Partial agonists for $\alpha_4\beta_2$ nicotinic receptors stimulate dopamine neuron firing with relatively enhanced maximal effects

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Receptor reserve enhances effects of partial agonists

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Summary

**Background and purpose.** Partial agonists selective for α4β2 nicotinic acetylcholine receptors have been developed for smoking cessation as they induce weak activation of native α4β2* receptors and inhibit effect of nicotine. However, it is unclear whether at brain functions there is an existence of receptor reserve that allows weak receptor activation to induce maximum physiological effects. We assessed the extent of α4β2 partial agonist-induced increase of firing rate in dopaminergic neurons and evaluated the influence of receptor reserve.

**Experimental approach.** The relative maximal effects and potencies of six nicotinic agonists were assessed on recombinant human α4β2 and α7 receptors expressed in mammalian cell lines by measuring calcium influx. Agonist-induced increase of the spontaneous firing rate of dopaminergic neurons was recorded using microelectrodes in the ventral tegmental area of rat brain slices.

**Key Results.** All α4β2 partial and full agonists increased the firing rate concentration-dependently. Their sensitivity to subtype-selective antagonists showed predominant activation of native α4β2* receptors. However, partial agonists with relative maximal effects as low as 33% on α4β2 receptors maximally increased the firing rate and induced additional depolarisation block of firing, demonstrating that partial activation of receptors caused the maximum increase in firing rate in the presence of a receptor reserve.

**Conclusions and implications.** Partial α4β2 agonists induced relatively enhanced effects on the firing rate of dopaminergic neurons, and the effect was mainly attributed to the existence of native α4β2* receptor reserve. The results have implications in the understanding of physiological effects and therapeutic efficacies of α4β2 partial agonists.

**Keywords:** receptor reserve, smoking cessation, dopamine neuron, nicotine addiction, nicotine, TC-2559, cytisine, 5-I-A85380, epibatidine, ABT-594

Abbreviations
ABT-594: (R)-5-(2-azetidinylmethoxy)-2-chloropyridine
ACH: acetylcholine
aCSF: artificial cerebrospinal fluid
DHβE: dihydro-β-erythroidine hydrobromide
Mec: mecamylamine
MLA: methyllycaconitine
nAChR: nicotinic acetylcholine receptor
TC-2559: 5-ethoxy-metanicotine
VTA: ventral tegmental area
5-I-A85380 (5-I-A): (3-[(2s)-2-azetidinylmethoxy]-5-iodopyridine
**Introduction**

The α4β2* nAChRs are the primary drug target for treating nicotine addiction, possibly because they directly mediate nicotine-induced increase in firing rate in dopamine neurons of the ventral tegmental area (VTA) (Picciotto et al., 1998; Chen et al., 2003; Mameli-Engvall et al., 2006) leading to dopamine release and nicotine addiction (Picciotto et al., 1998; Marubio et al., 2003; Maskos et al., 2005). Partial agonists for the α4β2 receptors show clinically efficacy for smoking cessation, presumably via the dual mechanisms of partial receptor activation and competitive antagonism against nicotine (Coe et al., 2005). While the former maintains a low level of nicotinic activity to prevent withdrawal symptoms during abstinence, the latter blocks effects of nicotine from smoking via receptor occupation. Cytisine, a high-affinity α4β2 receptor partial agonist, has been used in Eastern Europe for many years to treat tobacco dependence (Etter, 2006). Recently, varenicline (Coe et al., 2005), a cytisine derivative, has demonstrated even better clinical efficacy in maintaining abstinence in ex-smokers (Fagerstrom & Balfour, 2006; Cahill et al., 2011). However, to what extent the therapeutic delivery of α4β2 partial agonists elicit physiological effects remain controversial.

Chronic administration of partial agonists may predominantly desensitise native α4β2* receptors in the brain (Picciotto et al., 2008), as bath applications of agonists cause long-term decrease of response to agonists (Papke & Heinemann, 1994; Quick & Lester, 2002). Receptor activation is, however, transient and dependent on agonist concentration and the speed of agonist application. Prolonged application of α4β2 partial agonists may, therefore, only persistently activate receptors over a narrow “window” of concentrations where balance between activation and desensitisation is achieved (Lester, 2004; Rollemo et al., 2010; Papke et al., 2011).

However, bath applications of nicotine induce α4β2* receptor activation on dopamine neurons, and persistent increase in firing rate (Picciotto et al., 1998; Yin & French, 2000; Chen et al., 2003; de Filippi et al., 2010), showing steady-state α4β2* receptor activation of the reward pathway of the brain. We also found that TC-2559, a 33% α4β2 partial agonist (relative to epibatidine and ~50% relative to nicotine), stimulated dopamine neuron firing via prolonged bath application in VTA slices in a DHβE-sensitive manner (Chen et al., 2003). The effect was also mimicked by systemic administration in vivo (Wang et al., 2006), showing that α4β2 partial agonists also persistently stimulate native α4β2* receptors on dopamine neurons.

