Mansbach RS, Chambers LK, Rovetti CC, Schulz DW, Tingley FD 3rd, O'Neill BT. Varenicline: an alpha4beta2 nicotinic receptor partial agonist for smoking cessation. J Med Chem. 2005; 48:3474–3477. [PubMed: 15887955]

Damaj MI, Glassco W, Dukat M, Martin BR. Pharmacological characterization of nicotineinduced seizures in mice. J Pharmacol Exp Ther. 1999; 291:1284–1291. [PubMed: 10565853]

Damaj MI, Welch SP, Martin BR. Involvement of calcium and L-type channels in nicotineinduced antinociception. J. Pharmcol. Exp. Ther. 1993; 266:1330–1338.

Damaj MI, Welch SP, Martin BR. In vivo pharmacological effects of dihydro-betaerythroidine, a nicotinic antagonist, in mice. Psychopharmacology (Berl). 1995; 117:67–73. [PubMed: 7724704]

D'Amour FE, Smith DL. A method for determining loss of pain sensation . J. P. E. T. 1941; 72:74–79.

Dennis EW, Ford RV, Moyer JH, Hershberger RL. Mecamylamine in the treatment of hypertension. J Med Assoc Ga. 1957; 46:427–430. [PubMed: 13463512]

Fedorov NB, Benson LC, Graef J, Lippiello PM, Bencherif M. Differential pharmacologies of mecamylamine enantiomers: positive allosteric modulation and noncompetitive inhibition. J Pharmacol Exp Ther. 2009; 328:525–532. [PubMed: 18957576]

Fowler CD, Lu Q, Johnson PM, Marks MJ, Kenny PJ. Habenular alpha5 nicotinic receptor subunit signalling controls nicotine intake. Nature. 2011; 471:597–601. [PubMed: 21278726]

Frahm S, Slimak MA, Ferrarese L, Santos-Torres J, Antolin-Fontes B, Auer S, Filkin S, Pons S, Fontaine JF, Tsetlin V, Maskos U, Ibanez-Tallon I. Aversion to nicotine is regulated by the balanced activity of beta4 and alpha5 nicotinic receptor subunits in the medial habenula. Neuron. 2011; 70:522–535. [PubMed: 21555077]

Gotti C, Clementi F, Fornari A, Gaimarri A, Guiducci S, Manfredi I, Moretti M, Pedrazzi P, Pucci L, Zoli M. Structural and functional diversity of native brain neuronal nicotinic receptors. Biochem Pharmacol. 2009; 78:703–711. [PubMed: 19481063]

Gurley, DA.; Robbins, AK.; Gunyuzlu, PL.; Moore, WC.; Johnson, EC. Pharmacological properties of nicotinic agonists at the two stoichiometries of the alpha4beta2 and alpha2beta2 receptors, Nicotinic Acetylcholine Receptors. Hinxton, UK: Wellcome Trust; 2008.

Han ZY, Le Novere N, Zoli M, Hill JA Jr, Champtiaux N, Changeux JP. Localization of nAChR subunit mRNAs in the brain of Macaca mulatta. Eur J Neurosci. 2000; 12:3664–3674. [PubMed: 11029636]

Han ZY, Zoli M, Cardona A, Bourgeois JP, Changeux JP, Le Novere N. Localization of [3H]nicotine, [3H]cytisine, [3H]epibatidine, and [125I]alpha-bungarotoxin binding sites in the brain of Macaca mulatta. J Comp Neurol. 2003; 461:49–60. [PubMed: 12722104]

Harpsoe K, Ahring PK, Christensen JK, Jensen ML, Peters D, Balle T. Unraveling the high- and low-sensitivity agonist responses of nicotinic acetylcholine receptors. J Neurosci. 2011; 31:10759–10766. [PubMed: 21795528]

Jackson KJ, Marks MJ, Vann RE, Chen X, Gamage TF, Warner JA, Damaj MI. Role of alpha5 nicotinic acetylcholine receptors in pharmacological and behavioral effects of nicotine in mice. J Pharmacol Exp Ther. 2010; 334:137–146. [PubMed: 20400469]

Jackson KJ, Sanjakdar SS, Muldoon PP, McIntosh JM, Damaj MI. The alpha3beta4* nicotinic acetylcholine receptor subtype mediates nicotine reward and physical nicotine withdrawal signs independently of the alpha5 subunit in the mouse. Neuropharmacology. 2013; 70:228–235. [PubMed: 23416040]

Kuryatov A, Lindstrom J. Expression of functional human alpha6beta2beta3* acetylcholine receptors in Xenopus laevis oocytes achieved through subunit chimeras and concatamers. Mol Pharmacol. 2011; 79:126–140. [PubMed: 20923852]

Kuryatov A, Onksen J, Lindstrom J. Roles of accessory subunits in alpha4beta2(*) nicotinic receptors. Mol Pharmacol. 2008; 74:132–143. [PubMed: 18381563]

Lindsley CW. (S)-(+)-Mecamylamine (TC-5214): A Neuronal Nicotinic Receptor Modulator Enters Phase III Trials as an Adjunct Treatment for Major Depressive Disorder (MDD). ACS chemical neuroscience. 2010; 1:530–531. [PubMed: 22778844]

Lukas RJ, Muresan AZ, Damaj MI, Blough BE, Huang X, Navarro HA, Mascarella SW, Eaton JB, Marxer-Miller SK, Carroll FI. Synthesis and characterization of in vitro and in vivo profiles of hydroxybupropion analogues: aids to smoking cessation. J Med Chem. 2010; 53:4731–4748. [PubMed: 20509659]

Marubio LM, del Mar Arroyo-Jimenez M, Cordero-Erausquin M, Lena C, Le Novere N, de Kerchove d'Exaerde A, Huchet M, Damaj MI, Changeux JP. Reduced antinociception in mice lacking neuronal nicotinic receptor subunits. Nature. 1999; 398:805–810. [PubMed: 10235262]

