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RESEARCH PAPER

Partial agonists for α4β2 nicotinic receptors stimulate dopaminergic neuron firing with relatively enhanced maximal effects

Ying Chen[1], Lisa M Broad[2], Keith G Phillips[2] and Ruud Zwart[2]

[1]*Faculty of Health and Medical Sciences, University of Surrey, Guildford, UK, and* [2]*Eli Lilly & Co. Erl Wood Manor, Windlesham, Surrey, UK*

Correspondence
Dr Ying Chen, Faculty of Health and Medical Sciences, University of Surrey, Guildford, GU2 7XH, UK. E-mail: ying.chen@surrey.ac.uk

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BACKGROUND AND PURPOSE

Partial agonists selective for α4β2 nicotinic ACh 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 depolarization 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.

Abbreviations

ABT-594 (R)-5-(2-azetidinylmethoxy)-2-chloropyridine; aCSF, artificial cerebrospinal fluid; DHβE, dihydro-β-erythroidine hydrobromide; Mec, mecamylamine; MLA, methyllycaconitine; nAChR, nicotinic ACh 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* nicotinic ACh receptors (nAChRs) are the primary drug target for treating nicotine addiction, possibly because they directly mediate nicotine-induced increase in firing rate in dopaminergic 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 clinical

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 the effects of nicotine 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 (Cahill *et al.*, 2008). However, to what extent the therapeutic delivery of α4β2 partial agonists elicit physiological effects remains controversial.

Chronic administration of partial agonists may predominantly desensitize native α4β2* receptors in the brain (Picciotto *et al.*, 2008), as bath applications of agonists cause a long-term decrease in the response to agonists (Papke and Heinemann, 1994; Quick and 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 desensitization is achieved (Lester, 2004; Rollema *et al.*, 2010; Papke *et al.*, 2011).

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

TC-2559 also induces similar effects to nicotine on dopaminergic neuron firing (Chen *et al.*, 2003; Wang *et al.*, 2006), neurotransmitter release and several cognitive performances (Bencherif *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). Therefore, TC-2559 may still be a partial agonist on native rodent α4β2* receptors.

In the case where a partial agonist on recombinant human α4β2 receptors remains partial on native rodent receptors, its relatively enhanced effects may then be caused by the existence of receptor reserve (Stephenson, 1956; Furchgott and Bursztyn, 1967). Receptor reserve describes the 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 a case, a partial agonist may still weakly activate receptors but elicit the maximum effect by recruiting all the 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 depolarizes the membrane potential and increases the firing rate in dopaminergic neurons (Calabresi *et al.*, 1989), excessive receptor stimulation could depolarize the neurons to a level beyond the firing threshold and cause inactivation of Na^+ channels and failure to generate action potential, cumulating in depolarization block of firing (Grace and Bunney, 1984). Agonist-induced depolarization block may, then, indicate the existence of receptor reserve.

We systematically examined the relative maximal effects of six α4β2 agonists on recombinant α4β2 receptors and increase in dopaminergic neuron firing rate. The results showed relatively enhanced effects of partial agonists on dopaminergic neuron firing and suggest the existence of a nicotinic receptor reserve.

Methods

Calcium influx in mammalian cell lines expressing nAChRs

Human α4β2 nAChRs were stably expressed in HEK293 cells that were cultured in DMEM supplemented with 10% fetal calf serum, 100 $U \cdot mL^{-1}$ penicillin, 100 $\mu g \cdot mL^{-1}$ streptomycin, 4 mM glutamine and 50 $\mu g \cdot mL^{-1}$ geneticin. GH_4 cells that express endogenous Ric-3 were used to express α7 nAChRs (Lansdell *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 BJP's GRAC (Alexander *et al.*, 2011).