TC-2559 also induced similar effects to nicotine on dopamine neuron firing (Chen et al., 2003; Wang et al., 2006), neurotransmitter release and several cognitive performances (Benchert et al., 2000), indicating its potential to elicit relatively enhanced effects on native functions. However, quantitative pharmacological studies have shown that it is less effective than nicotine at inducing dopamine release (Smith et al., 2007). TC-2559 may, therefore, still be a partial agonist on native rodent α4β2* receptors.

In the case where a partial agonist induced relatively enhanced effect, a receptor reserve may be present (Stephenson, 1956; Furchgott & Bursztyn, 1967). Receptor reserve describes a situation where a full receptor agonist may produce the maximal effect by recruiting only part of the receptor pool, leaving the rest of the receptors in “reserve”. In such case, a partial agonist may still weakly activate receptors but elicit the maximum effect by recruiting all receptors.
available. Partial and full agonists may thus achieve the same maximum physiological effects by recruiting different proportions of the receptor pool. However, full agonists can further activate the receptors in reserve at high concentrations and induce additional effects. As the activation of nicotinic receptors depolarises membrane potential and increases firing rate in dopamine neurons (Calabresi et al., 1989), excessive receptor stimulation could depolarise to a level beyond the firing threshold and cause inactivation of Na\(^+\) channels and failure to generate action potential, cumulating in depolarisation block of firing (Grace & Bunney, 1984). Agonist-induced depolarisation block may, then, indicate the existence of receptor reserve.

We systematically examined the relative maximal effects of six \(\alpha_4\beta_2\) agonists on recombinant \(\alpha_4\beta_2\) receptors and dopamine neuron firing rate increase. Results show relatively enhanced effects of partial agonists on dopamine neuron firing and suggest an existence of nicotinic receptor reserve.

**Materials and Methods**

*Calcium influx in mammalian cell lines expressing nAChRs*

Human \(\alpha_4\beta_2\) nAChRs were stably expressed in HEK293 cells that were cultured in DMEM supplemented with 10% foetal calf serum, 100 units/ml penicillin, 100 \(\mu\)g/ml streptomycin, 4 mM glutamine and 50 \(\mu\)g/ml geneticin. GH\(_4\) cells that express endogenous Ric-3 were used to express \(\alpha_7\) nAChRs (Lansdell et al., 2005; Williams et al., 2005), and they were cultured in F-10 nutrient mixture with the same supplements used for HEK293 cells. The use of receptor nomenclature conforms to (Alexander et al., 2009).

Agonist-induced calcium dynamics through open channels (Kuntzweiler et al., 1998; Fucile et al., 2005) was measured using a FLIPR (Fluorescent Imaging Plate Reader, Molecular Devices, Winnersh, UK) (Chen et al., 2003; Broad et al., 2006). Cell lines were plated overnight in black-walled, transparent bottomed 96-well plates (Poly-D-lysine coated, Marathon Laboratories), at a density of 0.5x10\(^6\) cells/ml for HEK293 cells, and 1.0x10\(^6\) cells/ml for GH\(_4\) cells. All cells were grown in a humidified incubator maintained with 5% \(\text{CO}_2\) in air at 37\(^\circ\)C. Note that changes in incubation temperature may alter the subunit composition of \(\alpha_4\beta_2\) receptors and their sensitivity to ACh (Zwart et al., 2006). Cells were loaded with Fluo-3-AM (10 \(\mu\)M in Hepes-buffered saline solution) for 1 hour at room temperature before the dye was removed. The plates were then transferred to the FLIPR system for measurements.

Fluorescence emission was recorded every second for the first minute following agonist addition, with subsequent readings every 6 seconds for a further 2 minutes. Responses were measured as peak minus basal fluorescence intensity and expressed as percentage of the maximum response induced by 1 \(\mu\)M epibatidine in each experiment. Concentration-response relationships were obtained for each agonist with data from separate experiments and the \(\text{EC}_{50}\) and \(E_{\text{max}}\) values on \(\alpha_4\beta_2\), \(\alpha_3\beta_4\) and \(\alpha_7\) nAChRs were calculated using the Hill equation. Note that the \(E_{\text{max}}\) values represent the relative maximal responses of agonists on each receptor subtype, which may not be the absolute value of efficacy, the measure of the maximum probability of ion channels being open at high concentrations of agonist (Colquhoun, 1998).
Recording of neuronal firing in brain slices

Coronal midbrain slices (350 µm) containing the VTA were prepared from male Sprague-Dawley rats (25 - 40 days old) following procedures in compliance with the UK Animal (Scientific Procedure) Act 1986. Sections were cut using a Campden vibroslicer in ice-cold, oxygenated, artificial cerebrospinal fluid (aCSF), which contained (in mM) NaCl 123, NaHCO$_3$ 22, NaH$_2$PO$_4$ 1.25, KCl 3.75, D-Glucose 10, CaCl$_2$ 2.5 and MgSO$_4$ 1.2. 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 MΩ) (Yin & French, 2000; Chen et al., 2003; de Filippi et al., 2010). Under constant superfusion with oxygenated ACSF at a flow rate of ~3 ml/min at 34°C, the spontaneous neuronal firing can be recorded for more than 6 hours, which allow for the examination of prolonged applications of agonists at different concentrations. Yet, the frequency of spikes and the duration of the extracellular action potential waveform were similar to those recorded in vivo (Wang et al., 2006). Recording from single neurons was selected where spikes amplitude and duration were uniform. Signals were captured using Axopatch 1D in I = 0 mode with a low cut-off frequency of 2 kHz and then further amplified by 100 times in the AC mode using a Neurolog system (Digidata, Cambridge, UK) without any further filtering. Signals were then digitised using CED1401 plus (CED, Cambridge, UK) and captured using Spike 2 software (CED, Cambridge, UK).