Millar NS, Gotti C. Diversity of vertebrate nicotinic acetylcholine receptors. Neuropharmacology. 2009; 56:237–246. [PubMed: 18723036]

Miller C. Genetic manipulation of ion channels: a new approach to structure and mechanism. Neuron. 1989; 2:1195–1205. [PubMed: 2483110]

Mineur YS, Eibl C, Young G, Kochevar C, Papke RL, Gundisch D, Picciotto MR. Cytisine-based nicotinic partial agonists as novel antidepressant compounds. J Pharmacol Exp Ther. 2009; 329:377–386. [PubMed: 19164465]

Papke RL. Tricks of Perspective: Insights and limitations to the study of macroscopic currents for the analysis of nAChR activation and desensitization. Journal of Molecular Neuroscience. 2010; 40:77–86. [PubMed: 19672724]

Papke RL, Dwoskin LP, Crooks PA. The pharmacological activity of nicotine and nornicotine on nAChRs subtypes: relevance to nicotine dependence and drug discovery. J Neurochem. 2007; 101:160–167. [PubMed: 17241116]

Papke RL, Dwoskin LP, Crooks PA, Zheng G, Zhang Z, McIntosh JM, Stokes C. Extending the analysis of nicotinic receptor antagonists with the study of alpha6 nicotinic receptor subunit chimeras. Neuropharmacology. 2008; 54:1189–1200. [PubMed: 18448138]

Papke RL, Ono F, Stokes C, Urban JM, Boyd RT. The nicotinic acetylcholine receptors of zebrafish and an evaluation of pharmacological tools used for their study. Biochem Pharmacol. 2012; 84:352–365. [PubMed: 22580045]

Papke RL, Picciotto MR. Nicotine Dependence and Depression, What is the Future for Therapeutics? Journal of Addiction Research and Therapy. 2012; 2 1000e1105.

Papke RL, Sanberg PR, Shytle RD. Analysis of mecamylamine stereoisomers on human nicotinic receptor subtypes. J Pharmacol Exp Ther. 2001; 297:646–656. [PubMed: 11303054]

Papke RL, Wecker L, Stitzel JA. Activation and inhibition of mouse muscle and neuronal nicotinic acetylcholine receptors expressed in Xenopus oocytes. J Pharmacol Exp Ther. 2010; 333:501–518. [PubMed: 20100906]

Papke, RL.; Zheng, G.; Crooks, PA.; Dwoskin, LP. Novel trivalent antagonists with selectivity for alpha_ nicotinic acetylcholine receptors. 68th Annual Meeting College on Problems of Drug Dependence; Scottsdale, AZ. 2006.

Rabenstein RL, Caldarone BJ, Picciotto MR. The nicotinic antagonist mecamylamine has antidepressant-like effects in wild-type but not beta2- or alpha7-nicotinic acetylcholine receptor subunit knockout mice. Psychopharmacology (Berl). 2006; 189:395–401. [PubMed: 17016705]

Rollema H, Shrikhande A, Ward KM, Tingley FD 3rd, Coe JW, O'Neill BT, Tseng E, Wang EQ, Mather RJ, Hurst RS, Williams KE, de Vries M, Cremers T, Bertrand S, Bertrand D. Pre-clinical properties of the alpha4beta2 nicotinic acetylcholine receptor partial agonists varenicline, cytisine and dianicline translate to clinical efficacy for nicotine dependence. Br J Pharmacol. 2010; 160:334–345. [PubMed: 20331614]

Rose JE, Behm FM, Westman EC. Nicotine-mecamylamine treatment for smoking cessation: the role of pre- cessation therapy. Exp. Clin. Psychopharmacol. 1998; 6:331–343. [PubMed: 9725117]

Sanberg PR, Shytle RD, Silver AA. Treatment of Tourette’s syndrome with mecamylamine. Lancet. 1998; 352:705–706. [PubMed: 9728992]

Shytle, R.; Sheehan, D.; Sanberg, PR.; Arias, HR. Neuronal nicotinic receptors as therapeutic targets for mood disorders. In: Arias, HR., editor. Pharmacology of Nicotinic Acetylcholine Receptors from the Basic and Therapeutic Perspectives. India: Research Signpost; 2011. p. 187-198.

Silver, AA.; Shytle, RD.; Sheehan, DV.; Sheehan, KH.; Ramos, A.; Sanberg, PR. Safety and exploratory study of mecamylamine monotherapy for Tourette's syndrome. Neuropharmacology: Proc. Neur.nAChR Symposium; Nov. 2–4; New Orleans. 2000.

Silver AA, Shytle RD, Sheehan KH, Sheehan DV, Ramos A, Sanberg PR. Multicenter, double-blind, placebo-controlled study of mecamylamine monotherapy for Tourette's disorder. Journal of the American Academy of Child and Adolescent Psychiatry. 2001; 40:1103–1110. [PubMed: 11556635]

Singh A, Das DK, Kelley ME. Mecamylamine (Targacept). IDrugs. 2006; 9:205–217. [PubMed: 16523387]

Stokes C, Papke RL. Use of an alpha3-beta4 nicotinic acetylcholine receptor subunit concatamer to characterize ganglionic receptor subtypes with specific subunit composition reveals species-specific pharmacologic properties. Neuropharm. 2012; 63:538–546.

Suchocki JA, May EL, Martin TJ, George C, Martin BR. Synthesis of 2-exo- and 2-endomecamylamine analogues. Structure-activity relationships for nicotinic antagonism in the central nervous system. J Med Chem. 1991; 34:1003–1010. [PubMed: 2002445]

Tallarida, RJ.; Murray, RB. Manual of Pharmacological Calculations with Computer Programs. New York: Springer-Verlag; 1987.

