

# **The subtype-selective nicotinic acetylcholine receptor positive allosteric potentiator 2087101 differentially facilitates neurotransmission in the brain**

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## Abstract

Positive allosteric modulators of centrally expressed nicotinic acetylcholine receptors have therapeutic potentials in areas of cognition, motor function and reward. Several chemical classes of allosteric modulators that are selective for  $\alpha 7$  nicotinic receptors have been characterised, but potentiators for the most widely expressed  $\alpha 4\beta 2$  nicotinic receptor subtype are few and less defined, owing probably to the difficulty to achieve selectivity over other heteromeric receptor subtypes. 2087101 ((2-amino-5-keto)thiazole) is a potent potentiator of both  $\alpha 7$  and  $\alpha 4\beta 2$  receptors and it has selectivity against the  $\alpha 3\beta 4$  subtype, which may be responsible for the undesirable peripheral side effects. To further characterise its ability to differentiate between native nicotinic receptors, we examined the effects of 2087101 on  $\alpha 7$ ,  $\alpha 4\beta 2^*$  and  $\alpha 3\beta 4^*$  receptor-mediated responses in the rat brain in electrophysiological and neurochemical experiments. 2087101 significantly potentiated agonist-induced,  $\alpha 7$  and non- $\alpha 7$  receptor-mediated, GABAergic postsynaptic currents in cultured hippocampal neurones, but not the nicotine-stimulated [ $^3\text{H}$ ]noradrenaline release from hippocampal slices, which was primarily mediated by  $\alpha 3\beta 4^*$  receptors, confirming its selectivity for  $\alpha 7$  and  $\alpha 4\beta 2^*$  receptors in native systems. 2087101 also significantly enhanced nicotine-stimulated firing increase in dopamine neurones of the ventral tegmental area, an effect that was dihydro- $\beta$ -erythroidine-sensitive and thereby mediated by  $\alpha 4\beta 2^*$  nicotinic receptors. 2087101 can therefore enhance native nicotinic activities mediated by  $\alpha 7$  and  $\alpha 4\beta 2^*$ , but not  $\alpha 3\beta 4^*$  receptors, showing its unique ability to discriminate between native heteromeric nicotinic receptor subtypes and its therapeutic potential for treating brain disorders by concurrent modulation of both  $\alpha 7$  and  $\alpha 4\beta 2^*$  nicotinic receptors.

**Index words:** positive allosteric modulator, nicotinic acetylcholine receptors, ventral tegmental area, hippocampus, dopamine neuron firing, GABA, noradrenaline release,

## 1. Introduction

Native neuronal nicotinic acetylcholine receptors include the homomeric  $\alpha 7$  receptors and heteromeric  $\alpha 4\beta 2^*$ ,  $\alpha 3\beta 4^*$ ,  $\alpha 6\beta 2^*$  and  $\alpha 4\alpha 6\beta 2^*$  receptors which possibly contain additional subunits (\*) such as  $\alpha 5$ ,  $\alpha 2$  and  $\beta 3$  (Zoli et al., 1998, Grady et al., 2007). The  $\alpha 4\beta 2^*$  and  $\alpha 7$  receptors are the two main subtypes in the brain and their activation can modulate various neuronal processes and functions (Vizi and Kiss, 1998). In particular, loss of nicotinic receptor functions can cause deficits in cognitive, motor and reward functions in the elderly population and particularly in patients suffering from Alzheimer's disease (Perry et al., 2000) and schizophrenia (Freedman et al., 2000). Treatments that elevate levels of endogenous acetylcholine by inhibition of acetylcholinesterase are used clinically to alleviate symptoms of Alzheimer's disease. Recently, positive allosteric modulation has also been proposed as a favoured therapeutic strategy via functional enhancement of nicotinic receptors (Albuquerque et al., 2009).

Positive allosteric modulators induce conformational modifications of receptors at a site away from the orthosteric agonist binding site to enhance receptor function without causing direct receptor activation, inactivation or desensitisation, thus preserving the high temporal and spatial resolutions of endogenous nicotinic transmission (Albuquerque et al., 2009). Recently,  $\alpha 7$ -selective positive modulators such as PNU-120596 (Hurst et al., 2005), compound 6 (Ng et al., 2007) and NS-1738 (Timmermann et al., 2007) have been shown to enhance receptor current, improve cognitive functions and reduce hippocampal gating deficits in rodent experimental models, hence providing validation for the potential therapeutic values of nicotinic positive modulators.

In contrast, few selective modulators have been identified and characterised for the most widely expressed neuronal  $\alpha 4\beta 2^*$  receptors. Although the endogenous steroid 17 $\beta$ -estradiol (Curtis et al., 2002), a *Flustra foliacea* metabolite deformylflustrabromine (Sala et al., 2005), and a number of carbamates and piperidines (Albrecht et al., 2008, Springer et al., 2008) have been discovered, their molecular mechanisms and functional effects on native brain receptors are yet to be characterised. The difficulty to obtain  $\alpha 4\beta 2^*$ -selective compounds is probably owing to the lack of selectivity against other heteromeric receptors, especially the  $\alpha 3\beta 4^*$  receptors, which are predominantly expressed in ganglionic neurones to mediate many off-target side effects of nicotinic ligands.

2087101, a (2-amino-5-keto)thiazole, has been shown to potentiate  $\alpha 4\beta 2$ ,  $\alpha 7$ ,  $\alpha 4\beta 4$  and  $\alpha 2\beta 4$ , but not  $\alpha 3\beta 4$  or  $\alpha 1$ -containing muscle nicotinic receptors in mammalian cell lines and *Xenopus* oocytes (Broad et al., 2006). On  $\alpha 4\beta 2$  receptors, up to 8 fold potentiation of the receptor current was observed at a low acetylcholine concentration. Recently, the allosteric binding site of 2087101 on  $\alpha 7$  receptors has been located to an intrasubunit cavity at the transmembrane region, providing a molecular basis for its allosteric action (Young et al., 2008). In the central nervous system where the expression of  $\alpha 4\beta 4$  and  $\alpha 2\beta 4$  is limited, 2087101 may show considerable selectivity for  $\alpha 4\beta 2^*$  and  $\alpha 7$  but not  $\alpha 3\beta 4^*$  nicotinic receptors. To define its potential clinical applications we examined the effects of 2087101 on native  $\alpha 4\beta 2^*$ ,  $\alpha 7$  and  $\alpha 3\beta 4^*$  nicotinic receptor-mediated processes in the rat brain.