Two types of spontaneously firing neurons in the VTA were recorded and identified. The majority was the classically-defined dopamine neurons, which displayed spontaneous single-spike firing pattern at a rate between 0.5 and 4 Hz with action potential waveforms of 2.5 – 3 ms in duration, including a large negative phase (Grace & Onn, 1989; Johnson & North, 1992). The firing frequency of these neurons was significantly suppressed by 50 µM dopamine (- 74.2 ± 4.8%, n = 29) (Yin & French, 2000; Chen et al., 2003). The other type was known as the non-dopaminergic or GABAergic neurons (Grace & Onn, 1989; Johnson & North, 1992), which displayed higher firing frequency (4 – 15 Hz), regular firing pattern, and an action potential waveform of a shorter duration (~ 2ms). Dopamine did not inhibit the firing of these neurons (N = 18). A recent study confirmed that almost all dopaminergic neurons classified by their electrophysiological characteristics are tyrosine hydroxylase-positive (Brown et al., 2009), despite discrepancies found in other studies (Margolis et al., 2006; Lammel et al., 2008). Dopamine neurons referred to in this study conform to the electrophysiological and pharmacological criteria, but not necessarily the content of dopamine.

The baseline firing frequency of a neuron was established from a recording period of more than 4 min. Receptor agonists and antagonists were dissolved in aCSF and delivered to the slice via a switch from control aCSF. Agonists were applied for a minimum of 4 min to ensure that equilibrium was reached. The peak frequency was calculated using the mean of three consecutive 10s bins containing 15 - 120 spikes. Sufficient wash-out period (> 20 min) was given between consecutive agonist applications and was verified by obtaining repeatable responses (de Filippi et al., 2010). However, multiple
applications of agonists to a single slice were only performed to illustrate concentration-dependent effects or to compare effects of two agonists on the same neuron.

**Pharmacological agents**
Dopamine hydrochloride, (-)-nicotine hydrogen tartrate (nicotine), cytisine and dihydro-β-erythroidine hydrobromide (DHβE) were obtained from Sigma-RBI (Poole, UK). TC-2559 (5-ethoxy-metanicotine) and ABT-594 [(R)-5-(2-azetidinylmethoxy)-2-chloropyridine] were synthesised at Lilly Chemistry Synthesis Laboratory (UK). (±)-Epibatidine hydrochloride, 5-I-A85380 (3-[(2s)-2-azetidinylmethoxy]-5-iodopyridine), MLA (methyllycaconitine), α-conotoxin MII, CNQX and D-AP5 were purchased from Tocris (Avonmouth, UK).

**Statistics**
Numerical data in the text and figures are expressed as the mean ± standard error of the mean (S.E.M). ANOVA with appropriate posthoc tests, and Student’s t-test, unpaired or paired when appropriate, were used for statistical comparisons.

**Results:**

*Relative maximal effects of nicotinic agonists on recombinant α4β2 receptors*
Functional nicotinic receptors on dopamine neurons include both α4β2* (* may denote α5, α6 or both) and α7 subtypes (Pidoplichko et al., 1997; Picciotto et al., 1998; Klink et al., 2001; Champtiaux et al., 2003). Here, we examined the pharmacological profiles of six nicotinic agonists on recombinant human α4β2 and α7 receptors that are expressed in mammalian cell lines by measuring calcium influx (Chen et al., 2003). The EC$_{50}$ and normalised E$_{max}$ values for each agonist were obtained from concentration-response relationships (Table 1). Agonists displayed a range of EC$_{50}$ values on α4β2 receptors, showing their different potencies. EC$_{50}$ for cytisine could not be reliably calculated (N.D. Table 1) due to the low amplitude of the response. Indeed, cytisine displayed the lowest E$_{max}$ value of 21%, relative to epibatidine. Cytisine and TC-2559 (33%) are, therefore, partial α4β2 agonists, nicotine (75%) and 5-i-A85380 (73%, sum of both high and low-affinity components) more efficacious partial agonists, and epibatidine (100%) and ABT-594 (102%) are full agonists in this assay. The two components in the concentration-response curve for 5-i-A85380 may reflect its different potencies on the alternative stoichiometries of α4β2 receptors assembled with different α4 and β2 subunit ratios (Zwart et al., 2006). On α7 nAChRs, TC-2559 and 5-I-A85380 had no detectable activity at concentrations as high as 100 µM, showing their selectivity for α4β2 receptors. ABT-594 was also more than 20 fold more potent on α4β2 receptors. Nicotine, cytisine and epibatidine are α4β2 agonists, but also have significant activity on α7 receptors. We have, therefore, identified six α4β2 agonists with different relative maximal effects on recombinant human receptors, and TC-2559, 5-I-A85380 and ABT-594 are α4β2-selective.