Timmermann D, Sandager-Nielsen K, Dyhring T, Smith M, Jacobsen AM, Nielsen E, Grunnet M, Christensen J, Peters D, Kohlhaas K, Olsen G, Ahring P. Augmentation of cognitive function by NS9283, a stoichiometry-dependent positive allosteric modulator of alpha2- and alpha4-containing nicotinic acetylcholine receptors. Br J Pharmacol. 2012; 167:164–182. [PubMed: 22506660]

Wada E, Wada K, Boulter J, Deneris E, Heinemann S, Patrick J, Swanson LW. Distribution of alpha2, alpha3, alpha4, and beta2 neuronal nicotinic receptor subunit mRNAs in the central nervous system: a hybridization histochemical study in the rat. J. Comp. Neurol. 1989; 284:314–335. [PubMed: 2754038]

Webster JC, Francis MM, Porter JK, Robinson G, Stokes C, Horenstein B, Papke RL. Antagonist activities of mecamylamine and nicotine show reciprocal dependence on beta subunit sequence in the second transmembrane domain. Br. J. Pharmacol. 1999; 127:1337–1348. [PubMed: 10455283]

Zhou Y, Nelson ME, Kuryatov A, Choi C, Cooper J, Lindstrom J. Human alpha4beta2 acetylcholine receptors formed from linked subunits. J Neurosci. 2003; 23:9004–9015. [PubMed: 14534234]

Zwart R, Broad LM, Xi Q, Lee M, Moroni M, Bermudez I, Sher E. 5-I A-85380 and TC-2559 differentially activate heterologously expressed alpha4beta2 nicotinic receptors. Eur J Pharmacol. 2006; 539:10–17. [PubMed: 16674940]

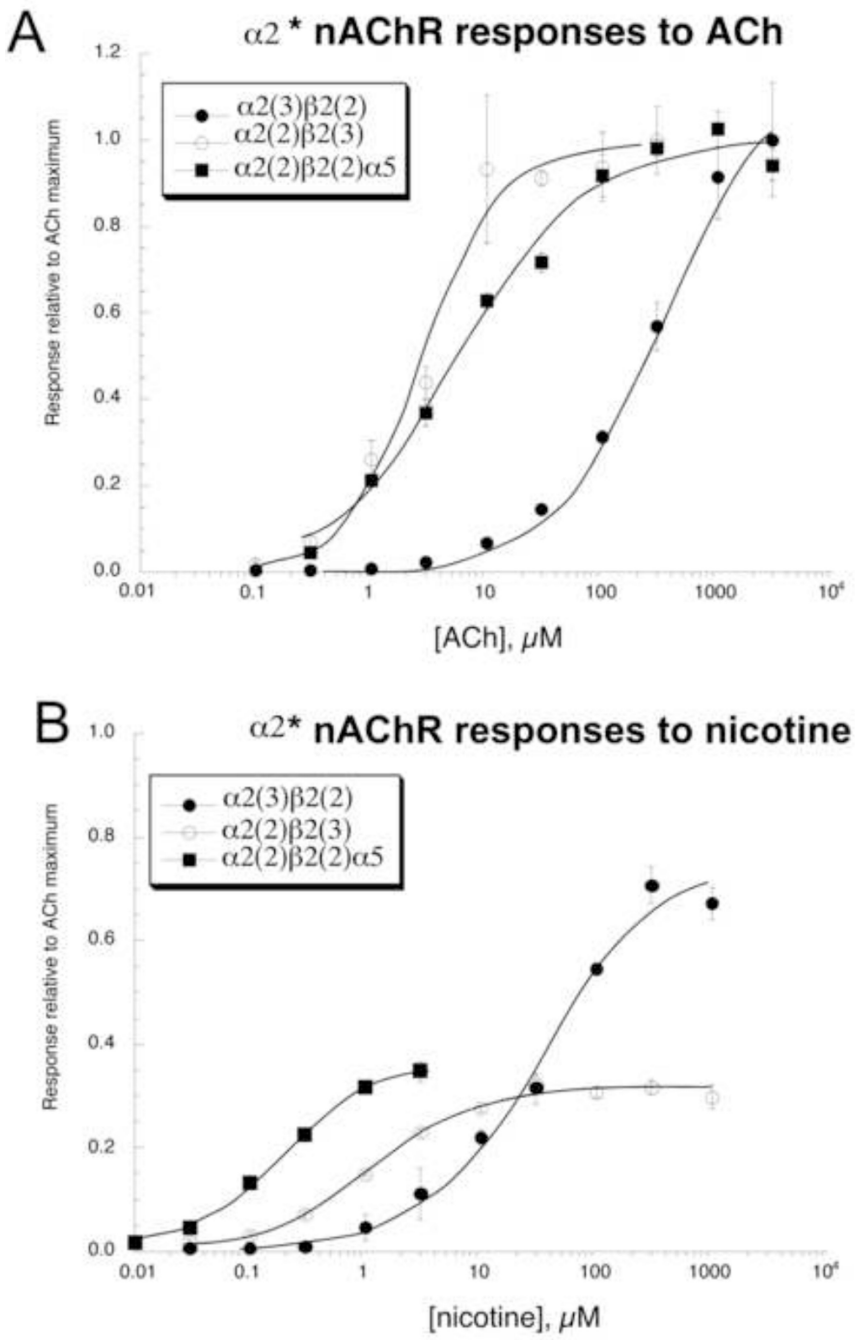

Fig. 1.

Characterization of the agonist-evoked responses of α2-containing nAChR with defined subunit composition. **A)** Responses to ACh. **B)** Responses to nicotine. The high (HS) and low (LS) sensitivity form of human α2β2 nAChR were generated by co-expressing the β2−6−α2 concatamer with monomeric β2 or α2 subunits, respectively. Receptors with the composition of α2(2)β2(2)α5 were formed by co-expressing the β2−6−α2 concatamer with monomeric α5. All data were normalized to ACh control responses obtained in the same oocytes and scaled relative to the ACh controls and the ACh maximum (see Methods). Data are the averages (± S.E.M.) of responses from at least 4 oocytes at each point.

