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RESEARCH PAPER

CC4, a dimer of cytisine, is a selective partial agonist at α4β2/α6β2 nAChR with improved selectivity for tobacco smoking cessation

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BACKGROUND AND PURPOSE

Many of the addictive and rewarding effects of nicotine are due to its actions on the neuronal nicotinic ACh receptor (nAChR) subtypes expressed in dopaminergic mesocorticolimbic cells. The partial agonists, cytisine and varenicline, are helpful smoking cessation aids. These drugs have a number of side effects that limit their usefulness. The aim of this study was to investigate the preclinical pharmacology of the cytisine dimer1,2-bis*N*-cytisinylethane (CC4).

EXPERIMENTAL APPROACH

The effects of CC4 on nAChRs were investigated using *in vitro* assays and animal behaviours.

KEY RESULTS

When electrophysiologically tested using heterologously expressed human subtypes, CC4 was less efficacious than cytisine on neuronal α4β2, α3β4, α7 and muscle-type receptors, and had no effect on 5-hydroxytryptamine3 receptors. Acting through α4β2 and α6β2 nAChRs, CC4 is a partial agonist of nAChR-mediated striatal dopamine release and, when co-incubated with nicotine, prevented nicotine's maximal effect on this response. In addition, it had low affinity for, and was less efficacious than nicotine and cytisine on the α3β4 and α7-nAChR subtypes. Like cytisine and nicotine, CC4-induced conditioned place preference (CPP), and its self-administration shows an inverted-U dose–response curve. Pretreatment with non-reinforcing doses of CC4 significantly reduced nicotine-induced self-administration and CPP without affecting motor functions.

CONCLUSION AND IMPLICATIONS

Our *in vitro* and *in vivo* findings reveal that CC4 selectively reduces behaviours associated with nicotine addiction consistent with the partial agonist selectivity of CC4 for β2-nAChRs. The results support the possible development of CC4 or its derivatives as a promising drug for tobacco smoking cessation.

Abbreviations

5-HT3, 5-hydroxytryptamine3; ACh, acetylcholine; CC4, 1,2-bisN-cytisinylethane; CPP, conditioned place preference; CSF, cerebrospinal sterile fluid; Cyt, cytisine; DA, dopamine; DhβE, dihydro-β-erythroidine; i.c.v., intracerebroventricular; IPn, nucleus interpeduncularis; MEC, mecamylamine; MII, αconotoxin MII; NA, noradrenaline; nAChR, neuronal nicotinic ACh receptor; PMSF, phenylmethanesulfonylfluoride

Introduction

Tobacco dependence, the most frequent preventable cause of death, is a chronic relapsing disorder in which compulsive tobacco use persists despite its known negative effects on health. Nicotine is the main psychoactive substance in tobacco, and its addictive properties are due to its interaction with neuronal nicotinic Ach receptors (nAChRs). nAChRs are a heterogeneous family of ligand-gated cation channels present in the CNS and in the peripheral nervous system. The most widely expressed nAChR subtype in brain is the heteromeric α4β2*-nAChR (* indicates the possibility of additional subunits). The homomeric α7 receptors is quite widely expressed while α6β2*-nAChRs are found only in selected areas [reviewed in (Gotti *et al.*, 2009)].

In the CNS whereas a few nAChR have somatodendritic post-synaptic localizations, most nAChR are presynaptic and/or preterminal where they modulate the release of many neurotransmitters, including dopamine (DA), noradrenaline (NA) ACh, GABA and glutamate, [reviewed in (Gotti *et al.*, 2006)]. Consequently, nicotine modifies a large number of physiological processes such as locomotion, nociception, anxiety, learning and memory, and induces a number of behavioural responses that are directly related to its addictive properties, including reinforcing effects and withdrawal symptoms (reviewed in Dani and Bertrand, 2007; De Biasi and Dani, 2011). nAChR expressed in the mesocorticolimbic pathway, one of the main reinforcement circuits of the brain, where they modulate DA release (reviewed in Livingstone and Wonnacott, 2009; Quik and Wonnacott, 2011) could be particularly important. Pharmacological and behavioural studies with animal models including mice with selective deletions of nAChR subunits have demonstrated that the α4β2* and α6β2* subtypes expressed in DA neurons play a pivotal role in mediating the addictive and rewarding properties of nicotine [reviewed in (Changeux, 2010)]. The α3β4*-nAChR is the predominant subtype in the autonomic ganglia and adrenal medulla, but is expressed in only a very limited number of areas of the CNS primarily the habenulo-interpeduncular system (reviewed in Gotti *et al.*, 2009). The α3β4*-nAChR plays a primary role in the manifestation of nicotine withdrawal (De Biasi and Dani, 2011) and in alcohol dependence (Chatterjee *et al.*, 2011).