*Concentration-dependent effects of α4β2 agonists on spontaneous firing rate of dopamine neurons*
We have previously shown that the α4β2 partial agonist TC-2559 increases the firing rate of dopamine neurons concentration-dependently in a DHβE-sensitive
manner (Chen et al., 2003). Here, we compared its relative maximal effects on neuronal firing with the other agonists by examining the concentration-dependent effects (n = 234 neurons, Fig. 1A and B). Nicotine induced effects are shown as an example (Fig. 1A). The concentration-dependent effects for each agonist spanned across concentration range of more than two orders of magnitude. Nicotine and other efficacious agonists, epibatidine, ABT-594, 5-I-A85380, also caused additional firing cessation (#) following the peak increase in firing rate (*) at high concentrations. The concentration-response relationships were fitted with the Hill equation (Fig. 1B).

\[ \alpha_4\beta_2 \text{-selective agonists TC-2559, 5-I-A85380 and ABT-594 showed similar } E_{\text{max}} \text{ values on neuronal firing (Table 1), despite their different } E_{\text{max}} \text{ values on recombinant } \alpha_4\beta_2 \text{ receptors. However, 5-I-A85380 and ABT-594 are more potent than TC-2559 on neuronal firing, which is in agreement with their respective } EC_{50} \text{ values on recombinant } \alpha_4\beta_2 \text{ receptors. The partial agonist TC-2559 is, therefore, relatively more effective on dopamine neuron firing. The } E_{\text{max}} \text{ values of nicotine and epibatidine were also not different from TC-2559 (Table 1 and Fig. 1B), indicating, again, its relatively enhanced effect on neuronal firing. Cytisine, however, induced a submaximal effect, of about 46% of epibatidine, which was enhanced from its 21% activity on recombinant } \alpha_4\beta_2 \text{ receptors. In addition, the maximal absolute increases in firing rate by TC-2559 and cytisine were also relatively larger (Fig. 1C).}

Plotting the \( E_{\text{max}} \) values of agonists on neuronal firing against those on recombinant \( \alpha_4\beta_2 \) nAChRs, hence, found most data points located above the diagonal line (dashed, Fig. 1D), showing relatively enhanced effects of partial agonists on neuronal firing. In addition, TC-2559, nicotine, epibatidine, ABT-594 and 5-I-A85380 show similar \( E_{\text{max}} \) values, with the mean (91.6 ± 3.7%) shown as the dotted line (Fig. 1D), illustrating the similar maximal effects of partial agonists TC-2559, nicotine and 5-I-A85380 to full agonists epibatidine and ABT-594 on neuronal firing.

A linear correlation was, however, found between the \( EC_{50} \) values on neuronal firing and recombinant \( \alpha_4\beta_2 \) receptors (Fig. 1E, \( r = 0.79 \) and slope = 1, the \( EC_{50} \) for 5-I-A85380 was taken from the higher affinity component on recombinant \( \alpha_4\beta_2 \) nAChRs), indicating that agonists stimulate firing rate increase and recombinant \( \alpha_4\beta_2 \) receptors with similar potencies.

**Native \( \alpha_4\beta_2^* \) receptors and firing rate increase in dopamine neurons**

Firing rate increase induced by \( \alpha_4\beta_2 \)-selective agonist TC-2559 was shown to be mediated by (non-\( \alpha_6/\alpha_7 \) )\( \alpha_4\beta_2^* \) receptors previously (Chen et al., 2003). Here, we examined receptor subtypes mediating the effects of non-selective \( \alpha_4\beta_2 \) agonists (Fig. 2A). Cytisine (Cyt, 10 µM, upper panel), nicotine (0.3 µM, middle panel), and epibatidine (Epi, 0.1 µM, lower panel) all caused steady-state increase in firing rate. Nicotinic antagonists mecamylamine (Mec, 10 µM) and DHβE (2 µM) inhibited the effects, confirming the activation of nicotinic receptors. The \( \alpha_3/\alpha_6 \)-selective antagonist α-conotoxin MII (CTX MII, 100 nM) and the \( \alpha_7 \)-selective antagonist methyllycaconitine (MLA, 10 nM) (Fig. 2B) did not cause significant inhibition. This suggests the predominant activation of native \( \alpha_4\beta_2^* \) receptors, with lack of effects by \( \alpha_3/\alpha_6^* \) or \( \alpha_7^* \) receptors.

Co-application of ionotropic glutamate receptor antagonists CNQX (20 µM) and D-AP5 (50 µM) did not significantly affect agonist-induced firing increase (Fig. 2B), showing a lack of contribution from glutamate release. \( \alpha_4\beta_2^* \) receptors
that are located extrasynaptically on somatodendritic sites of dopamine neurons (Lendvai & Vizi, 2008), may thus play a predominant role.

**Agonist-induced depolarisation block of firing**

The relatively enhanced effects of partial agonists could result from increased relative effects on native α4β2* nAChRs. However, a presence of receptor reserve may also enhance the effects of partial agonists. In the latter case, full agonist may induce an additional effect at high concentration when receptors in reserve are activated.