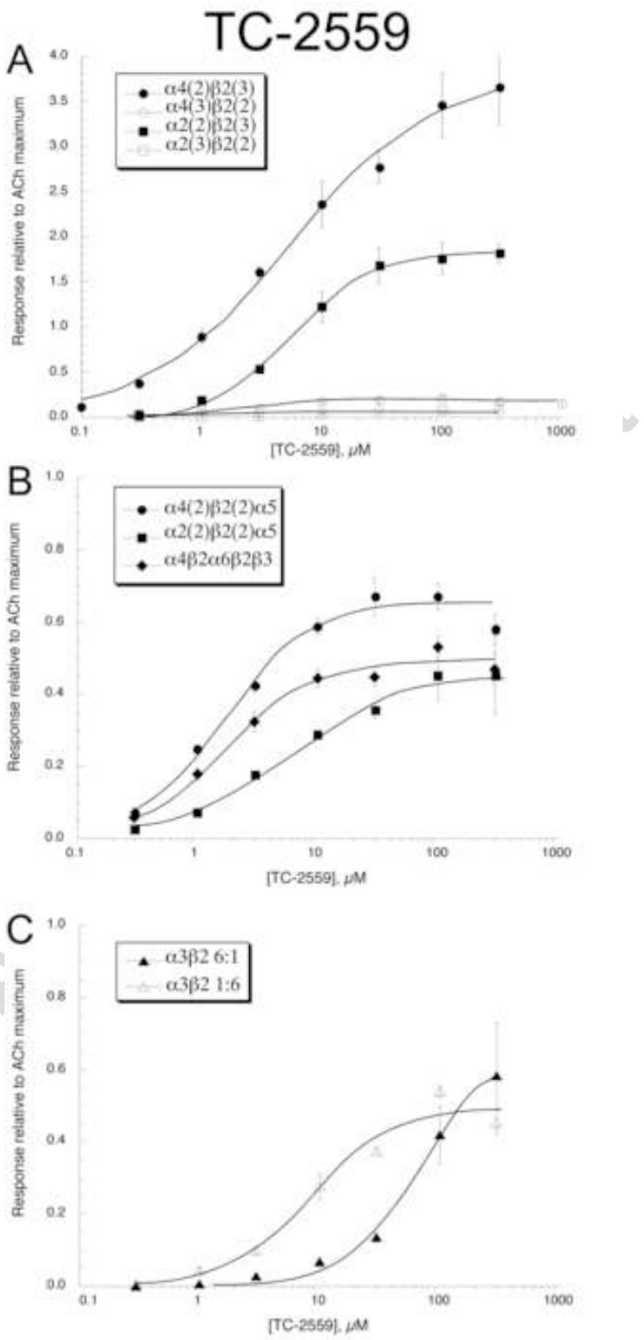

Fig. 2.

Evaluation of TC-2559 as a selective activator of HS α4β2 nAChR subtypes. **A)** TC-2559-evoked responses of receptors formed with the co-expression of the β2−6−α4 or β2−6−α2 concatamers with monomeric β2 (to form HS receptors) or alpha subunits (to form LS receptors). **B)** TC-2559-evoked responses of receptors formed with the co-expression of the β2−6−α4 or β2−6−α2 concatamers with monomeric α5 subunits, or formed with the expression of α4β2α6β2β3 concatamer. **C)** TC-2559-evoked responses of receptors formed with the co-expression of α3 and β2 subunits with RNA injections at either 6:1 or 1:6 ratios. All data were normalized to ACh control responses obtained in the same oocytes and scaled relative to the ACh controls and the ACh maximum (see methods). Data are the averages (± S.E.M.) of responses from at least 4 oocytes at each point.