## 2. Materials and Methods

Animal tissues were prepared following procedures in compliance with the UK Animal (Scientific Procedure) Act 1986.

### ***2.1 Patch-clamp recordings in rat hippocampal neuronal culture***

Primary cultures of hippocampal neurones were prepared from newborn (1- to 2-day old) Wistar rats (Harlan, UK) with modified procedures (Alkondon and Albuquerque, 1993). The brains were removed following decapitation and hippocampi were rapidly dissected and cut for enzymatic digestion in PBS solution containing 0.5 mg/ml papain (Sigma-Aldrich, Poole, UK) and 0.25 mg/ml deoxyribonuclease (DNAse I, Sigma-Aldrich, Poole, UK) for 20 min at 37°C. The papain solution was replaced with 2 ml Dulbecco's Modified Eagle's Medium (DMEM, PAA Laboratories GmbH) supplemented with 10% heat-inactivated foetal bovine serum (FBS, Sigma), 10000 Units/ml penicillin, 10000 µg/ml streptomycin, and 29.2 mg/ml L-glutamine (PSG, Invitrogen, Paisley, UK). The digested tissue was then gently triturated by suction using a sterile glass Pasteur pipette with flamed tip. Dissociated cells were plated at a density of approximately  $4 \times 10^5$  cells/ml on glass cover slips pre-coated with poly-D-lysine and mouse laminin (Becton Dickinson Biosciences, Oxford, UK), and maintained in an incubator (37°C and humidified atmosphere of 95% O<sub>2</sub> and 5% CO<sub>2</sub>) in serum-deprived medium containing B-27 supplement (Invitrogen, Paisley, UK) to maintain neuronal survival. Between 5 and 9 days in culture, 5 µM cytosine β-D-arabinofuranoside (AraC, Sigma-Aldrich, Poole, UK) was supplemented to the medium to reduce the proliferation of non-neuronal cells. A heterogeneous population of hippocampal neurones was thus maintained for 4 weeks in culture. Neurones selected for patch-clamp recordings had somatic diameters of 15-30 µm, with neurite extensions.

Whole-cell voltage-clamp recordings were carried out 18-25 days after plating. During recordings, cells were continuously superfused (2 ml/min) with extracellular solution containing (in mM): NaCl 145, KCl 2, KH<sub>2</sub>PO<sub>4</sub> 1.18, HEPES 10, MgSO<sub>4</sub> 1.2, CaCl<sub>2</sub> 2, D-glucose 11, kynurenic acid 5 (glutamate receptor antagonist), atropine 0.1 µM (muscarinic ACh receptor antagonist) at room temperature (pH adjusted to 7.2 with NaOH). Recordings were carried out using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, output cut-off frequency = 5 kHz) at holding potential (V<sub>h</sub>) of +20 mV for GABAergic postsynaptic currents (PSCs). Signals were digitised every 50 µs and analysed off-line with PClamp8 software (Axon Instruments, Foster City, CA). Patch pipettes were pulled from borosilicate glass capillaries (1.5 mm outer diameter, Harvard apparatus, UK), and had resistances between 5 and 7 MΩ when filled with the intracellular solution containing (in mM): Cs<sub>2</sub>SO<sub>4</sub> 81, NaCl 4, MgSO<sub>4</sub> 2, CaCl<sub>2</sub> 0.02, BAPTA 0.1, D-glucose 15, HEPES 10, ATP 3, GTP 0.1, Na-phosphocreatine 5 (pH adjusted to 7.2 with CsOH). For the recording of somatic K<sup>+</sup> current, K-gluconate (130 mM) was used instead of Cs<sub>2</sub>SO<sub>4</sub> in pipettes, and 300 µM CdCl<sub>2</sub> and 300 nM tetrodotoxin (TTX) were added to the extracellular solution to block voltage-gated Ca<sup>2+</sup> and Na<sup>+</sup> channels, respectively. Series resistance of the recordings was monitored before and after each drug application, and recordings were excluded from analysis when series resistance changed by more than 10%. Changes in amplitude, frequency and duration of PSCs were summed by measuring the total charge transfer for a 20 s period from the onset of agonist perfusion.

Drugs were diluted at their final concentration in the extracellular solution and nicotinic agonists were applied via a multibarrel pipette positioned 150  $\mu\text{m}$  away from the recorded neuron.

## ***2.2 Electrophysiological recordings of neuronal firing in the ventral tegmental area***

Coronal midbrain slices (350  $\mu\text{m}$ ) containing the ventral tegmental area (VTA) from male Sprague-Dawley rats (25 - 30 days old) were prepared in ice-cold, oxygenated, artificial cerebrospinal fluid (aCSF), which contained (in mM) NaCl 123,  $\text{NaH}_2\text{CO}_3$  22,  $\text{NaH}_2\text{PO}_4$  1.25, KCl 3.75, D-Glucose 10,  $\text{CaCl}_2$  2.5 and  $\text{MgSO}_4$  1.2. During recording, slices were constantly superfused with oxygenated aCSF at a flow rate of 3 ml/min at 34°C. The VTA was visually identified as a grey area medial to the substantia nigra and the medial lemniscus, a white fibre tract. Single cell extracellular recordings were made using glass microelectrodes filled with aCSF (impedance of 3 - 6  $\text{M}\Omega$ ). Action potentials were recorded using an Axopatch 1D amplifier (Axon Instruments, Foster City, CA), digitised using CED1401 plus (CED, Cambridge, UK) and captured and stored on a PC using Spike 2 software (CED, Cambridge, UK). Recordings from single neurones displayed constant action potential amplitude and waveform. For details, see (Chen et al., 2003).

Spontaneously firing neurones in the VTA were distinguished by both electrophysiological and pharmacological characteristics (Grace and Onn, 1989, Johnson and North, 1992). The most frequently recorded neurones are the classically defined dopaminergic neurones, which fire spontaneous action potentials at a frequency ranging from 0.5 to 4 Hz and whose extracellular action potential waveform has large negative phase and 2.5–3 ms duration. The firing rate can be significantly suppressed by 50  $\mu\text{M}$  dopamine. The other main type of VTA neurones exhibits shorter duration ( $\sim$  2 ms) action potentials at higher firing frequencies (4 – 15 Hz). Dopamine does not inhibit the firing rate of these classically defined non-dopaminergic, GABAergic interneurones. The identification of dopaminergic neurones by the electrophysiological and pharmacological characteristics has been challenged recently as projecting dopaminergic neurones in the VTA were found to exhibit more diverse electrophysiological and pharmacological characteristics (Margolis et al., 2006, Lammel et al., 2008). For the study of nicotinic modulation of VTA neuron firing, we focused on the classically defined low-frequency firing, putative dopaminergic neurones, which have been shown to have higher sensitivity to nicotine than the high-frequency firing, putative non-dopaminergic neurones (Yin and French, 2000).