Agonist-induced calcium dynamics through open channels (Kuntzweiler *et al.*, 1998; Fucile *et al.*, 2005) were measured using a fluorescent imaging plate reader (FLIPR; Molecular Devices, Winnersh, UK) (Chen *et al.*, 2003; Zwart *et al.*, 2006). Cell lines were plated overnight in black-walled, transparent bottomed 96-well plates (Poly-D-lysine coated, Marathon Laboratories, London, UK), at a density of 0.5×10^6 $cells \cdot mL^{-1}$ for HEK293 cells, and 1.0×10^6 $cells \cdot mL^{-1}$ for GH_4 cells. All cells were grown in a humidified incubator maintained with 5% CO_2 in air at 37°C. Note that changes in incubation temperature may alter the subunit composition of α4β2 receptors and their sensitivity to ACh (Zwart *et al.*, 2006). Cells were loaded with Fluo-3-AM (10 μM in HEPES-buffered saline solution) for 1 h 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 s for a further 2 min. Responses were measured as peak minus basal fluorescence intensity and expressed as percentage of the maximum response induced by 1 μM epibatidine in each experiment. Concentration-response relationships were obtained for each agonist with data from separate experiments, and the EC_{50} and E_{max} values on α4β2, α3β4 and α7 nAChRs were calculated using the Hill equation. Note that the E_{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 (Loughborough, UK) in ice-cold, oxygenated, artificial CSF (aCSF), which contained (in mM): NaCl 123, $NaHCO_3$ 22, NaH_2PO_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 substantial 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 and 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^{-1} at 34°C, the spontaneous neuronal firing can be recorded for more than 6 h, which allows 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 digitized using CED1401 plus (CED, Cambridge, UK) and captured using Spike 2 software (CED).

Two types of spontaneously firing neurons in the VTA were recorded and identified. The majority was the classically defined dopaminergic 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 and Onn, 1989; Johnson and North, 1992). The firing frequency of these neurons was significantly suppressed by 50 μM dopamine (–74.2 ± 4.8%, n = 29) (Yin and French, 2000; Chen *et al.*, 2003). The other type, known as the non-dopaminergic or GABAergic neurons (Grace and Onn, 1989; Johnson and North, 1992), displayed higher firing frequency (4–15 Hz), regular firing pattern and an action potential waveform of a shorter duration (~2 ms); 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). The dopamine neurons referred to in this study conform to these 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 10 s bins containing 15–120 spikes. Sufficient washout 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 synthesized at Lilly Chemistry Synthesis Laboratory (Windlesham, UK). (+/-)-Epibatidine hydrochloride, 5-I-A85380 (3-[(2s)-2-azetidinylmethoxy]-5-iodopyridine), methyllycaconitine (MLA), 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 ± SEM. ANOVA with appropriate *post hoc* 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 dopaminergic 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 normalized 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 (ND in 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) are 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,

Table 1

EC_{50} and E_{max} values of nicotinic agonists on human α4β2 and α7 nAChRs that are stably expressed in mammalian cell lines, and on firing rate enhancement of dopaminergic neurons in the VTA

	Calcium influx in mammalian cells[a]				Increase in firing rate in dopaminergic neurons[d]	
	α4β2		α7			
	EC_{50} (nM)	E_{max} (%)	EC_{50} (nM)	E_{max} (%)	EC_{50} (nM)	E_{max} (%)
Epibatidine	16 ± 2	100	70	100	24 ± 8	84 ± 10
ABT-594	20 ± 2	102 ± 2	560	100	6 ± 3	94 ± 12
Nicotine	830 ± 176	75	2 600	100	279 ± 69	83 ± 11
5-i-A85380	2 and 2 600[b]	29 and 44[b]	>100 000	ND	13 ± 4	103 ± 12
TC-2559[b,c]	180 ± 30	33 ± 2	>100 000	ND	1 036 ± 200	99 ± 10
Cytisine	N.D.	21 ± 3	3 100	100	40 ± 30	39 ± 6*

*P < 0.05; one-way ANOVA followed by Tukey–Kramer multiple comparisons was performed between the E_{max} values on firing rate enhancement.

ND denotes 'not detected' due to the signal being too small.

[a]Fluo-3-AM loaded HEK293 cells in culture were challenged with nicotinic agonists at concentrations ranging from 0.03 nM to 1 mM to obtain full concentration-response curves. Cell population calcium measurements were recorded using a FLIPR, and normalized to the response induced by 1 μM epibatidine. The EC_{50} and Emax values were obtained by fitting the Hill equation to the data (the Hill slope was fixed at 1). Each value represents the mean or mean ± SEM from fitting.

[b]Zwart *et al.*, 2006.

[c]Chen *et al.*, 2003.