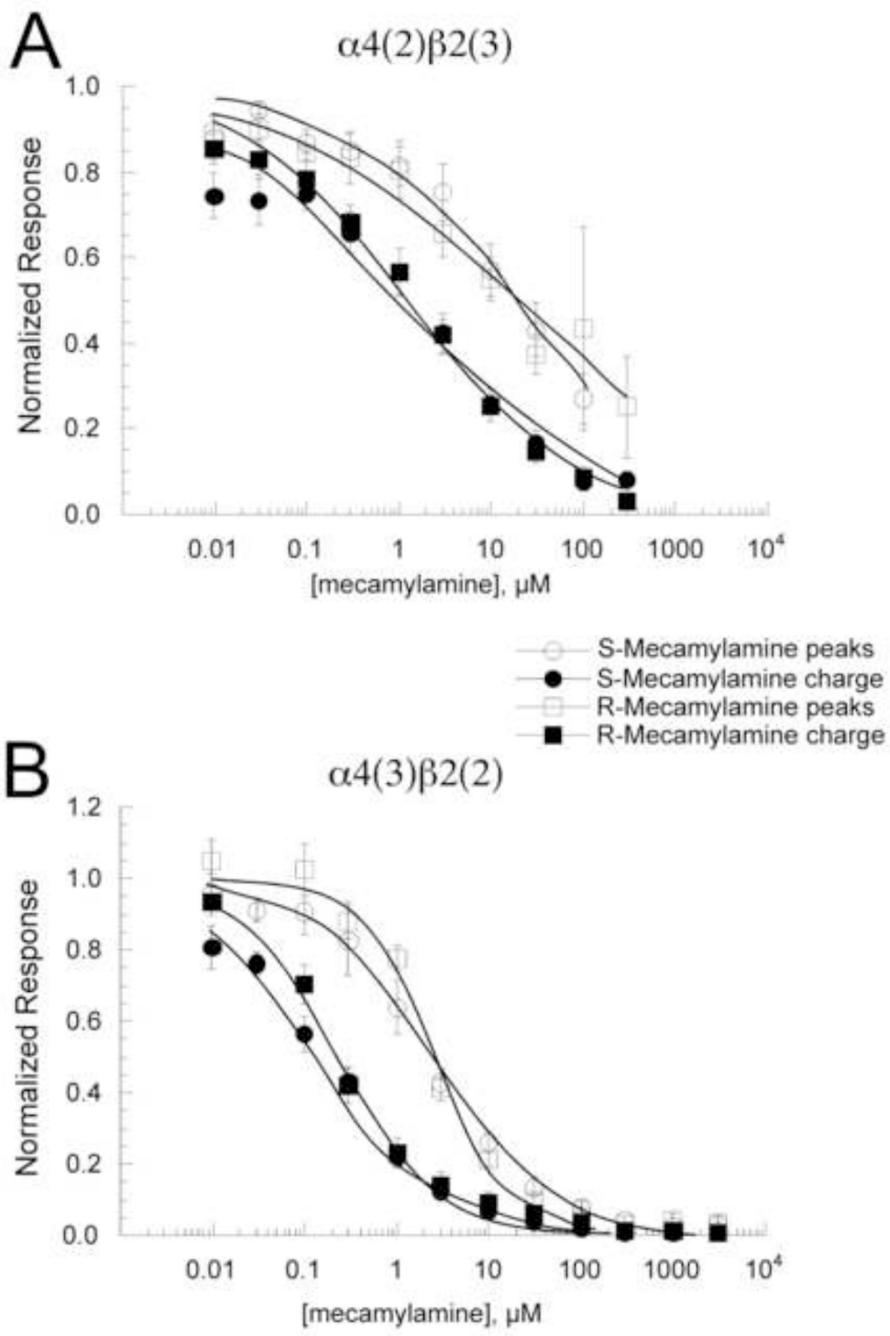

Fig. 3.

Inhibition of wild-type HS and LS α4β2 receptors by S- and R-mecamylamine. **A**) The high sensitivity (HS) form of human α4β2 nAChR was generated by co-expressing the β2−6−α4 concatamer with monomeric β2 subunits. The mecamylamine stereoisomers were then tested for their ability to inhibit the peak currents and net charge evoked by co-application with 10 μM ACh. **B**) The low sensitivity (LS) form of human α4β2 nAChR was generated by co-expressing the β2−6−α4 concatamer with monomeric α4 subunits. The mecamylamine stereoisomers were then tested for their ability to inhibit the peak currents and net charge evoked by co-application with 100 μM ACh. The data plotted are the averages of at least 4 oocytes (± S.E.M.) for each condition.

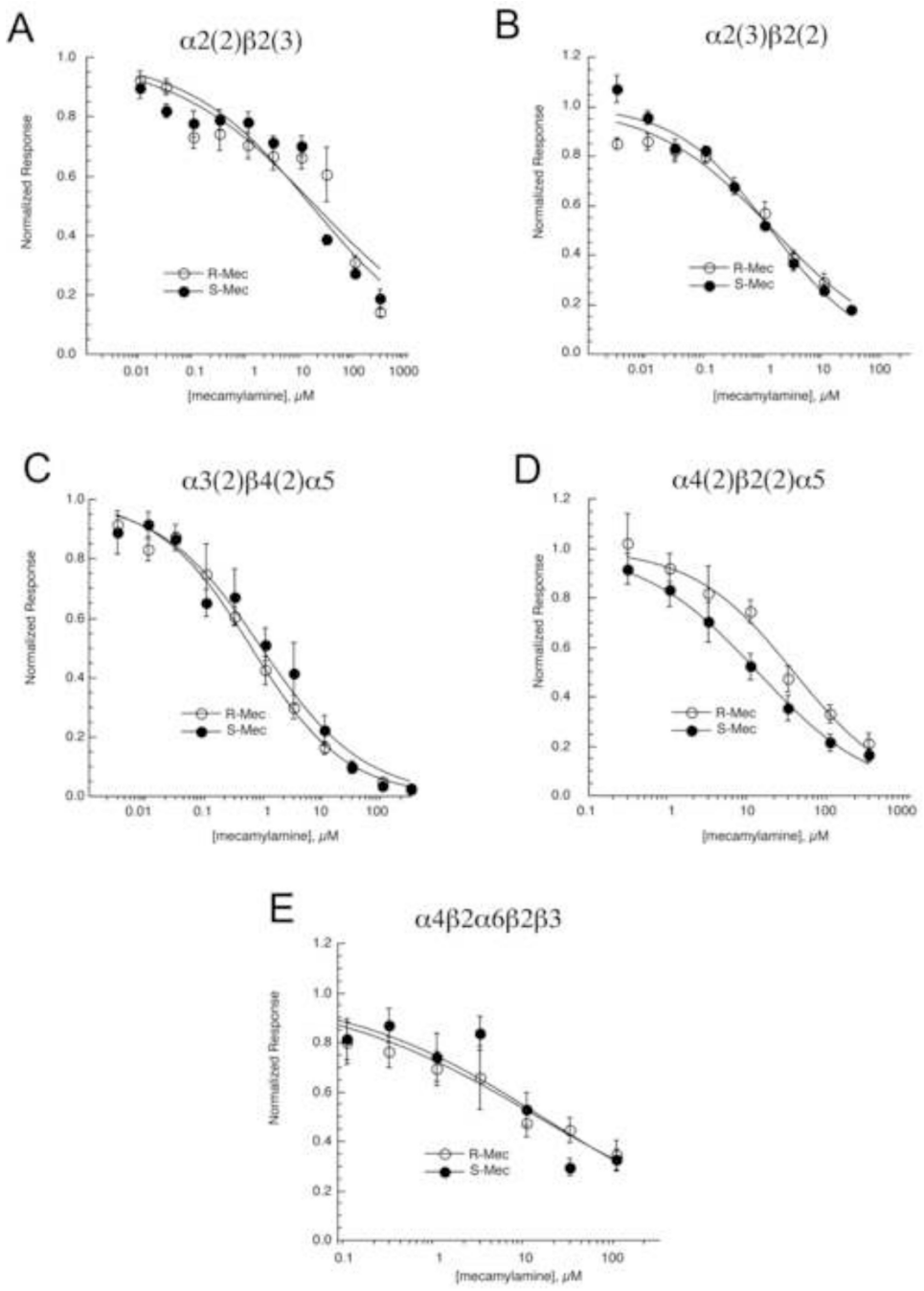

Fig. 4.