Drugs were dissolved in aCSF and delivered to the slice via a switch from control aCSF. An initial recording period of > 4 min was usually allowed to establish the baseline firing frequency of a neuron. Peak changes of frequency caused by drug application were calculated using at least three consecutive 10 s bins containing 15 - 120 spikes. A washing period (> 20 min) was given between subsequent drug applications. The effect of 2087101 was examined by comparing the effects of nicotine before and after the application of 2087101 in the same neuron.

## ***2.3 [ $^3\text{H}$ ]Noradrenaline release from rat hippocampal slices***

Hippocampi slices (150  $\mu\text{m}$ ) from 3 rats (male Lister-Hooded rats (250 – 350 g)) were chopped using a McIlwain tissue chopper, each time rotating the tissue through 60°.

Slices were dispersed in Krebs bicarbonate buffer (in mM): NaCl 118, KCl, 2.4, CaCl<sub>2</sub>·2H<sub>2</sub>O 2.4, KH<sub>2</sub>PO<sub>4</sub> 1.2; MgSO<sub>4</sub>·7H<sub>2</sub>O 1.2, NaHCO<sub>3</sub> 25, glucose 10, ascorbic acid 1 (oxygenated with 5% CO<sub>2</sub>/95% O<sub>2</sub> for 1h), and then incubated with 10 μM pargyline, a monoamine oxidase inhibitor, and incubated with [<sup>3</sup>H]noradrenaline (100 nM) for 30 min at 37°C (Dajas-Bailador et al., 2003, Barik and Wonnacott, 2006). Following loading, slices were washed with Krebs buffer containing 1 μM nomifensine (a monoamine uptake inhibitor) and 10 μM pargyline (Barik and Wonnacott, 2006). After washing, slices were loaded in each well of a 96-well GF/C filter plate (Millipore), and 70 μl of buffer ± antagonist/potentiator was added to each well for 5 minutes at 37°C. After this, buffer containing released radioactivity was removed by filtration into a 96-well collection plate. Slices were then stimulated for 5 min with buffer containing agonist (± antagonist/potentiator; 70 μl/well), and the stimulating buffer was removed into another 96-well collection plate.

Optiphase Supermix (Wallac) scintillation fluid (170 μl) was added to each well of the collection plates prior to being heat sealed and radioactivity quantified using a Wallac 1450 Microbeta 96-well plate counter (Wallac Oy, Turku, Finland, counting efficiency 25%). Radioactivity remaining in the slices was measured by digestion of the tissue in 0.2 ml Solvable (Packard Biosciences) for 1 h followed by an addition of 0.5 ml isopropyl alcohol and 4.5 ml Hionic Fluor scintillation fluid (Packard Biosciences). Radioactivity was then quantified using a Wallac 1410 scintillation counter (Wallac Oy, Turku, Finland, counting efficiency 35%).

Release of [<sup>3</sup>H]noradrenaline was expressed as a fraction of the total radioactivity contained within the slices at the time of stimulation. To compare the concentration-dependent effect of nicotine and the effect of 2087101, the release of [<sup>3</sup>H]noradrenaline induced by different nicotine concentration before and during the addition of 2087101 was normalised to that evoked by 100 μM nicotine.

## **2.4 Reagents**

ACh chloride, (-)-nicotine hydrogen tartrate (nicotine), mecamylamine, atropine sulfate, choline chloride, kynurenic acid, bicuculline methochloride, nomifensine, pargyline and dopamine hydrochloride were from Sigma (Poole, Dorset, UK). Dihydro-β-erythroidine hydrobromide (DHβE) was from Sigma-RBI, and methyllycaconitine citrate (MLA), D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5), 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) and (2S)-(+)-5,5-Dimethyl-2-morpholineacetic acid (SCH 50911) from Tocris Cookson Ltd (Bristol, UK). [7,8-<sup>3</sup>H]noradrenaline (8-15 Ci/mmol) was purchased from Amersham Biosciences (Amersham, Bucks, UK). 2087101 was synthesised by Lilly Research Laboratories (courtesy of S. Hollinshead). Stock solution of 10 mM were made up in DMSO, stored at -20 °C and diluted in the extracellular recording solution at the final concentration before use.

## **2.5 Statistics**

Numerical data in the text and figures are expressed as mean ± standard error of the mean (S.E.M). Statistical comparisons were made with ANOVA or Student's *t*-test,

paired or unpaired when appropriate. Values of  $P < 0.05$  were taken to be statistically significant.

### 3. Results

#### 3.1 Effects of 2087101 on nicotinic agonist induced postsynaptic currents in cultured hippocampal neurones.

Activation of  $\alpha 7$  and non- $\alpha 7$  nicotinic receptors in hippocampal neurones has been shown to stimulate somatodendritic currents (Alkondon and Albuquerque, 1993) and to induce action potential-dependent GABA release, which was recorded as GABA<sub>A</sub> receptor-mediated postsynaptic currents (PSCs) (Alkondon et al., 1999, Albuquerque et al., 2009). In our cultured hippocampal neurones, ACh induced somatodendritic current in the presence of TTX, and the effect was completely inhibited by 10 nM MLA, showing a predominant  $\alpha 7$  receptor-mediated effect (Fig. 1A), which is in agreement with the low prevalence of non- $\alpha 7$  heteromeric receptor-mediated somatodendritic currents shown previously (Alkondon and Albuquerque, 1993, Hurst et al., 2005, Ng et al., 2007).