[d]Agonist-induced concentration-dependent increase in the spontaneous firing rate (% from baseline) were recorded in putative dopaminergic neurons of the VTA in brain slices. The EC_{50} and E_{max} values were obtained by fitting the Hill equation to data (the Hill slope was fixed at 1). The mean ± SEM are shown.

cytisine and epibatidine are not only α4β2 agonists, but they 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 dopaminergic neurons

We have previously shown that the α4β2 partial agonist TC-2559 increases the firing rate of dopaminergic 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, Figure 1A,B). Nicotine-induced effects are shown as an example (Figure 1A). The concentration-dependent effects for each agonist spanned a 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 (Figure 1B).

The α4β2-selective agonists TC-2559, 5-I-A85380 and ABT-594 showed similar E_{max} values on neuronal firing (Table 1) despite their different E_{max} values on recombinant α4β2 receptors. However, 5-I-A85380 and ABT-594 were more potent than TC-2559 on neuronal firing, which is in agreement with their respective EC_{50} values on recombinant α4β2 receptors. The partial agonist TC-2559 is, therefore, relatively more effective on dopaminergic neuron firing. The E_{max} values of nicotine and epibatidine were also not different from TC-2559 (Table 1 and Figure 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 α4β2 receptors. In addition, the maximal absolute increases in firing rate by TC-2559 and cytisine were also relatively larger (Figure 1C).

Plotting the E_{max} values of agonists on neuronal firing against those on recombinant α4β2 nAChRs, hence, found most data points located above the diagonal line (dashed, Figure 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_{max} values, with the mean (91.6 ± 3.7%) shown as the dotted line (Figure 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 α4β2 receptors (Figure 1E, r = 0.79 and slope = 1, the EC_{50} for 5-I-A85380 was taken from the higher affinity component on recombinant α4β2 nAChRs), indicating that agonists stimulate firing rate increase and recombinant α4β2 receptors with similar potencies.

Native α4β2 receptors and firing rate increase in dopaminergic neurons*

The firing rate increase induced by the α4β2-selective agonist TC-2559 was shown to be mediated by (non-α6/α7)α4β2* receptors previously (Chen *et al.*, 2003). Here we examined

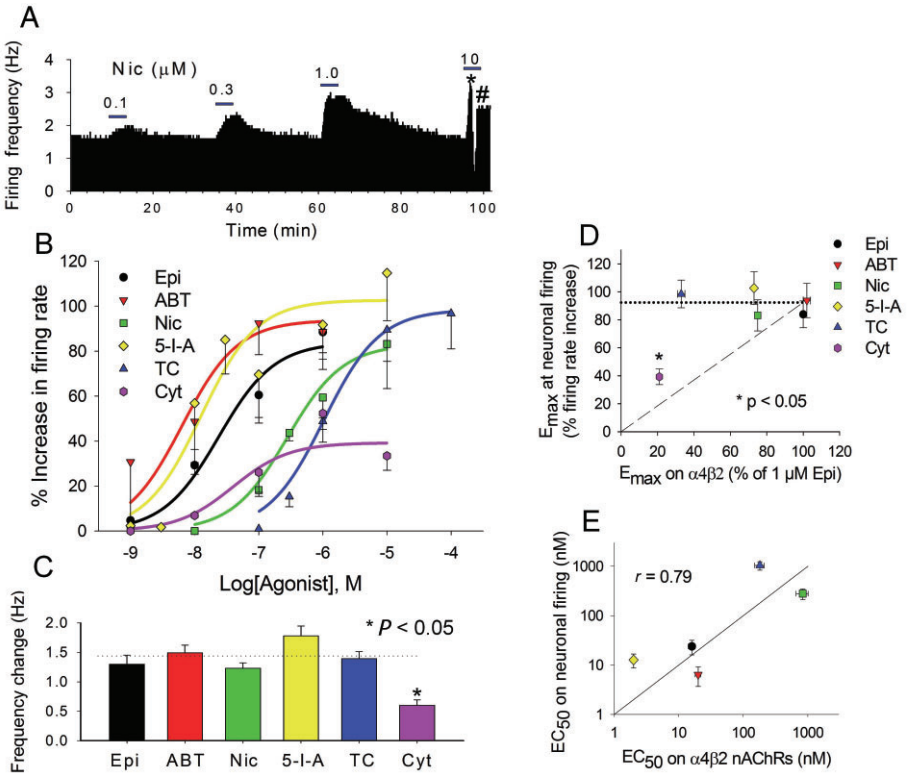

Figure 1

Agonists for α4β2 nAChRs induced a concentration-dependent firing rate increase in dopaminergic neurons. (A) Nicotine (Nic)-induced a concentration-dependent increase in the spontaneous firing rate. Rate histogram is plotted in 10 s 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 ($n \geq 4$ 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 maximum concentrations of each agonist are compared and the effect of Cyt was significantly smaller compared with the other agonists (*$P < 0.05$, one-way ANOVA followed by Tukey–Kramer multiple comparisons). (D) The E_{max} of α4β2 partial agonists is 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 shown using the same colored symbols as in D.