Inhibition of additional nAChR subtypes by S- and R-mecamylamine. **A**) The HS form of human α2β2 nAChR was generated by co-expressing the β2−6−α2 concatamer with monomeric β2 subunits. The mecamylamine stereoisomers were then tested for their ability to inhibit the peak currents evoked by co-application with 10 μM ACh. **B**) The LS form of human α2β2 nAChR was generated by co-expressing the β2−6−α2 concatamer with monomeric α2 subunits. The mecamylamine stereoisomers were tested for their ability to inhibit the peak currents evoked by co-application with 100 μM ACh. **C**). Inhibition of α3(2)β4(2)α5 receptor peak current responses by mecamylamine stereoisomers. **D**) Inhibition of α4(2)β2(2)α5 receptor peak current responses by mecamylamine stereoisomers. **E**) Inhibition of α4α6β2(2)β3 receptor peak current responses by mecamylamine

stereoisomers. The IC_{50} values for these experiments are given in Table 3, along with the IC_{50} values for inhibition of net charge (net charge data not plotted). The data plotted are the averages of at least 4 oocytes (± S.E.M.) for each condition.

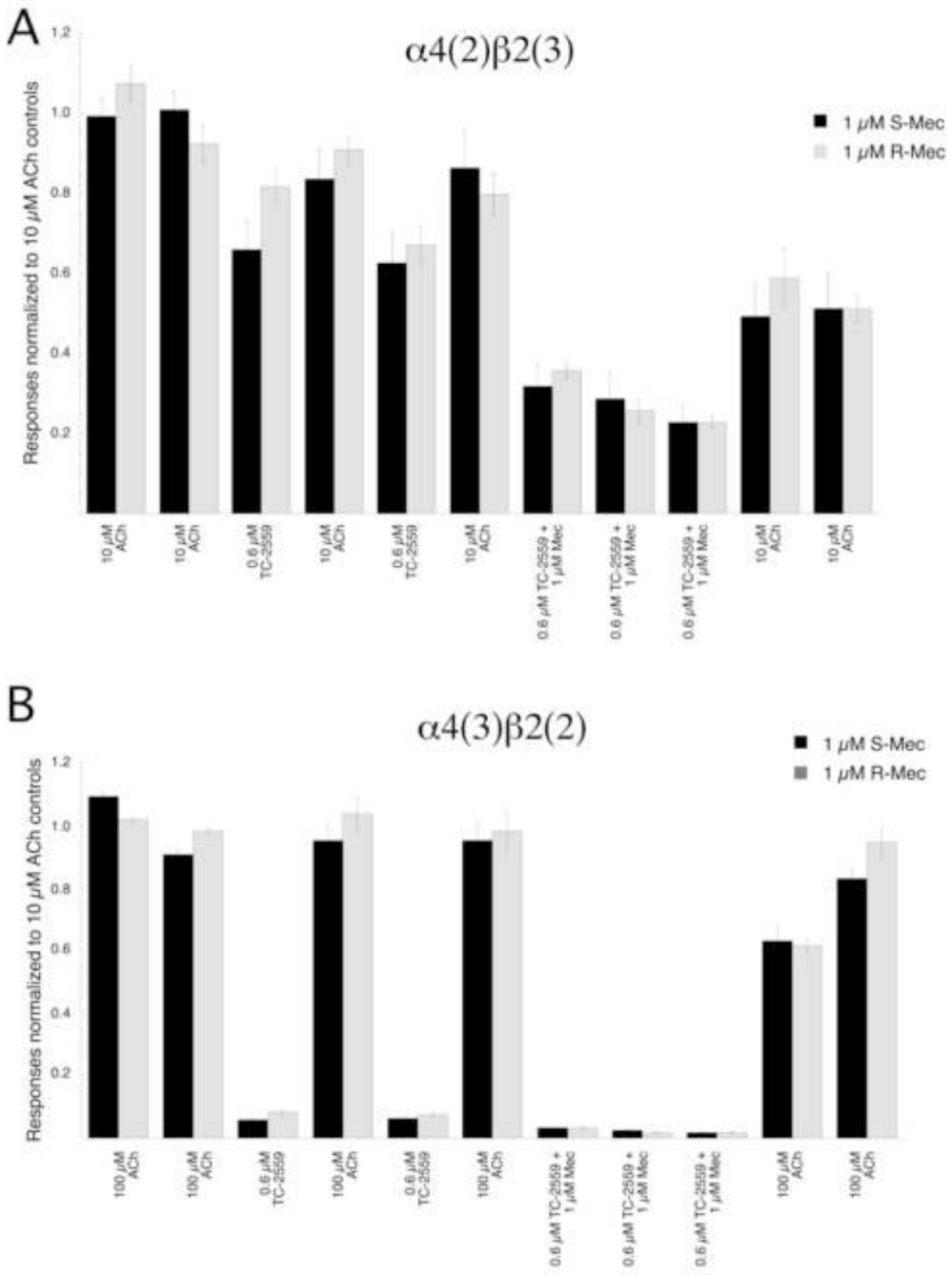

Fig. 5.

Evaluation of the inhibition TC-2559-evoked responses of HS (**A**) and LS (**B**) α4β2 nAChR with the two stereoisomers of mecamylamine. The columns represent sequential responses to a sequence of drug applications and co-applications designed to emulate the protocol reported by Fedorov et al. (Fedorov et al., 2009) to reveal the selective potentiation of HS α4β2 responses by S-Mec (TC-5214).