In the search for new drugs that selectively act on DA release, particular attention has been given to cytisine (Cyt), a nicotinic agonist, that has been used as a smoking cessation drug in Eastern and Central European countries since the 1960s (Etter and Stapleton, 2006). Cyt is an interesting lead because it has an inherent preference for neuronal nAChR over muscle-type nAChR. Cyt has a high affinity for many nAChR subtypes, but its agonist properties vary markedly among these subtypes and can functionally distinguish them (Chavez-Noriega *et al.*, 1997). A number of Cyt-related compounds have also been prepared (Rollema *et al.*, 2007a,b).

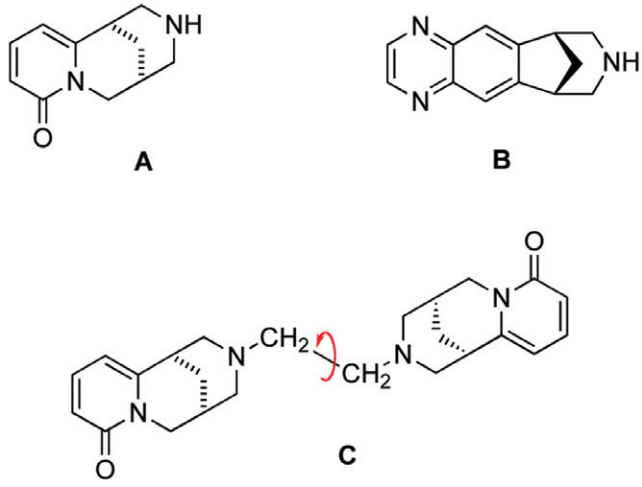

Figure 1
Chemical structure of cytisine (A), varenicline (B) and CC4 (C).

One of them [varenicline (Chantix) (Coe *et al.*, 2005)] has high binding affinity for the α4β2 subtype and has demonstrated therapeutic potential for smoking cessation (reviewed in Rollema *et al.*, 2007b; 2010), as well as for the treatment of alcohol dependence (McKee *et al.*, 2009) and depression (reviewed in Mineur and Picciotto, 2010). However, serious side effects have been reported in patients taking varenicline including adverse cardiovascular effects and/or neuropsychiatric events such as (but not limited to) depression, suicidal ideation, suicide attempts and completed suicide (Freedman, 2007; Moore *et al.*, 2011; Singh *et al.*, 2011).

In our search for new Cyt-derived subtype-selective drugs, with improved brain penetration and a longer half-life, we concentrated on 1,2-bis*N*-cytisinylethane (CC4) (see Figure 1), which was synthesized and has been partially characterized by our group (Canu Boido and Sparatore, 1999; Carbonnelle *et al.*, 2003). It is part of a set of compounds containing two Cyt molecules joined by linkers of different length and nature (Canu Boido *et al.*, 2003).

In the current study, we tested CC4 for its affinity and potency at various nAChR subtypes *in vitro* in relation to the mesolimbic nAChR α4β2* and α6β2* subtypes. The effects of CC4 were subsequently compared with the effects of varenicline, Cyt and nicotine. We also assessed *in vivo* ability of CC4 to reverse the reinforcing effect of nicotine using conditioned place preference (CPP) and i.c.v self-administration paradigms.

Consistent with the pharmacological properties identified with *in vitro* experiments, *in vivo* experiments demonstrated that CC4 induced a slight reinforcing effect *per se*, decreased nicotine-induced CPP and nicotine self-administration.

Methods

Receptor nomenclature is conformed to BJP's Guide to Receptors and Channels (Alexander *et al.*, 2011).

Animals

All of the rat experimental procedures were carried out in accordance with the European Community Council Directive no. 86/609/EEC and the subsequent Italian Law on the 'Protection of animals used for experimental and other scientific reasons'. The mouse experiments carried out at the Institute for Behavioral Genetics, University of Colorado, Boulder, CO, USA, were in accordance with the Guidelines of the National Institutes of Health, USA, and with approval of the Animal Care and Utilization Committee of the University of Colorado, Boulder, CO USA. Every effort was made to minimize the number of animals used and their discomfort.