receptor subtypes mediating the effects of the non-selective α4β2 agonists (Figure 2A). Cytisine (Cyt; 10 μM, upper panel), nicotine (Nic; 0.3 μM, middle panel) and epibatidine (Epi; 0.1 μM, lower panel) all caused a steady-state increase in firing rate. Nicotinic antagonists mecamylamine (Mec; 10 μM) and DHβE (2 μM) inhibited these effects, confirming the activation of nicotinic receptors. The α3/6-selective antagonist α-conotoxin MII (CTX MII, 100 nM) and the α7-selective antagonist MLA (10 nM) (Figure 2B) did not cause significant inhibition. This suggests the predominant activation of native α4β2* receptors, with lack of effects on α3/6* or α7* receptors.

Co-application of the ionotropic glutamate receptor antagonists CNQX (20 μM) and D-AP5 (50 μM) did not significantly affect the agonist-induced firing increase (Figure 2B), showing a lack of contribution from glutamate release. α4β2* receptors, located extrasynaptically on somatodendritic sites of dopaminergic neurons (Lendvai and Vizi, 2008), may thus play a predominant role in the effects of these non-selective nicotinic agonists.

Agonist-induced depolarization block of firing

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

The maximal effects of cytisine and TC-2559 were characterized by a persistent increase in firing rate [Figure 2A upper panel and Chen *et al.*, (2003)], but the maximal effect of nicotine (10 μM) showed an abrupt firing cessation (# in

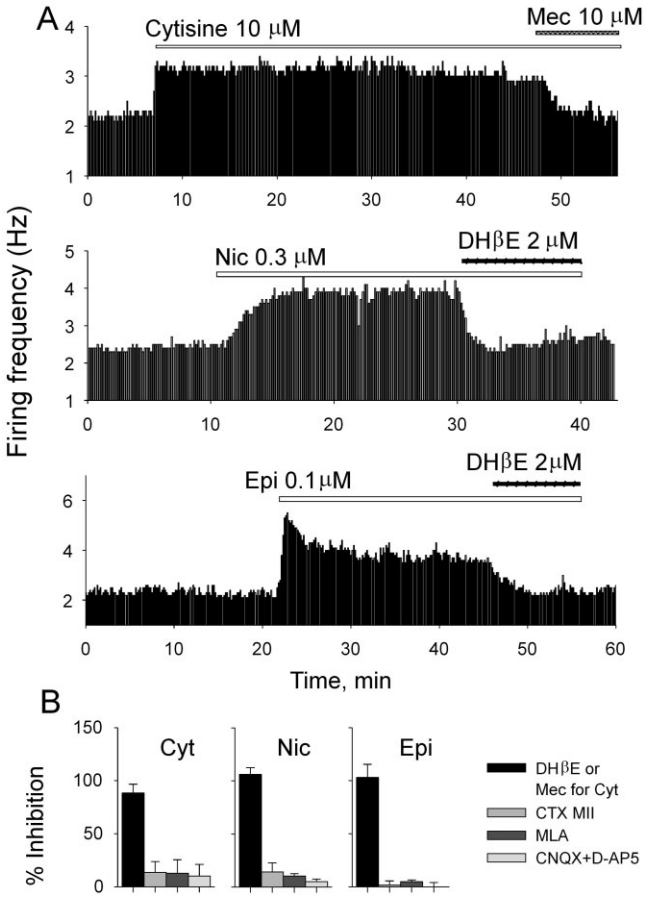

Figure 2

Inhibitory effects of nicotinic antagonists on agonist-induced increase in firing rate in dopaminergic 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 dopaminergic 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 10 s 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; 100 nM), α7 antagonist methyllycaconitine (MLA; 10 nM) 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.