In order to obtain both  $\alpha 7$  and  $\alpha 4\beta 2^*$  receptor-mediated responses by the same mechanism, we recorded ACh-induced PSCs (Fig. 1B) in the absence of TTX and at the holding potential ( $V_h$ ) of +20 mV to minimise the amplitude of  $\alpha 7$ -mediated somatodendritic currents that show inward rectification (Alkondon et al., 1999). 1 mM kynurenic acid and 10  $\mu$ M atropine were present continuously to pharmacologically block ionotropic glutamate receptors and muscarinic ACh receptors. Under these conditions, the PSCs were sensitive to the GABA<sub>A</sub> receptor antagonist bicuculline, hence resulted from presynaptic GABA release (Alkondon et al., 1999). The  $\alpha 7$ -selective agonist choline (10 mM) was able to induce PSCs in most cultured neurones (Fig. 1C top trace & D left trace) and the effects was inhibited by 10 nM MLA (Fig. 1C bottom trace), confirming the presence of an  $\alpha 7$  component. In the presence of 10 nM MLA to block the  $\alpha 7$  receptors, ACh also evoked PSCs (Fig. 1F), showing a non- $\alpha 7$  mediated component due to the activation  $\alpha 4\beta 2^*$  receptors (Alkondon et al., 1999, Albuquerque et al., 2009). The overall ACh-induced response was significantly inhibited by MLA at a higher concentration (100 nM) (Fig. 1B, bottom trace) that inhibits both  $\alpha 7$  and non- $\alpha 7$  receptors (Alkondon and Albuquerque, 1993). We have therefore examined the effects of 2087101 on PSCs induced by choline and ACh + MLA (10 nM) to activate the  $\alpha 7$  and the non-  $\alpha 7$ ,  $\alpha 4\beta 2^*$  components separately.

The application of 2087101 (3  $\mu$ M) caused no currents in hippocampal neurones on its own, but in the presence of 3  $\mu$ M 2087101, the frequency and duration of PSCs induced by both choline and ACh + MLA were enhanced in a reversible manner (Fig. 1D and F, middle and right traces). To quantify the changes in PSCs, total charge transfer for a 20 s period was measured starting from the application of agonist (Alkondon et al., 1999). By comparing to agonist-induced responses before the addition of 2087101 in each neuron, we found significantly enhanced agonist-evoked effects in the presence of 2087101 (10 min) and the effect was washed out (Fig. 1E and G, \*  $P < 0.05$ , One-way ANOVA with Dunnett's posthoc test for multiple comparisons against the control). Averaged enhancements for choline and ACh + MLA induced responses were  $247 \pm 14\%$  ( $n = 5$ ) and  $235 \pm 27\%$  of control ( $n = 6$ ), respectively. The results show that 2087101 significantly potentiated agonist-induced  $\alpha 7$  and  $\alpha 4\beta 2^*$  receptor-mediated GABA release.

In order to ascertain that 2087101 indeed potentiated nicotinic acetylcholine receptors but not GABA<sub>A</sub> receptors to facilitate the PSCs, we examined its effect on postsynaptic GABA<sub>A</sub> receptors in hippocampal neurones. It is particularly important to examine this possible cross reactivity because the binding site for 2087101 on  $\alpha 7$  receptors has been shown to be a conserved allosteric site within the whole family of Cys-loop ligand-gated ion channel receptors, which include both nicotinic and GABA<sub>A</sub> receptors (Hosie et al., 2006, Young et al., 2008)

Using whole-cell patch clamp recordings at the  $V_h$  of +20 mV and in the presence of 10  $\mu$ M SCH-50911, a GABA<sub>B</sub> receptor-selective antagonist, direct application of GABA (5  $\mu$ M) induced an outward whole-cell current mediated by GABA<sub>A</sub> receptors. Application of 2087101 (3  $\mu$ M for 7 min) did not cause any whole-cell current by itself, nor any significant alteration of GABA-induced currents ( $P > 0.05$ ,  $n = 5$ ), showing a lack of direct activity of 2087101 on GABA<sub>A</sub> receptors.

We have shown previously that 2087101 has no effect on voltage-gated Ca<sup>2+</sup> or Na<sup>+</sup> channels or ionotropic glutamate receptors in cultured cortical neurones (Broad et al., 2006). We have now examined its effects on voltage-gated K<sup>+</sup> channels, since their modulation may indirectly affect GABA release. Outward currents were activated by a depolarising voltage step from -70 to +20 mV using a K<sup>+</sup>-gluconate containing patch pipette in the extracellular solution containing 300  $\mu$ M CdCl<sub>2</sub> and 300 nM TTX to block the voltage-gated Ca<sup>2+</sup> and Na<sup>+</sup> channels, respectively. The application of 2087101 (3  $\mu$ M for 7 min) did not affect these voltage-gated K<sup>+</sup> current ( $n = 5$ ). Altogether, these experiments show a lack of effects of 2087101 on either GABA<sub>A</sub> receptors or voltage-gated K<sup>+</sup> channels, and thus further support the evidence of selective modulation of  $\alpha 4\beta 2^*$  and  $\alpha 7$  nicotinic acetylcholine receptors.

### ***3.2 Effects of 2087101 on nicotine-stimulated [<sup>3</sup>H]noradrenaline release in the hippocampus.***

In contrast to the nicotinic acetylcholine receptor mediated GABA release, nicotine-stimulated noradrenaline release in the hippocampus is not sensitive to the  $\alpha 7$  antagonist MLA or the  $\beta 2$ -preferring antagonist dihydro- $\beta$ -erythroidine (DH $\beta$ E), but is partially inhibited by the  $\alpha 3$ -preferring antagonist  $\alpha$ -conotoxin AuIB (Clarke and Reuben, 1996, Luo et al., 1998), so that  $\alpha 3\beta 4^*$  receptors could be the main subtype mediating this process (Vizi and Kiss, 1998). Because 2087101 has been shown to have no allosteric effect on recombinant  $\alpha 3\beta 4$  receptors (Broad et al., 2006), we examined its effect on nicotine-stimulated [<sup>3</sup>H]noradrenaline release in rat hippocampal slices.

Nicotine caused a concentration-dependent increase of fractional [<sup>3</sup>H]noradrenaline release (Fig. 2 filled circles) with an EC<sub>50</sub> of 5.8  $\mu$ M, which is very similar to that reported for nicotine-induced [<sup>3</sup>H]noradrenaline release from synaptosomes (6.5  $\mu$ M) (Clarke and Reuben, 1996). The non-competitive nicotinic antagonist mecamylamine (MEC, 10  $\mu$ M,  $n = 6$ ), but not DH $\beta$ E (2  $\mu$ M,  $n = 6$ ), blocked nicotine-induced [<sup>3</sup>H]noradrenaline release, in agreement with the notion that  $\alpha 3\beta 4^*$ , but not  $\alpha 4\beta 2^*$ , receptors are the predominant subtype in this process (Vizi and Kiss, 1998).