Male Wistar rats (Charles River, Calco, Como, Italy) weighing 250–300 g were housed in individual cages in a climatically controlled colony room with a 12/12-h light/dark cycle (lights on at 08:00 h).

Mice (C57BL/6J) were bred and maintained at the Institute for Behavioral Genetics, University of Colorado, Boulder, CO, USA. After weaning, mice were housed five to a cage with same sex littermates and allowed free access to food and water. A 12/12-h light dark cycle (lights on at 08:00 h) was used. The vivarium was maintained at 22°C.

Binding to nicotinic receptor subtypes

The brain tissue preparations, immunoimmobilized α6β2̃nAChR receptors and heterologously expressed human α3β4-nAChR were prepared as previously described (Bolchi *et al.*, 2011). For [^{125}I]-αbungarotoxin, non-specific binding was determined in parallel by means of incubation in the presence of 1 μM unlabelled αbungarotoxin and for [^{3}H]-epibatidine, non-specific binding was determined by incubation with 10 nM epibatidine The inhibition of [^{3}H]-epibatidine and [^{125}I]-αbungarotoxin binding by nicotine, Cyt, varenicline and CC4 was measured by incubating samples with increasing concentrations of each compound for 5 min, followed by overnight incubation at 4°C, with 0.1 nM [^{3}H]-epibatidine (in the case of the α4β2-nAChR and α6β2-nAChR subtypes) or 0.25 nM (in the case of the α3β4̃nAChR subtype) and at room temperature with 1 nM [^{125}I]-αbungarotoxin for the α7-nAChR subtype. After incubation, the wells containing the α6β2-nAChR subtype or the membrane-bound α4β2̃, α3β4̃ or α7̃nAChR subtypes were washed five times with ice-cold PBS. [^{3}H]-Epibatidine binding was determined by means of liquid scintillation counting in a β counter, and [^{125}I]-αbungarotoxin samples by direct counting in a γ counter.

The LIGAND program was used to calculate K_i values of all the tested compounds using data obtained from at least three independent saturation and competition-binding experiments.

Electrophysiological measurements

cDNAs coding for human neuronal nicotinc subunits were obtained from Janssen Pharmaceuticals (Titusville, NJ, USA), for human muscle nicotinic subunits from Andrew G. Engel (Mayo Clinic, Rochester, MN, USA) and for human 5-hydroxytryptamine$_3$ (5-HT$_3$) subunits from The Institute for Genomic Research (TIGR, Rockville, MD, USA).

Human neuronal α7, α3β4-, α4β2-, and muscle $(\alpha1)_2\beta1\varepsilon\tilde{\delta}$nAChRs, and also human 5-HT$_3$ receptors 5-HT$_3$A and 5-HT$_3$B were expressed by transient transfection in the rat anterior pituitary GH4C1 cell line (Fucile *et al.*, 2003) by adding 1 μg of each subunit cDNA to each dish, together with 4 μL lipofectamine.

The electrophysiological measurements were made as previously described: for human muscle (Fucile *et al.*, 2006; Piccari *et al.*, 2011), for the human α4β2- and α3β4-nAChR (Lax *et al.*, 2002; Dallanoce *et al.*, 2011) and for the 5-HT$_3$ (Davies *et al.*, 1999). Briefly, whole-cell currents were recorded using borosilicate glass patch pipettes (3–6 MΩ tip resistance) connected to an Axopatch 200A amplifier (Molecular Devices, Union City, CA, USA). Data were recorded and analysed using pCLAMP10 software (Molecular Devices). Whole-cell capacitance and patch series resistance (5–15 MΩ) were estimated from slow-transient compensations, with a series-resistance compensation of 70–90%. Cells were voltage clamped at a holding potential of –70 mV and continuously superfused using a gravity-driven perfusion system with independent tubes for standard and agonist-containing solutions, positioned 50–100 μm from the patched cell. A fast exchanger system (RSC-100, BioLogique, Nice, France) allowed complete solution exchange in less than 50 ms. Data are given as means ± standard error of the mean (SEM) dose–response curves were constructed by sequentially applying different concentrations of agonists, and normalizing the current amplitudes to the value obtained by using 1 mM ACh on the same cell. Data were best-fitted to a Hill equation.