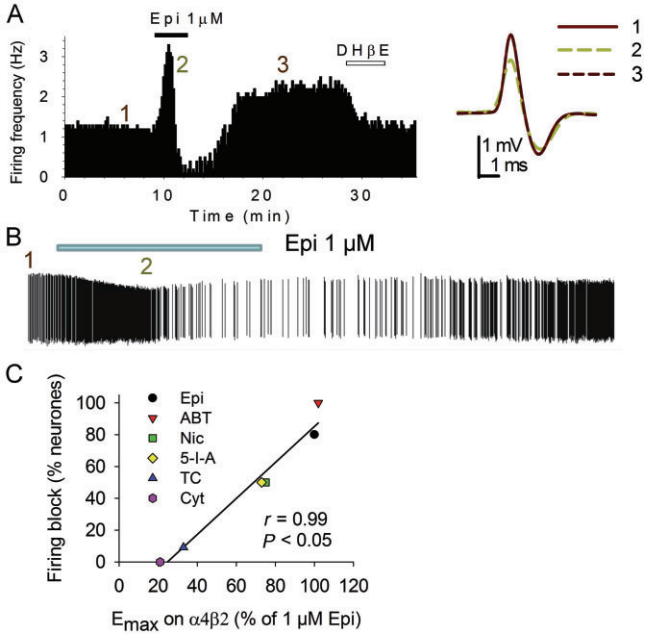

Figure 3

Agonist-induced firing rate cessation in dopaminergic 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 depolarization. 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 that underwent agonist-induced depolarization block of firing is plotted against the E_{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 depolarization block. ABT, ABT-594; 5-I-A, 5-I-A85380; Nic, nicotine; TC, TC-2559 and Cyt, cytisine.

Figure 1A) after the initial firing rate increase (* in Figure 1A). Furthermore, epibatidine (1 μM, n = 14, Figure 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 depolarization block due to excessive receptor activation and membrane depolarization. The reduced spike amplitude seen during firing increase confirms the underlying membrane depolarization (Figure 3A inset, spike waveform 2). Washing out of epibatidine rapidly relieved firing blockade and the decreased spike amplitude (Figure 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, Figure 3A), showing receptor activation.

The depolarization 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_{max} values of agonists on recombinant α4β2 receptors revealed a linear correlation (P < 0.05, r = 0.99, slope = 1.1, Figure 3C), showing that the more effective agonists on α4β2 receptors are more likely to induce depolarization blockade, presumably by further activation of α4β2* receptors that were surplus for the maximum firing increase, that is, the 'receptor reserve'. Conversely, the partial α4β2 agonists, cytisine and TC-2559, showed a much lowered incidence of depolarization block, indicating that they activate all receptors to induce the maximal increase without any reserve. Note that receptor desensitization may cause a 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.

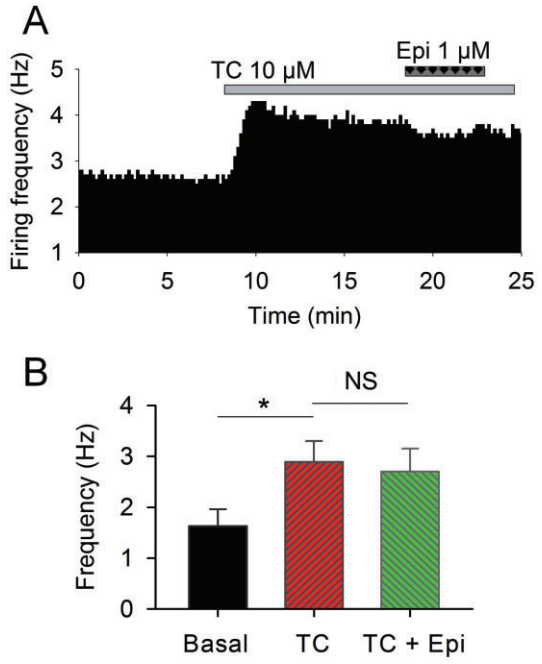

Figure 4

The stimulant effects of epibatidine are inhibited by TC-2559. (A and B) Both increase and cessation of firing rate induced by epibatidine (Epi; 1 μM) were 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).