Application of 3  $\mu\text{M}$  2087101 alone did not induce any [ $^3\text{H}$ ]noradrenaline release in hippocampal slices. In the presence of the potentiator, nicotine-induced concentration-dependent release (Fig. 2 open circles) was found similar to that of nicotine alone ( $\text{EC}_{50}$ : 4.2  $\mu\text{M}$  versus 5.8  $\mu\text{M}$ , and  $E_{\text{Max}}$ : 94.0  $\pm$  7.9 % versus 99.5  $\pm$  4.1 %,  $n = 6$  each). Mecamylamine (10  $\mu\text{M}$ ) also blocked the effects of nicotine + 2087101 (Fig. 2 open diamond). Two-way ANOVA analysis of the two curves showed significant ( $F[10, 110] = 51.2$ ,  $P < 0.001$ ) concentration-dependent effects of nicotine, but no significant effect of 2087101 ( $F[1, 110] = 0.12$ ,  $P > 0.05$ ).

The nicotine concentration-response curves were fitted by a one-site sigmoidal equation, showing no evidence for a low affinity  $\alpha 7$  component that was shown previously to be activated by choline or anatoxin in hippocampal slices (Barik and Wonnacott, 2006). The lack of significant effect of 2087101 on nicotine-induced [ $^3\text{H}$ ]noradrenaline release thereby agrees with its lack of effect on  $\alpha 3\beta 4$  receptors.

### **3.3 Effects of 2087101 on nicotine-stimulated neuronal firing in the VTA.**

The spontaneous firing activity of midbrain dopamine neurones in rat brain slices can be enhanced by submicromolar concentrations of nicotine via the activation of  $\beta 2^*$  nicotinic receptors (Picciotto et al., 1998), which may include the  $\alpha 4\beta 2$ ,  $\alpha 6\beta 2$ ,  $\alpha 4\alpha 5\beta 2$  and  $\alpha 4\alpha 6\beta 2\beta 3$  subtypes in the VTA (Klink et al., 2001, Champtiaux et al., 2003). Albeit at a lower density,  $\alpha 7$  receptors are also expressed in the VTA at pre- and postsynaptic locations to modulate glutamate release and somatodendritic currents (Mansvelder and McGehee, 2000, Klink et al., 2001). Given the essential role of nicotinic receptors in the VTA for nicotine re-enforcement, we examined whether 2087101 can enhance nicotine-stimulated neuronal firing activity in rat brain slices.

Bath-applied nicotine at 0.3  $\mu\text{M}$  significantly increased the firing rate of the putative dopamine neurones by 46.4  $\pm$  4.4% from baseline (mean baseline firing frequency = 1.9  $\pm$  0.1 Hz, range 0.7 – 3.5 Hz,  $n = 61$ , Fig. 3A). The effect of nicotine was concentration-dependent with 0.3  $\mu\text{M}$  nicotine producing a sub-maximal effect (Fig. 3B). In contrast, the firing frequency of the putative non-dopamine neurones was much less sensitive to bath-applied nicotine, where 1  $\mu\text{M}$  nicotine caused no significant firing rate increase (1.4  $\pm$  1.4%, baseline frequency = 6.7  $\pm$  0.7 Hz, range 5.4 – 8.4 Hz,  $n = 4$ ), in agreement with a previous finding (Yin and French, 2000). Subsequently, we examined the effect of 2087101 on nicotine-stimulated firing rate increase in the putative dopamine neurones.

Repeated applications of 0.3  $\mu\text{M}$  nicotine with a 20 min washing period in-between produced identical increases in firing rate in dopamine neurones (44.3  $\pm$  7.6 % vs. 45.4  $\pm$  7.3 %,  $n = 9$ ,  $P = 0.5$ , paired  $t$ -test, Fig. 3A). Pre-application (10 min) of 10  $\mu\text{M}$  2087101 before the second application of nicotine significantly enhanced the response by 29  $\pm$  11% compared to the control nicotine response ( $n = 10$ , \*  $P < 0.05$ , paired  $t$ -test, Fig. 3C). However, the effect of 3  $\mu\text{M}$  2087101 was not statistically significant ( $P > 0.05$ ,  $n = 16$ , paired  $t$ -test). Fig. 3D shows the % enhancement of 0.3  $\mu\text{M}$  nicotine-stimulated response by 0, 3 and 10  $\mu\text{M}$  2087101. The nicotinic potentiator can thus concentration-dependently facilitate the effect of nicotine on dopamine neurones in the VTA.

2087101 (at 3 and 10  $\mu\text{M}$ ) had no significant effects on baseline firing rate ( $2.2 \pm 1.6$  % change,  $n = 27$ , see Fig. 3C), or action potential waveform (data not shown), showing no direct activation of nicotinic receptors or other receptors/channels. The potential nicotinic receptor subtypes activated by 0.3  $\mu\text{M}$  nicotine was examined using nicotinic antagonists. DH $\beta$ E (2  $\mu\text{M}$ ) significantly inhibited the response ( $91 \pm 5\%$  inhibition,  $n = 3$ , Fig. 3E), but  $\alpha$ -conotoxin MII (CTX, 100 nM, an  $\alpha 6$  antagonist) or MLA (10 nM, an  $\alpha 7$  antagonist) had no significant effects ( $14 \pm 8$  % inhibition,  $n = 4$  and  $4.8 \pm 2.1\%$  inhibition,  $n = 3$ , respectively, Fig. 3E), suggesting a major role for the  $\alpha 4\beta 2^*$  receptors under the experimental conditions. The effect of 2087101 could therefore be caused by potentiation of  $\alpha 4\beta 2^*$  receptors in the VTA.

Because the excitatory effect of nicotine could arise not only directly from the activation of somatodendritic nicotinic receptors, but also indirectly from the release of excitatory neurotransmitters glutamate or ACh, we examined the effects of glutamate receptor antagonists (50  $\mu\text{M}$  D-AP5, an NMDA receptor antagonist, and 20  $\mu\text{M}$  CNQX, an AMPA receptor antagonist,  $n = 3$ ) and the muscarinic ACh receptor antagonist atropine (1  $\mu\text{M}$ ,  $n = 4$ ). The antagonists had no significant effect on 0.3  $\mu\text{M}$  nicotine-induced response or the basal spontaneous firing rate. The result is in support of a direct somatodendritic effect by nicotine, in agreement with the effect by a  $\alpha 4\beta 2$ -selective agonist TC-2559 shown by us previously (Chen et al., 2003).