The maximal effects of cytisine and TC-2559 were characterised by persistent increase in firing rate (Fig. 2A upper panel and Chen et al. (2003)), but the maximal effect of nicotine (10 µM) showed an abrupt firing cessation (# in Fig. 1A) after the initial firing rate increase (* in Fig. 1A). Furthermore, epibatidine (1 µM, n = 14, Fig. 3A and B) and ABT-594 (1 µM, n = 7, data not shown) triggered firing blockade following the maximal increase in all neurons.

The firing blockade resembles depolarisation block due to excessive receptor activation and membrane depolarisation. The reduced spike amplitude seen during firing increase confirms the underlying membrane depolarisation (Fig. 3A inset, spike waveform 2). Washing-out of epibatidine rapidly relieved firing blockade and the decreased spike amplitude (Fig. 3A and the overlapping waveforms 1 and 3 in the inset), and the residual firing rate increase was inhibited by DHβE (2 µM, n = 5, Fig. 3A), showing receptor activation.

The depolarisation block of firing by efficacious agonists may indicate their ability to further activate receptors at high concentrations. Plotting agonist-induced incidence of firing blockade (% neurons) against the E\textsubscript{max} values of agonists on recombinant α4β2 receptors revealed a linear correlation (P < 0.05, r = 0.99, slope = 1.1, Fig. 3C), showing that the more effective agonists on α4β2 receptors are more likely to induce depolarisation blockade, presumably by further activation of α4β2* receptors that were surplus for the maximum firing increase, i.e. the “receptor reserve”. Conversely, partial α4β2 agonists, cytisine and TC-2559, showed much lowered incidence of depolarisation block, indicating that they activated all receptors to induce the maximal increase without any reserve. Note that receptor desensitisation may cause rapid decrease of effects similar to receptor antagonism, but not complete cessation of neuronal firing.

Furthermore, for the receptor reserve hypothesis to be true, all agonists must activate the same pool of receptors. Indeed, both firing increase and cessation induced by 1 µM epibatidine were fully blocked by the pre-application of 10 µM TC-2559 (Fig. 4A, B). Partial and full agonists may thus activate the same population of α4β2* receptors for both firing increase and depolarisation block of firing.

As α4β2* nAChRs are also expressed on GABAergic neurons in the VTA (Klink et al. 2001; Mansvelder et al., 2002), agonists may stimulate GABAergic neurons and cause feed-forward inhibition of dopamine neuron firing. Epibatidine (1 µM), however, did not affect firing rate (11.4 ± 12.1% increase, basal frequency = 5.9 ± 2.0 Hz, n = 3, p > 0.05, Fig. 5A), or spike amplitude (Fig. 5A inset, waveform 1 and 2) of GABAergic neurons. Comparing the effects of 1 µM epibatidine, 1 and 100 µM TC-2559, on firing rate of GABAergic neurons (Fig. 5B bar plot) with dopamine neurons (Fig. 5B scatter plot) showed significantly reduced effects (** P<0.01, one-way ANOVA with Dunn’s pair-wise comparisons), in agreement with the previously reported low sensitivity of GABAergic neurons.
to nicotine (Yin & French, 2000). A small receptor pool on GABAergic neurons could contribute to the lower sensitivity to agonists.

**Effects of partial agonist and size of receptor pool**

The presence of a large receptor pool is likely to be essential for the existence of receptor reserve and for partial agonists to exert relatively enhanced effects. We further evaluated the relative size of the receptor pool between dopamine neurons. As the relative maximal effects of cytisine were induced presumably by activating all receptors, they may indicate the relative sizes of the receptor pool in individual dopamine neurons. The effects ranged between 13 and 89% of firing rate increase (n = 10 dopamine neurons), with a coefficient of variance (CV) of 61%, demonstrating considerable variability between the size of the receptor pool.

In dopamine neurons with a larger receptor pool, partial agonists may exert relatively larger effects. When TC-2559 (1 µM, an EC\(_{50}\) concentration) was examined in the same neuron as cytisine (10 µM) (Fig. 6A), the effects were correlated (r = 0.90, slope = 1, ** p < 0.01, n = 10, Fig. 6B), indicating that TC-2559 was more potent in neurons that express larger pool of receptors. In these experiments, TC-2559 was applied before cytisine (Fig. 6C), because its effect was rapidly washed out and repeatable in consecutive applications (Fig. 6C and D). Receptor availability may, therefore, modulate the effects of partial agonists.

**Antagonist effects of partial agonists**

Partial agonists are also thought to be effective antagonists against nicotine due to their potential to occupy a large proportion of the receptor pool. The presence of 10 µM TC-2559 was shown in Fig. 4A was indeed shown to inhibit the effect of epibatidine, demonstrating the antagonist effect of the partial agonist. Pre-application of TC-2559 at the EC\(_{50}\) concentration of 1 µM was also found to inhibit the effect of nicotine (Fig. 7), at the concentration (1 µM) estimated to be maximal in the blood of smokers (Henningfield et al., 1997). Potent antagonist effects of partial agonists are, therefore, shown alongside their relatively enhanced agonist effects.