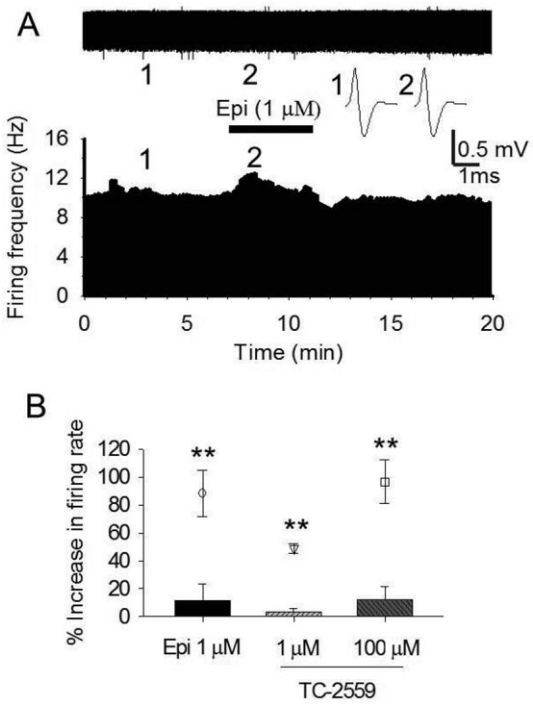

Figure 5

Reduced effects of agonists on GABAergic neurons. (A) Epibatidine (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 (1 and 100 μM) on GABAergic neurons (histogram) were significantly smaller than on dopaminergic neurons (scatter plot, $^{**}P < 0.01$, one-way ANOVA with Dunn's pair-wise comparisons).

Indeed, both firing increase and cessation induced by 1 μM epibatidine were fully blocked by the pre-application of 10 μM TC-2559 (Figure 4A,B). Partial and full agonists thus activate the same population of α4β2* receptors for both firing increase and depolarization 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 dopaminergic neuron firing. Epibatidine (1 μM), however, did not affect firing rate (11.4 ± 12.1% increase, basal frequency = 5.9 ± 2.0 Hz, $n = 3$, $P > 0.05$, Figure 5A), or spike amplitude (Figure 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 (Figure 5B histogram) with dopaminergic neurons (Figure 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 and 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 dopaminergic 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 dopaminergic neurons. The effects ranged between 13 and 89% of firing rate increase ($n = 10$ dopaminergic neurons), with a coefficient of variance of 61%, demonstrating considerable variability between the size of the receptor pool.

In dopaminergic 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) (Figure 6A), the effects were correlated ($r = 0.90$, slope = 1, $^{**}P < 0.01$, $n = 10$, Figure 6B), indicating that TC-2559 was more potent in neurons that express a larger pool of receptors. In these experiments, TC-2559 was applied before cytisine (Figure 6C), because its effect was rapidly washed out and repeatable for consecutive applications (Figure 6C,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

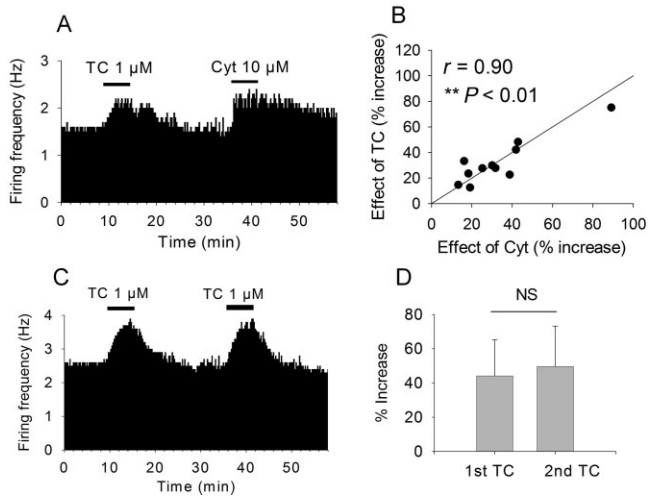

Figure 6

Correlation between the effects of TC-2559 (TC) and cytisine (Cyt) in individual dopaminergic neurons. Effects of 1 μM TC and 10 μM 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 (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. NS denotes no statistical significance ($P > 0.05$). Agonists are applied for 6 min as shown by the horizontal bars.