(Figure 3 near here)

#### 4. Discussion

We showed that the nicotinic allosteric modulator 2087101 significantly facilitated nicotinic stimulation of GABA release in the hippocampus and dopamine neuron firing in the VTA, but it had no effect on nicotine-induced noradrenaline release in the hippocampus. The differential facilitation of these nicotinic processes agrees with its selectivity for different receptor subtypes (Broad et al., 2006). Based on the present knowledge of native nicotinic receptor subunit compositions, our results suggest that 2087101 is active on neuronal processes mediated by  $\alpha 4\beta 2^*$  and  $\alpha 7$ , but not  $\alpha 3\beta 4^*$  nicotinic receptors. The selectivity profile of 2087101 on recombinant receptors therefore translates very well to native nicotinic receptor subtypes.

The activity of 2087101 on native  $\alpha 7$  receptors has been clearly demonstrated in hippocampal neurones. The  $\alpha 7$  receptor selective agonist choline (Alkondon et al., 1999) was used to induce GABAergic PSCs, and the process was enhanced by  $\sim 1.5$  fold by 2087101, without any effect on GABA $_A$  receptors, demonstrating unequivocally the potentiator's activity on native  $\alpha 7$  receptors. Restricted by the lack of subtype selective agonists and antagonists for heteromeric nicotinic receptors (Albuquerque et al., 2009), we examined the effects of 2087101 on non- $\alpha 7$  nicotinic receptor-mediated hippocampal GABA release using ACh in the presence of MLA, an  $\alpha 7$ -selective antagonist. This process was also enhanced by 2087101. As the non- $\alpha 7$  component of hippocampal GABA release was shown to be mainly mediated by  $\alpha 4\beta 2^*$  nicotinic receptors (Alkondon et al., 1999) and amongst those 80% were identified to be of the  $\alpha 4\beta 2$  composition (McClure-Begley et al., 2009), 2087101 may have therefore potentiated the hippocampal  $\alpha 4\beta 2$  receptors.

Although the subunit composition has yet to be confirmed at the molecular level, nicotine-induced hippocampal [<sup>3</sup>H]noradrenaline release has been proposed to be mediated by (non- $\alpha 7$ , non- $\alpha 4\beta 2$ )  $\alpha 3\beta 4^*$  nicotinic receptors (Vizi and Kiss, 1998), with the pharmacological characteristics very similar to those recently identified  $\alpha 3\beta 4^*$  receptors expressed in the habenulo-interpeduncular pathway mediating ACh release (Grady et al., 2009). 2087101 was, however, not able to potentiate nicotine-induced [<sup>3</sup>H]noradrenaline release, a finding in agreement with its lack of activity on recombinant  $\alpha 3\beta 4$  receptors in heterologous expression systems (Broad et al., 2006). These differential effects of 2087101 on hippocampal GABA and noradrenaline release have therefore suggested its selectivity for native  $\alpha 4\beta 2^*$  and  $\alpha 7$ , but not  $\alpha 3\beta 4^*$  receptors.

We have also further demonstrated the activity of 2087101 on nicotinic receptors in the VTA. The stimulatory effect of 0.3  $\mu$ M nicotine on putative dopamine neurones was significantly enhanced by 2087101. The nicotinic receptor subtypes underlying the effect were investigated using antagonists. DH $\beta$ E, but not MLA or  $\alpha$ -conotoxin MII, produced significant inhibition, suggesting the activation of  $\alpha 4\beta 2^*$  receptor subtypes, the predominant subtype in the VTA (Zoli et al., 1998, Klink et al., 2001, Champtiaux et al., 2003). The lack of a significant contribution by  $\alpha 7$  receptors could be explained by the relatively low density of  $\alpha 7$  receptors in the brain area (Zoli et al., 1998). Although nicotine can stimulate glutamate release via presynaptic  $\alpha 7$  receptors and increased the frequency of excitatory postsynaptic currents in postsynaptic neurones (Mansvelder et al., 2000), we found that the block of glutamatergic transmission had no influence on nicotine-stimulated firing rate increase. The increased glutamate release may therefore have negligible effect on the firing rate of putative dopamine neurones when compared with the direct somatodendritic depolarisation induced by nicotine. It is also noted that a slightly higher concentration of 2087101 is required to significantly facilitate nicotine-induced dopamine neuron firing rate increase. It may be speculated that the incorporation of additional subunits, i.e.  $\alpha 5$  and  $\beta 3$ , by the  $\alpha 4\beta 2^*$  nicotinic receptors in the VTA (Zoli et al., 1998, Klink et al., 2001, Champtiaux et al., 2003) may reduce the potency of 2087101.

Receptor subunit composition could indeed influence the potency and/or efficacy of allosteric modulators. Several intrasubunit (Curtis et al., 2002) and intersubunit sites (Hansen and Taylor, 2007) have been identified on  $\alpha 4\beta 2$  receptors that were shown to specifically interact with certain allosteric modulators. The allosteric binding site of 2087101 on  $\alpha 7$  receptors was identified as an intrasubunit hydrophobic cavity (Young et al., 2008). As this binding site is a conserved allosteric site present in various members of the family of cys-loop ligand-gated ion channels receptors (Young et al., 2008), it is possible to envisage a corresponding allosteric site on the  $\alpha 4$  subunit. However, the lack of activity on  $\alpha 3\beta 4$  receptors in heterologous expression systems (Broad et al., 2006) and in the rat hippocampus may suggest that the modulator does not bind the  $\alpha 3$  subunit.

The ability of 2087101 to enhance GABAergic activity could be beneficial for many severe neurological disorders, including Alzheimer's disease and schizophrenia (Freedman et al., 2000, Perry et al., 2000). Indeed, the  $\alpha 7$ -selective potentiators were also shown to facilitate GABA release in the hippocampus to normalise sensory gating deficits in animal models (Hurst et al., 2005) and to improve cognitive functions in behavioural paradigms (Ng et al., 2007, Timmermann et al., 2007). An  $\alpha 7$  and  $\alpha 4\beta 2$  dual potentiator may thus not only enhance  $\alpha 7$ -mediated GABA release but also  $\alpha 4\beta 2$ -mediated effects.