**Discussion**

Our experiments show that α4β2 partial agonists stimulate dopamine neuron firing via the activation of native α4β2* receptors and induce relatively enhanced maximal effects which are equivalent to full agonists and nicotine. The enhanced effect is predominantly attributed to the presence of receptor reserve, as efficacious α4β2 agonists, but not partial agonists, induced depolarisation block of firing in addition to the maximum firing increase via the activation of receptors in reserve. The maximum firing increase may, therefore, be induced by partial activation of the receptor pool. Consequently, partial agonists elicit maximum firing increase by activating all receptors without reserve or depolarisation block. The existence of native receptor reserve is, therefore, likely to affect the physiological effects of α4β2 partial agonists.

**α4β2 agonist-induced firing rate increase**

Nicotine and nicotinic agonists activate nAChRs on dopamine neurons to induce inward currents and membrane depolarisation (Calabresi et al., 1989; Pidoplichko et al., 1997; Klink et al., 2001), resulting in increased spontaneous firing rate (Picciotto et al., 1998; Yin & French, 2000; Chen et al., 2003; de Filippi
et al., 2010). However, bath application of agonists induced only narrow “window” currents on recombinant αβ2 receptors (Rollema et al., 2010; Papke et al., 2011) and native α3β4* receptors (Lester, 2004) due to profound desensitisation of receptors by agonists at both low and high concentrations. The therapeutic delivery of α4β2 partial agonists may thus not be able to cause any significant agonist effects. However, we confirmed here that bath application of nicotine and α4β2 agonists activate α4β2* receptors and persistently stimulate dopamine neuron firing (Fig. 2) across a wide range of concentrations (Fig. 1), in contrast to the narrow “window” current on recombinant α4β2 receptors. The results may, therefore, indicate reduced, agonist-induced receptor desensitisation on dopamine neurons. Native receptor subtypes and associated proteins (Araud et al., 2010) in dopamine neurons may contribute to the effect.

Both α4β2* (* may denote α5, α6 or both) and α7 subtypes are functional receptors on dopamine neurons (Pidoplichko et al., 1997; Picciotto et al., 1998; Klink et al., 2001; Champtiaux et al., 2003). However, neither α6* nor α7 receptors were activated by α4β2-selective agonist TC-2559, nicotine, epibatidine or cytisine, indicating the predominant effect of (non-α6/α7)α4β2* receptors. In agreement with these findings, α6* nAChRs were shown to be predominantly expressed in the terminal regions of dopamine neurons (Champtiaux et al., 2003) and α7 receptors in less than half of the dopamine neurons (Klink et al., 2001). In addition, native α4β2* receptor currents in dopamine neurons are relative slow and longer-lasting (Pidoplichko et al., 1997; Klink et al., 2001), which may, therefore, underlie the persistent firing increase induced by bath-application of agonists. More interestingly, the incorporation of α5 subunits with α4 and β2 increased the “window” current in oocytes (Papke et al., 2011), suggesting that native α4β2α5 receptors in dopamine neurons (Klink et al., 2001; Champtiaux et al., 2003) could also contribute to agonist-induced, persistent effects. Specific associated proteins that modulate desensitisation of native α4β2* receptors in dopamine neurons are currently unknown.

**Enhanced relative maximal effects of α4β2 partial agonists**

More importantly, this study revealed enhanced relative maximal effects of α4β2 partial agonists on neuronal firing. Our systematic analyses of concentration-dependent effects of six partial and full α4β2 agonists on dopamine neuron firing show that all partial agonists induced larger maximal effects relative to full agonists. Most significantly, the 33% agonist TC-2559 elicited similar maximal firing rate enhancement to nicotine and full agonists, and the 21% agonist cytisine induced 46% of the maximum firing increase (Table 1 and Fig. 1A).

α4β2 agonists increase the firing rate by depolarising the membrane potential upon receptor activation. When full agonists at high concentrations induced additional depolarisation block of firing following the maximum firing increase (Fig. 3), larger membrane depolarisation that inactivates sodium channels was highly likely induced by further activation of receptors. As the α4β2* receptor subtype were shown to be predominantly activated by the agonists (Fig. 2) and partial and full agonists share the same α4β2* receptor pool (Fig. 4), full agonists, therefore, caused the maximum firing increase by activating only a proportion of the receptor pool, and depolarisation block by activating the
rest of the receptors in “reserve” at high concentrations. Partial activation of receptors on dopamine neurons is, therefore, sufficient for the maximum firing increase and a receptor reserve exists for this function. As the less efficacious agonists on recombinant α4β2 receptors induced less frequent depolarisation block (Fig. 3C), partial agonists may only weakly activate the native α4β2* receptors. Partial agonists on recombinant α4β2 receptors could, therefore, still be partial on native rodent receptors. However, by activating all receptors available without leaving any reserve, partial agonists may induce the equivalent partial receptor activation and cause the same maximum effect as full agonists. The experimental results are, therefore, in support of the existence of receptor reserve.

The existence of receptor reserve can enhance the relative maximal effects of partial agonist, and the size of the reserve can further determine which partial agonist to become maximally effective. On dopamine neuron firing, the size of the reserve was large so that the 33% agonist TC-2559 was fully effective, while the 21% partial agonist cytisine was submaximal. On dopamine release from striatal slices, however, both cytisine and TC-2559 exerted submaximal effects compared to nicotine and epibatidine (Smith et al., 2007), indicating a smaller or lack of receptor reserve there. The lower density of α4β2* receptor expression in the striatum (Marks et al., 1983) may be responsible for the smaller receptor pool and receptor reserve.