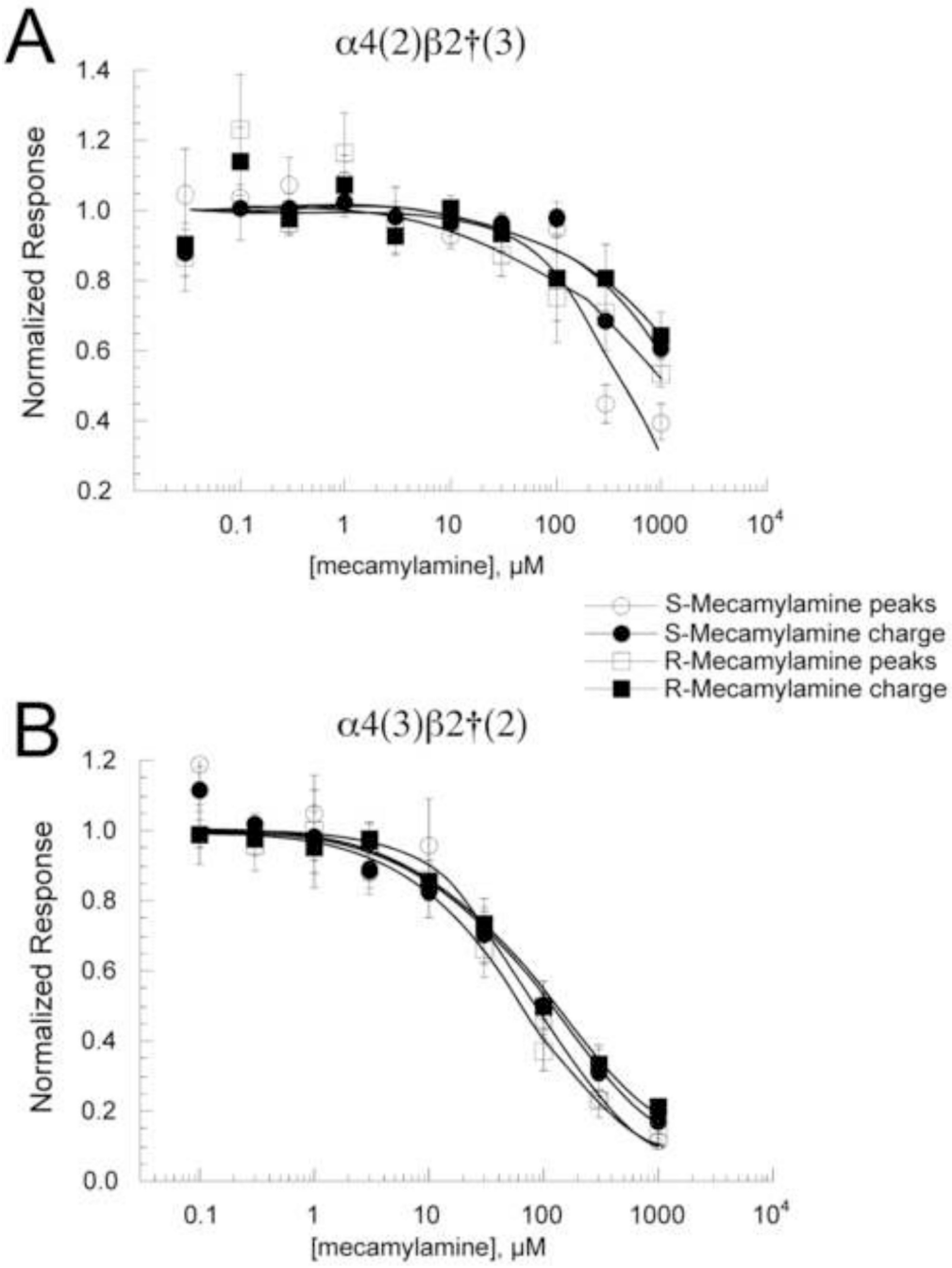

Fig. 6.

The mecamylamine stereoisomers were tested for their ability to inhibit the peak currents and net charge of mutant α4β2 nAChR evoked by co-application with 100 μM ACh. **A**) Mutations of the 6' and 10' positions in the TM2 (Webster et al. 1999) were made in the β2 element of the β2−6−α4 concatamer (β2†-6-α4), which was co-expressed with the 6'-10' mutant form of β2 (β2†) to generate a homolog of HS α4β2 receptors with a greatly reduced sensitivity to inhibition by mecamylamine. The mecamylamine stereoisomers were then tested for their ability to inhibit the peak currents and net charge evoked by co-application with 10 μM ACh. **B**) Mutations of the 6' and 10' positions in the TM2 (Webster et al. 1999) were made in the β2

element of the β2†−6−α4 concatamer, which was co-expressed with wild-type α4 to generate a homolog of LS α4β2 receptors with greatly reduced sensitivity to inhibition by mecamylamine.