The latter may be particularly beneficial to schizophrenia patients, because the  $\alpha 7$  receptor expression and functions are significantly reduced in this disorder (Freedman et al., 2000). GABA release in many brain regions has been shown to be predominantly modulated by both  $\alpha 7$  and  $\alpha 4\beta 2^*$  receptors (Albuquerque et al., 2009), so that 2087101 and similar compounds could exert significant concurrent activity across many brain areas to offer better therapeutic potencies for some indications than subtype-selective allosteric potentiators.

The dual facilitation of both the GABAergic and dopaminergic systems may also ameliorate cognitive and psychiatric deficits. Dopaminergic transmission in the brain is known to modulate cognitive and reward processes as well as neuropsychiatric symptoms (Wise, 2004). A synergy between GABAergic and dopaminergic systems may thus be explored for cognitive enhancement.

This *in vitro* demonstration of the potentiator activity may encourage future *in vivo* behavioural evaluations for its therapeutic potentials. Because of its lack of activity on acetylcholinesterase (Broad et al., 2006) or  $\alpha 3\beta 4^*$  receptors, 2087101 may show a much improved side effect profile compared to galantamine, an acetylcholinesterase inhibitor and a non-selective nicotinic receptor allosteric modulator, which is used clinically for the symptomatic treatment of Alzheimer's disease.

In summary, the nicotinic positive allosteric modulator, 2087101, significantly potentiated native nicotinic processes mediated by  $\alpha 4\beta 2^*$  and  $\alpha 7$ , but not  $\alpha 3\beta 4^*$  nicotinic receptors. The selectivity profile of 2087101 may be further explored to differentiate the allosteric binding sites between heteromeric receptors. In addition, this potentiator can be used to further evaluate the therapeutic potential of concurrent enhancement of both  $\alpha 7$  and  $\alpha 4\beta 2^*$ -mediated nicotinic processes in the brain for the improvements of cognitive, motor and reward functions.

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## Figure legends

**Fig. 1** 2087101 enhanced nicotinic receptor agonist-induced GABA release in cultured rat hippocampal neurones. **A.** Acetylcholine (ACh, 1mM) in the presence 1  $\mu$ M TTX induced somatodendritic current (top trace) and the effect was inhibited by 10 nM MLA, a  $\alpha 7$  selective antagonist (bottom trace). **B.** ACh in the absence of TTX induced postsynaptic currents (PSCs), which were recorded as outward current at a holding potential of +20 mV to minimise the amplitude of somatodendritic current as it inward rectifies (top trace). ACh-induced PSCs were inhibited by MLA at the high concentration of 100 nM, which antagonised both  $\alpha 7$  and non- $\alpha 7$  nicotinic components (bottom trace). **C.** The  $\alpha 7$ -selective agonist choline (10 mM) induced PSCs (top trace) that were sensitive to 10 nM MLA (bottom trace). Choline (**D**) and ACh in the presence of 10 nM MLA (ACh + MLA) (**F**) induced PSCs in the control aCSF (left traces), during the 10 min application of 2087101 (3  $\mu$ M, middle traces) and after washing out (right traces). Filled horizontal bars indicating agonist application periods and the open horizontal bars show the presence of 3  $\mu$ M 2087101. During the experiments, agonists were applied every two minutes. Changes in PSC frequency and duration were measured by total charge transfer for a 20 s period from the start of each agonist application and the values were normalised to agonist's effect prior to the addition of 2087101 in each experiment. Averaged time courses for the effects of 2087101 on PSCs induced by 10 mM choline (**E**, n = 5) or 1 mM ACh + 10 nM MLA (**G**, n = 6) are shown. During the application of 2087101 (marked as a grey shadowed area), significant increases of agonist-induced PSCs were found (\*P < 0.05, One-way ANOVA with Dunnett's posthoc test), and recovery was seen during the washing out of 2087101. 1 mM kynurenic acid and 10  $\mu$ M atropine were present in the aCSF throughout the experiments. PSCs were recorded at the holding potential of +20 mV. Values are shown as mean  $\pm$  S.E.M.

**Fig. 2.** Nicotine-induced concentration-dependent [ $^3$ H]noradrenaline release in rat hippocampal slices in the presence (open circles, Nic + 2087101) and absence (filled circles, Nic) of 3  $\mu$ M 2087101. Fractional release of preloaded [ $^3$ H]noradrenaline (mean  $\pm$  S.E.M from 6 independent experiments) was measured following 5 min application of nicotine with or without 2087101 in 96-well plates. The concentration-response curves were fitted to a one-site sigmoidal equation and the EC<sub>50</sub> for nicotine + 2087101 and nicotine alone were obtained at 4.2  $\mu$ M (LogEC<sub>50</sub> = -6.63  $\pm$  0.16 M) and 5.8  $\mu$ M (LogEC<sub>50</sub> = -6.77  $\pm$  0.07 M), respectively. Maximal fractional release relative to that induced by 100  $\mu$ M nicotine were found at 99.5  $\pm$  4.1 % for nicotine and 94.0  $\pm$  7.9 % for nicotine + 2087101. No statistical difference was found for the effect of 2087101 (P > 0.05), although there was a significant effect for nicotine concentration (P < 0.05, Two-way ANOVA). Mecamylamine (MEC) at 10  $\mu$ M completely inhibited 100  $\mu$ M nicotine-induced release in the presence and absence of 2087101 (diamond). Data points are mean  $\pm$  S.E.M.

**Fig. 3** 2087101 enhanced nicotine-induced firing rate increase of putative dopamine neurones in the VTA in rat brain slices. **A.** Bath applications of 0.3  $\mu$ M nicotine (filled horizontal bars) increased the spontaneous firing rate of dopamine neurones. Firing frequencies were calculated from the number of spikes in three 10s bins before and after nicotine application in the rate histogram. **B.** A plot of nicotine-induced % increases of firing rates at the indicated concentrations shows that 0.3  $\mu$ M nicotine elicited sub-maximal effects on firing frequency. **C.** The presence of 10  $\mu$ M 2087101 (open bar) enhanced nicotine (filled bars)-induced firing frequency increase without causing any effect on its own. **D.** % changes from paired control applications of nicotine in the presence 0, 3 and 10  $\mu$ M 2087101 are shown. \*  $P < 0.05$  indicates significantly enhanced nicotine-induced firing rate increase in the presence of 10  $\mu$ M 2087101. **E.** % inhibition caused by nicotinic receptor antagonists dihydro-beta-erythroidine (DH $\beta$ E),  $\alpha$ -conotoxin MII (CTX) and methyllycaconitine (MLA) on 0.3  $\mu$ M nicotine induced response. All values are presented as mean  $\pm$  S.E.M.