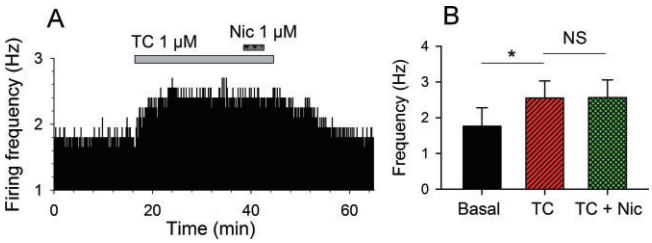

Figure 7

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

proportion of the receptor pool. The presence of 10 μM TC-2559 was shown in Figure 4A 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 (Figure 7), at a 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 dopaminergic neuron firing via the activation of native α4β2* receptors and induce relatively enhanced maximal effects that 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 depolarization 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 depolarization 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 dopaminergic neurons to induce inward currents and membrane depolarization (Calabresi *et al.*, 1989; Pidoplichko *et al.*, 1997; Klink *et al.*, 2001), resulting in increased spontaneous firing rate (Picciotto *et al.*, 1998; Yin and French, 2000; Chen *et al.*, 2003; de Filippi *et al.*, 2010). However, bath application of agonists induced only narrow 'window' currents on recombinant α4β2 receptors (Rollema *et al.*, 2010; Papke *et al.*, 2011) and native α3β4* receptors (Lester, 2004) due to profound desensitization 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 dopaminergic neuron firing (Figure 2) across a wide range of concentrations (Figure 1), in contrast to the narrow 'window' current on recombinant α4β2 receptors. The results may, therefore, indicate reduced, agonist-induced receptor desensitization on dopaminergic neurons. Native receptor subtypes and associated proteins (Araud *et al.*, 2010) in dopaminergic neurons may contribute to the effect.

Both α4β2* (* may denote α5, α6 or both) and α7 subtypes are functional receptors on dopaminergic 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 dopaminergic neurons (Champtiaux *et al.*, 2003) and α7 receptors in less than half of the dopaminergic neurons (Pidoplichko *et al.*, 1997; Klink *et al.*, 2001). In addition, native α4β2* receptor currents in dopaminergic 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 dopaminergic neurons (Klink *et al.*,

2001; Champtiaux *et al.*, 2003) could also contribute to agonist-induced, persistent effects. Specific associated proteins that modulate desensitization of native α4β2* receptors in dopaminergic 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 dopaminergic 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 Figure 1A).

α4β2 agonists increase the firing rate by depolarizing the membrane potential upon receptor activation. When full agonists at high concentrations induced additional depolarization block of firing following the maximum firing increase (Figure 3), larger membrane depolarization that inactivates sodium channels was highly likely induced by further activation of receptors. As the α4β2* receptor subtype was shown to be predominantly activated by the agonists (Figure 2) and partial and full agonists share the same α4β2* receptor pool (Figure 4), full agonists, therefore, caused the maximum firing increase by activating only a proportion of the receptor pool, and depolarization block by activating the rest of the receptors in 'reserve' at high concentrations. Partial activation of receptors on dopaminergic 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 depolarization block (Figure 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 a partial agonist, and the size of the reserve can further determine which partial agonist becomes maximally effective. On dopaminergic 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 with nicotine and epibatidine (Grady *et al.*, 2007; 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 generalized 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 dopaminergic 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 depolarization 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 dopaminergic neurons may be optimized for reward, high doses of nicotine cause tonic-clonic seizures in rodents (Salas *et al.*, 2003), possibly due to excessive membrane depolarization. Efficacious agonists may also excessively stimulate human nicotinic receptors, as smokers are also known to self-regulate nicotine intake to maximize the desirable effects (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 desensitization depends not only on agonist concentration but also on 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 a persistent increase in dopaminergic neuron firing rate and prevent the 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 the existence of a receptor reserve. For example, for the nicotinic stimulation of dopaminergic 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 optimized, taking into account native receptor availability. We now know that for dopaminergic neuron firing, only partial agonists with relative maximum effects lower than 33% on recombinant human α4β2 receptors, such as cytisine, elicit partial activity. This is in agreement with cytisine and its derivative, varenicline, being used currently as smoking cessation aids, although it is not known to what extent receptor reserve may exist in a smokers' brain. Our results obtained in rodent brain suggest that the interpretation of clinical efficacies of different α4β2 partial agonists requires receptor reserve to be taken into account.

In conclusion, this study demonstrated that nicotinic α4β2 partial agonists are more effective at neuronal functions

where there is receptor reserve. The size of the receptor reserve at targeted neuronal functions may influence the physiological effects and clinical efficacies of partial agonists.

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Conflicts of interest

The authors declare no conflicts of interest. Eli Lilly does not sell any of the drugs or devices mentioned in the article.

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