[^{3}H]Neurotransmitter release

Slices. [^{3}H]-DA in rat striatal slices and [^{3}H]-NA in rat hippocampal slices were measured using 96-well assays as previously described (Pucci *et al.*, 2011). Striatal DA and hippocampal NA release were expressed as the fractional percentage of total radioactivity in each well. Basal release was subtracted from stimulated release in each sample, and the results were normalized to the maximal release evoked by nicotine 10 μM (striatum) or 100 μM (hippocampus).

Concentration–response parameters were determined by means of nonlinear regression analysis using Prism 4.0 (GraphPad, San Diego, CA, USA). Pooled normalized data were analysed in Prism to determine EC_{50}, IC_{50} and E_{max} as a percentage of nicotine response.

Synaptosomes. To measure both [^{3}H]-DA and [^{3}H]-ACh release, crude mouse synaptosomes were prepared from freshly dissected striatum and nucleus interpeduncularis (IPn). The methods of (Salminen *et al.*, 2004; 2007) were used to measure [^{3}H]-DA release. To measure the [^{3}H]-ACh release, the methods of (Grady *et al.*, 2001; 2009) were followed.

^{86}Rb^{+} efflux.rubidium efflux

^{86}Rb^{+} efflux was measured as described previously (Marks *et al.*, 1999; 2010). Crude synaptosomes prepared from mouse thalamus were loaded with ^{86}Rb^{+} efflux and superfused with buffer (NaCl, 135 mM; CsCl, 5 mM; KCl, 1.5 mM; $CaCl_2$,

2 mM; $MgSO_4$, 1 mM; HEPES ½Na, 25 mM; glucose, 25 mM; atropine, 1 μM; tetrodotoxin, 50 nM; BSA, 1 $g{\cdot}L^{-1}$). Agonist activity was determined by stimulating the synaptosomes with varying concentrations of CC4 for 5 s. Inhibition was measured by exposing the synaptosomes to varying concentrations of CC4 for 8 min before stimulation with 10 μM ACh for 5 s. $^{86}Rb^+$ was measured by online detection with IN-US β-ram equipped with Cherenkov cells (LabLogic, Bradenton, FL, USA). EC_{50}, maximal efflux (for activation) and DC_{50} values (for desensitization) were determined by least squares nonlinear regression of the results from six separate preparations as described earlier for neurotransmitter release.

CPP

Apparatus. CPP was tested in a shuttle box as described elsewhere (Braida *et al*., 2008). Briefly, the apparatus was divided into two equally sized compartments separated by a guillotine door. Each compartment had different visual and textured cues in the form of brown and white horizontal lines or circles and rough or smooth wooden floor. The visual and tactile cues were balanced such that no evident preference was exhibited before conditioning.

Procedure. The task consisted of three phases: preconditioning, conditioning and post-conditioning.

Preconditioning. On Days 1–2, the rats were allowed to explore the two compartments for 15 min each day. To check for any initial unconditioned preference for either of the two sites, the time spent by each animal in the two compartments on the third day was recorded.

Conditioning. Conditioning sessions (four for nicotine or nicotinic partial agonists and four for vehicle) were conducted once daily (09:00 h) for 8 days. Five minutes after the i.p. injection of nicotine, CC4 or Cyt animals were confined in the conditioned compartment for 30 min, with the door closed. On alternate days, animals receiving vehicle (saline) were confined in the opposite compartment for 30 min. For the antagonism studies, rats received, before each drug pairing, an i.p. injection of different partial agonists or mecamylamine (MEC). After treatment, animals were placed in the conditioned compartment for 30 min. On alternate days, animals receiving double injection of appropriate vehicle were confined to the opposite compartment for 30 min. Drug–texture pairings were always counterbalanced.

Post-conditioning. On the test day, neither drug nor vehicle was injected. Each rat was put at the intersection of the two compartments, with access to both sides, and the time spent in each of the two compartments was measured over a 15-min period as an indicator of rewarding properties.