Does the existence of receptor reserve affect behavioural effects of partial agonists? It is interesting to note that cytisine and TC-2559 were partially and fully generalised to a nicotine cue in a drug discrimination paradigm, respectively (Smith et al., 2007), suggesting that the behavioural responses may be translated from their respective submaximal and maximal effects on dopamine neuron firing. Increased dopamine neuron firing is associated with increased dopamine release from terminals, resulting in greater reward potential. Although efficacious agonists are also effective at stimulating firing increase, high concentrations may also induce depolarisation block of firing, reduce the release of dopamine in the brain and cause adverse behavioural effects. Indeed, while an optimal dose is often observed in nicotine self-administration (Picciotto, 1995; Pons et al., 2008), where the firing rate of dopamine neurons may be optimised for reward, high doses of nicotine cause tonic-clonic seizures in rodents (Salas et al., 2003), possibly due to excessive membrane depolarisation. Efficacious agonists may also excessively stimulate human nicotinic receptors, as smokers are also known to self-regulate nicotine intake to maximize the desirable effects (Benowitz & Jacob, 1985; Kassel et al., 2007). Conversely, partial agonists may also behave predominantly like an antagonist when acting on a nicotinic function without receptor reserve. On the treatment of anxiety, for example, cytisine behaved like an antagonist with negligible agonist effect (Picciotto et al., 2008). Behavioural effects of nicotinic partial agonists are, therefore, determined by their physiological effects, which are modulated by the existence of receptor reserve.

Nicotinic partial agonists as smoking cessation aids

The therapeutic efficacy of a drug stems from the translation of its pharmacological properties on receptors to physiological effects. The translational processes for a receptor agonist are complicated as the balance between receptor activation and desensitisation depends not only on agonist concentration but also the speed and duration of delivery at target receptors. Our
findings show that therapeutic delivery of α4β2 partial agonists may be able to activate native α4β2* receptors on neurons, because bath-applied agonists induced persistent dopamine neuron firing rate increase and prevented effects of nicotine. α4β2 partial agonists can, therefore, both mimic and compete against nicotine to preclude its rewarding effects, in support for the dual mechanisms for smoking cessation.

However, our results further show that partial agonists can become fully effective on neuronal function because of an existence of receptor reserve. For example, on the nicotinic stimulation of dopamine neuron firing, the 33% α4β2 partial agonist TC-2559 is fully effective. Therefore, to achieve partial activity for therapeutic purposes, the relative efficacies of agonists may need to be optimised taking into account of native receptor availability. We now know that on dopamine neuron firing, only partial agonists with relative maximum effects lower than 33% on recombinant human α4β2 receptors, such as cytisine, may elicit partial activity. This is in agreement with cytisine and its derivative, varenicline, being used currently as smoking cessation aids, although it is unknown to what extent receptor reserve may exist in smokers' brain. The results from rodent brain may raise the issue that the interpretation of clinical efficacies of different α4β2 partial agonists requires taking into account of receptor reserve.

In conclusion, this study demonstrated that nicotinic α4β2 partial agonists are more effective at neuronal functions where there is receptor reserve. The size of receptor reserve at targeted neuronal functions may influence the physiological effects and clinical efficacies of partial agonists.

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Statement of conflicts of interest
Authors declare no conflicts of interest. Eli Lilly does not sell any of the drugs or devices mentioned in the article.
Figure legends

Figure 1. Agonists for α4β2 nAChRs induced concentration-dependent firing rate increase in dopamine neurons. A. Nicotine (Nic)-induced concentration-dependent increase of the spontaneous firing rate. Rate histogram is plotted in 10s bins. Increases in firing rate by different concentrations of nicotine are shown. The increase induced by 10 µM nicotine (∗) was followed by a period of firing cessation (#). B. α4β2-selective agonists TC-2559 (TC), 5-i-A85380 (5-i-A) and ABT-594 (ABT), and non-selective agonists, epibatidine (Epi), nicotine (Nic) and cytisine (Cyt), all induced firing increase with increasing concentrations. Concentration-response curves were fitted with the Hill equation (the Hill slope was fixed at 1) and EC_{50} and E_{max} values for each agonist were obtained and shown in Table 1. Mean % firing rate increases (n ≥ 4 for each data point) were obtained for each agonist at the indicated concentrations. Error bars are shown at the negative direction only for clarity. C. The mean maximal increases of firing frequency (Hz) induced by top concentrations of each agonist are compared and the effect of Cyt was significantly smaller compared to the other agonists (* P < 0.05, one-way ANOVA followed by Tukey-Kramer multiple comparisons). D. The E_{max} of α4β2 partial agonists are relatively enhanced on neuronal firing as the data points for Cyt, TC and 5-i-A are located above the diagonal line (dashed). The E_{max} values for Epi, ABT, 5-i-A, Nic and TC are not different from each other and their mean is shown as the dotted line. The E_{max} value for Cyt is significantly lower than the other agonists (* p < 0.05, one-way ANOVA with Dunn’s pair-wise comparisons). E. EC_{50} values of agonists on neuronal firing and on recombinant α4β2 nAChRs are linearly correlated (slope = 1, and the regression line passes the origin), indicating the similar rank order of potencies for agonists on both functions. Cyt is omitted from the plot because its EC_{50} value for recombinant α4β2 nAChRs was not obtained. The other agonists are plotted using the same colored symbols as in D.