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**Statement of conflicts of interest.**

The manuscript is approved for publication by Eli Lilly & Co. and there are no other conflicts of interest known to all authors.

Fig. 1

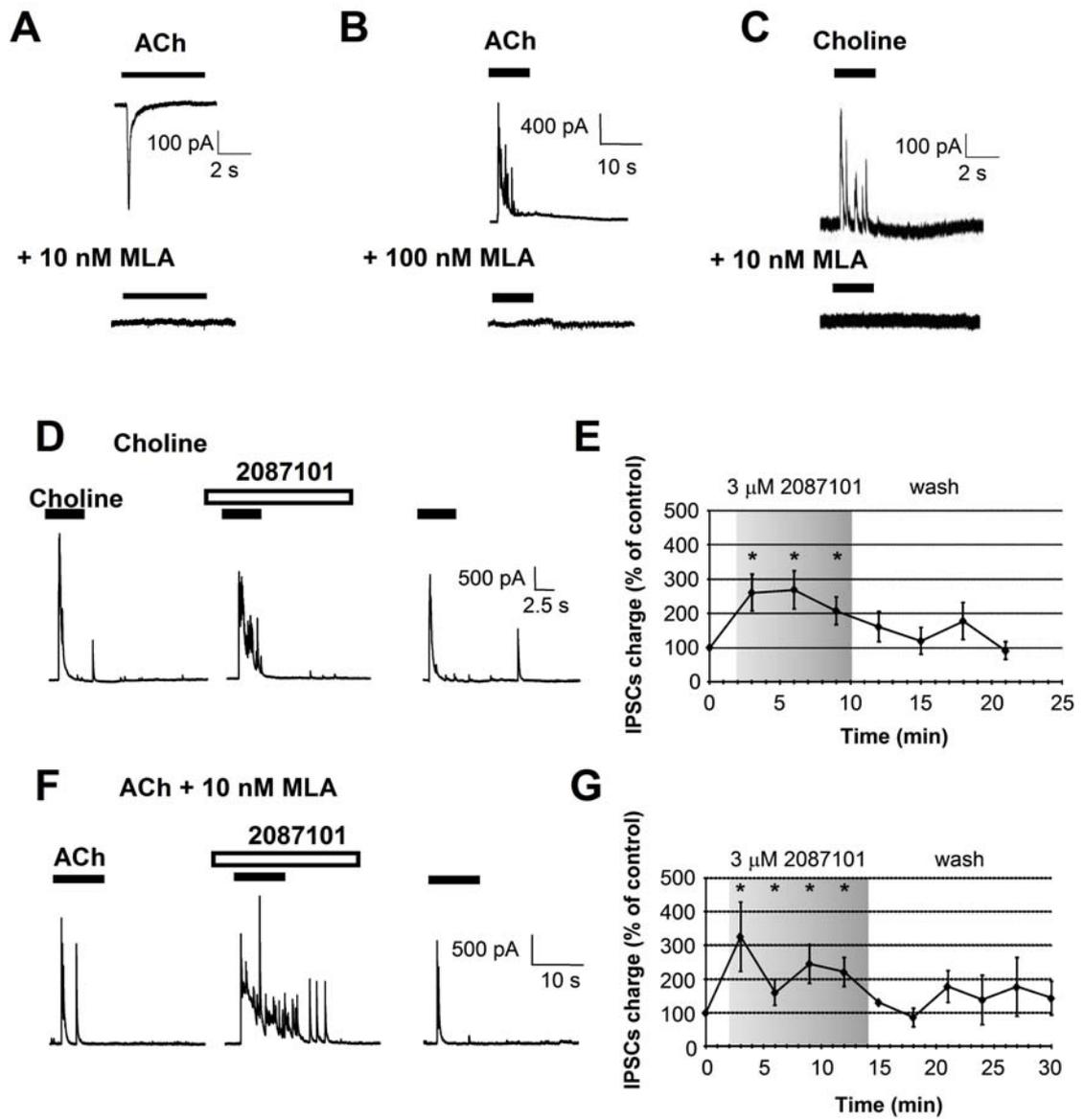


Fig. 2

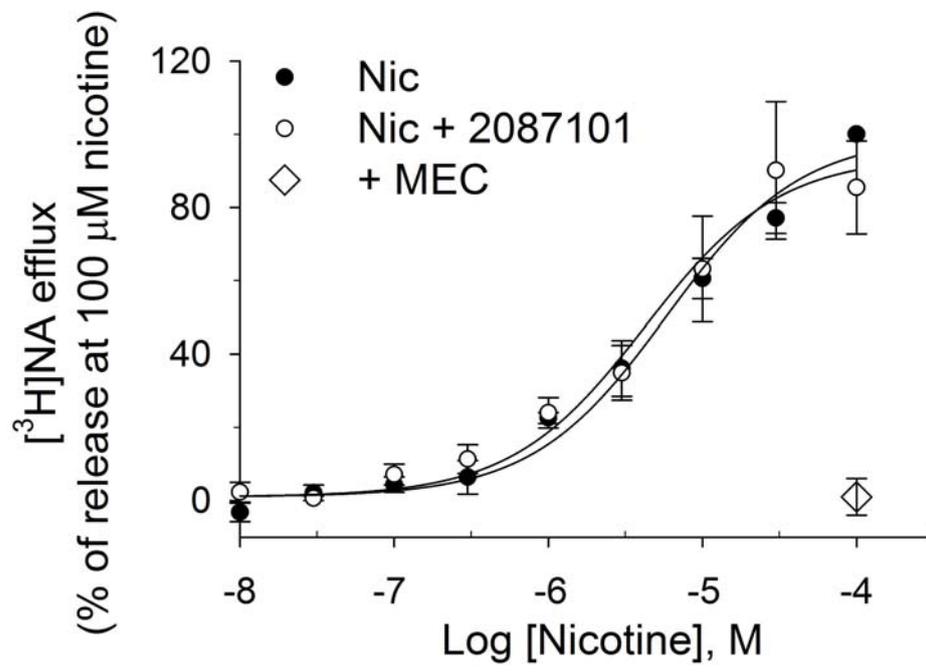


Fig. 3