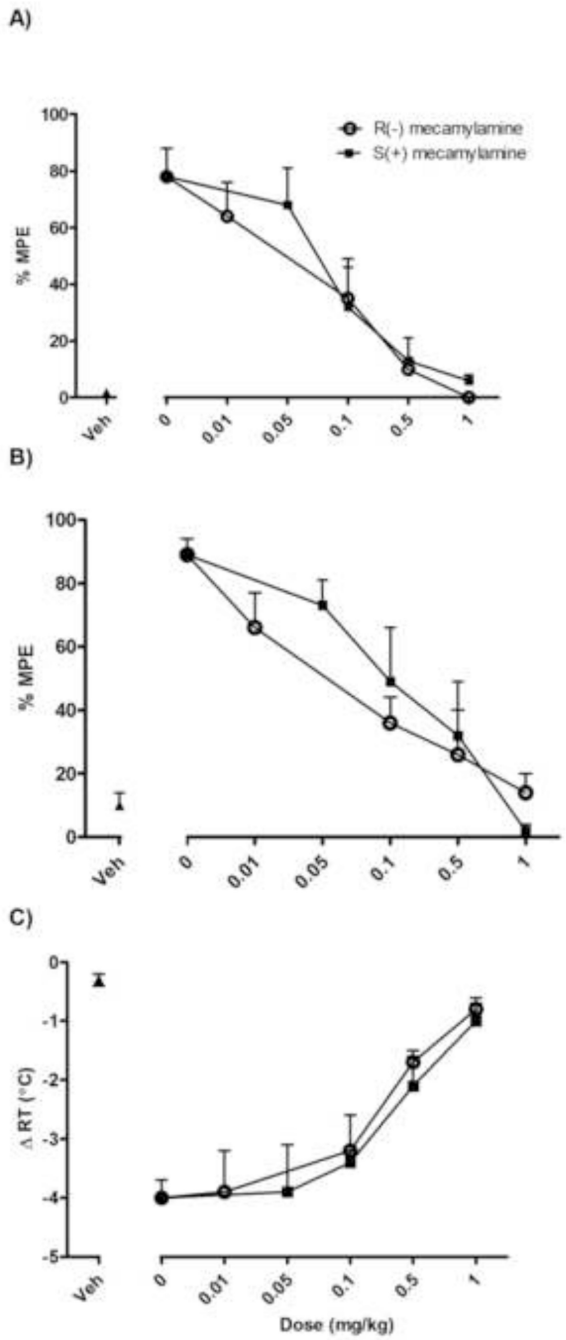

Fig. 7.

Blockade of nicotine's effects (2.5mg/kg s.c.) in the **(A)** hot-plate test, **(B)** tail-flick test and **(C)** body temperature by R(-) mecamylamine and S(+) mecamylamine. Antagonists were administered s.c. 10 min before nicotine-induced antinociception in the tail-flick and hot-plate tests and hypothermia. Mice were tested 5 min after nicotine injection for antinociception and 30 min for hypothermia. Each point represents the mean ± S.E.M. of 6 mice.

Table 1

ACh and nicotine activation of HS and LS forms of α4β2 [a] and α2β2 nAChR

Receptor	ACh		Nicotine	
	EC_{50}	I_{max}	EC_{50}	I_{max}
α4(2)β2(3)	1.5 ± 0.3*	1.0	0.14 ± 0.04	0.26 ± 0.01
α4(3)β2(2)	155 ± 23*	1.0	22.6 ± 9.6	0.54 ± 0.06
α4(2)β2(2)α5	1.5 ± 0.4*	1.0	0.21 ± 0.02	0.37 ± 0.01
α2(2)β2(3)	2.7 ± 0.5	1.0	1.1 ± 0.1	0.31 ± 0.01
α2(3)β2(2)	270 ± 37	1.0	33 ± 9	0.75 ± 0.05
α2(2)β2(2)α5	6.0 ± 1.0	1.0	1.6 ± 0.2	0.58 ± 0.01

EC_{50} values are in μM.

[a] ACh data for α4β2* receptors taken from Papke et al. JPET, 2010.

Table 2

TC-2559 activity

	EC_{50}	I_{max} [a]
α4(2)β2(3)	5.6 ± 1.0 μM	3.8 ± 0.2
α4(3)β2(2)	1.6 ± 0.6 μM	0.18 ± 0.2
α2(2)β2(3)	5.7 ± 0.2 μM	1.8 ± 0.1
α2(3)β2(2)	24 ± 10 μM	0.09 ± 0.01
α4(2)β2(2)α5	1.6 ± 0.3 μM	0.65 ± 0.1
α2(2)β2(2)α5	6.2 ± 1.0 μM	0.47 ± 0.02
α4β2α6β2β3	1.7 ± 0.3 μM	0.49 ± 0.02
α3β2 (1:6)	0.50 ± 0.04	9.1 ± 2.6
α3β2 (6:1)	0.67 ± 0.05	72 ± 13

[a] relative to ACh maximum

Table 3

Effects of mecamylamine stereoisomers on nAChR

IC_{50}s μM	Peak current		Net charge	
Receptor	**R(-)Mecamylamine**	**S(+)Mecamylamine**	**R(-)Mecamylamine**	**S(+)Mecamylamine**
α3(2)β4(2)α5	0.60 ± 0.07	0.89 ± 0.18	0.64 ± 0.18	0.67 ± 0.19
α4(2)β2(3)	19 ± 5	17 ± 4	1.3 ± 0.2	1.0 ± 0.3
α4(3)β2(2)	2.6 ± 0.3	2.2 ± 0.2	0.24 ± 0.02	0.16 ± 0.01
α2(2)β2(3)	19 ± 9	15 ± 8	0.30 ± 0.18	0.90 ± 0.64
α2(3)β2(2)	1.4 ± 0.3	1.3 ± 0.2	0.44 ± 0.08	0.23 ± 0.03
α4(2)β2(2)α5	35 ± 5	12 ± 1	0.55 ± 0.06	0.37 ± 0.05
α4β2α6β2β3	13 ± 5	14 ± 7	0.40 ± 0.06	0.14 ± 0.02
α4(2)β2(3) [a]	1130 ± 810	450 ± 120	2800 ± 2300	1500 ± 570
(IC_{50} ratio)	(59)	(26)	(2200)	(1500)
α4(3)β2(2) [a]	100 ± 16	88 ± 21	117 ± 9	66 ± 6
(IC_{50} ratio)	(38)	(40)	(487)	(412)

[a] β2 mutants containing 6' and 10' mutation to the sequence of the muscle β1 subunit.

Table 4

Summary of the potency of mecamylamine stereoisomers in blocking nicotine's effects in the tail-flick, hot plate, and body temperature tests after acute nicotine administration (2.5 mg/kg, s.c.) in male ICR mice. Potency is expressed as AD_{50} ± confidence limits (mg/kg). Each group contained 6–8 mice.

Test	*R(-)Mecamylamine*	*S(+)Mecamylamine*
Tail flick	0.03 (0.002–0.05)	0.08 (0.06–0.10) [a]
Hot Plate	0.07 (0.03–0.20)	0.15 (0.05–0.72)
Body Temperature	0.48 (0.20–0.60)	0.61 (0.06–0.90)

[a] denotes significance vs. the opposite stereoisomer.