Figure 2. Inhibitory effects of nicotinic antagonists on agonist-induced increase in firing rate in dopamine neurons. A. Bath applications of α4β2 partial agonists cytisine (Cyt, 10 µM), nicotine (Nic, 1 µM), and epibatidine (Epi, 0.1 µM), significantly and persistently increased dopamine neuron firing frequency in rat VTA slices. Application periods are indicated by horizontal bars. Agonist-induced effects were inhibited by nicotinic antagonists mecamylamine (Mec 10 µM) and DHβE (2 µM) as indicated. The firing frequency was calculated from 10s bins. B. Effects of antagonists were compared. Agonist-induced effects were inhibited by Mec (10 µM) or DHβE (2 µM), but not by the α3/6 antagonist α-conotoxin MII (CTX MII, 100nM), α7 antagonist methyllycaconitine (MLA, 10nM), or the ionotropic glutamate receptor antagonists CNQX (20 µM) or D-AP5 (50 µM), indicating the predominant involvement of somatodendritic α4β2* nAChRs. Data are presented as mean ± SEM (n = 3-6) for each antagonist.

Figure 3. Agonist-induced firing rate cessation in dopamine neurons. A and B. Epibatidine (Epi, 1 µM) induced an initial firing rate increase and subsequent firing cessation. Spike waveforms before (1), during (2) and after (3) Epi are compared in the inset. The reduced spike amplitude during firing rate increase (2) indicates significant underlying membrane depolarisation. Washing-out of Epi
allowed rapid recovery from firing blockade and reduced spike amplitude. Application of nicotinic antagonist DHβE reduced the firing rate to the baseline level, indicating residual receptor activation. C. % of neurons undergone agonist-induced depolarisation block of firing is plotted against the $E_{\text{max}}$ values of agonists on recombinant α4β2 nAChRs. A statistically significant correlation ($r = 0.99$, slope = 1.1, $P < 0.05$) is found between the effects, showing that the more effective agonists on α4β2 nAChRs are more likely to induce depolarisation block. Abbreviations: Epi for epibatidine; ABT, ABT-594; 5-i-A, 5-I-A85380; Nic, nicotine; TC, TC-2559 and Cyt, cytisine.

Figure 4. The stimulatory effects of epibatidine are inhibited by TC-2559. A and B. Both increase and cessation of firing rate induced by epibatidine (Epi 1 µM) are inhibited by the pre-application of 10 µM TC-2559 (TC 10 µM) (TC+Epi in B, n = 3, * $P < 0.05$, NS $P > 0.05$, one-way ANOVA with Dunn’s pair-wise comparisons).

Figure 5. Reduced effects of agonists on GABAergic neurons. A. Epi at 1 µM did not affect the firing rate or the spike amplitude (inset) of GABAergic neurons. B. Firing rate changes induced by Epi (1 µM) and TC-2559 (TC, 1 and 100 µM) on GABAergic neurons (bar plot) are significantly smaller than on dopamine neurons (scatter plot, **$P < 0.01$ compared to that bar plot, one-way ANOVA with Dunn’s pair-wise comparisons).

Figure 6. Correlation between the effects of TC-2559 and cytisine in individual dopamine neurons. Effects of 1 µM TC-2559 (TC) and 10 µM cytisine (Cyt) were compared in the same neuron (A) and statistically significant correlation between the effects is shown (B. slope = 1 and the regression line passes the origin, **$p < 0.01$, n = 10), demonstrating larger effects of TC in neurons having larger responses to Cyt, which represent the relative size of the receptor pool in each neuron. Two consecutive applications of TC-2559 (1 µM) (1st TC and 2nd TC, n = 7) show repeatable responses (C and D), so that TC is less likely to have residual effects on subsequent Cyt. N.S. denotes no statistical significance ($p > 0.05$). Agonists are applied for 6 min as shown by the horizontal bars.

Figure 7. The antagonist effect of partial agonist. Prolonged applications of TC-2559 (TC, 1 µM) induced persistent increase of firing rate (A and B, * $p < 0.05$, one-way ANOVA with Dunn’s pair-wise comparisons) and inhibited effects of nicotine (Nic 1 µM, A and B, $P > 0.05$, n = 4, N.S. denotes no statistical significance), demonstrating the antagonist effect by receptor occupation.
References


Figure 1
Figure 2

A. 

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B. 

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- DHβE or Mec for Cyt
- CTX MII
- MLA
- CNQX+D-AP5
Figure 3

A

B

C

Epi 1 μM

D

E

Firing frequency (Hz)

Time (min)

1 mV

1 ms

1

2

3

Firing block (% of neurons)

$E_{max}$ on α4β2 (% of 1 μM Epi)

$r = 0.99$

$p < 0.05$
Figure 5

A

B

** P < 0.01

TC-2559
Figure 6
Figure 7

A

Time (min)

B

Frequency (Hz)

Basal  TC  TC + Nic

N.S.