I.c.v. self-administration

This method has been previously described (Braida *et al*., 1998). Briefly, animals were individually trained for 1 h a day to press two active levers in an operant chamber to obtain drinking water as reinforcer for 1 week in a continuous reinforcement schedule. Then, animals were anaesthetized with chloral hydrate (450 $mg{\cdot}kg^{-1}$ i.p.) and implanted with i.c.v. double guide stainless steel cannulas (22 gauge), anchored to a pedestal as described elsewhere (Braida *et al*., 1998). One week after implantation with i.c.v. double guide stainless steel cannulas, animals were daily trained to receive an infusion of 2 $\mu L{\cdot}8\ s^{-1}$ of vehicle each time they pressed either lever, through a bilateral injection cannula inserted in the double guide and connected to two infusion pumps. Each infusion delivered artificial sterile CSF prepared according to Silvia *et al*. (1994) as follows: 124 mM NaCl, 1 mM KCl, 1.24 mM KH_2PO_4, 1.3 mM $MgSO_4$, 26 mM $NaHCO_3$, 2.4 mM $CaCl_2$, 10 mM glucose. When a stable baseline was reached (at least 5 days with no more than 15% difference across the session), drug sessions were carried out. On the basis of individual preference, the preferred lever was always associated with the vehicle and the non-preferred one with the drug. Within this group, each unit dose was given in a counterbalanced order and only when the baseline response for the preceding unit dose was stable. Then each rat, already checked during training for its preference for one of the two levers, was evaluated in a continuous reinforcement schedule for operant responding after self-administration of the different drugs during a 1-h daily session. During the testing procedure, water was delivered after each lever pressing. The experiment continued until a stable baseline on five consecutive days was again achieved (about 2 weeks).

Spontaneous motor activity

Spontaneous motor activity was evaluated as previously described (Braida *et al*., 2002) for more details, see the Supporting Information.

Drugs

For *in vitro* studies, non-radioactive epibatidine, dihydro-β-erythroidine (DhβE), 5-hydroxytryptamine, MEC, nicotine, nomifensine, pargyline, varenicline, PMSF, Tris, Triton X-100, glucose, NaCl, KCl, $MgSO_4$, $CaCl_2$, KH_2PO4, $NaHCO_3$, ascorbic acid and protease inhibitors were purchased from Sigma-Aldrich (St. Louis, MO, USA). CC4 and Cyt were synthesized as described by (Carbonnelle *et al*., 2003) $\tilde{\alpha}$conotoxin MII was synthesized as described by (Pucci *et al*., 2011). α-Bungarotoxin was supplied by Tocris (Bristol, UK). (±)-[^{3}H]-Epibatidine (specific activity 45–65 $Ci{\cdot}mmol^{-1}$), [^{3}H]-DA (specific activity 50–62 $Ci{\cdot}mmol^{-1}$), [^{3}H]-NA (specific activity 40–50 $Ci{\cdot}mmol^{-1}$), [^{3}H]-choline (methyl-^{3}H, 60–90 $Ci{\cdot}mmol^{-1}$), and [^{125}I]-αbungarotoxin (10–20 $\mu C{\cdot}mg^{-1}$) were purchased from Perkin-Elmer (Boston, MA, USA).

For CPP test, nicotine (0.01–0.2 $mg{\cdot}kg^{-1}$), Cyt (0.1–2.5 $mg{\cdot}kg^{-1}$) and CC4 (0.05–10 $mg{\cdot}kg^{-1}$) were dissolved in saline and given i.p. in a volume of 5 $mL{\cdot}kg^{-1}$. For antagonism studies, MEC (10 $mg{\cdot}kg^{-1}$), CC4 (5 or 10 $mg{\cdot}kg^{-1}$) and Cyt (2.5 $mg{\cdot}kg^{-1}$) were given i.p. 10 min before the maximal reinforcing dose of nicotine (0.1 $mg{\cdot}kg^{-1}$). For i.c.v. self-administration, increasing concentrations of nicotine (0.003–0.3 μg·2 μL), CC4 (0.003–0.3 μg·2 μL) and Cyt (0.001–0.5 μg·2 μL) were delivered after pressing the less preferred lever. MEC (10 $mg{\cdot}kg^{-1}$) or CC4 (5 $mg{\cdot}kg^{-1}$) were given i.p. 30 min before each daily session during which nicotine (0.1 μg per infusion) was delivered.

Statistical analysis

Data from behavioural experiments were expressed as mean ± SEM and analysed by one-way ANOVA followed by *post hoc* Tukey's test. Owing to the individual animal's baseline in the self-administration experiments, different numbers of sessions (from 15 to 20) were needed to reach a stable baseline of lever pressing (less than 15% difference across the sessions) with each drug unit dose. Thus, statistical analyses involved only the last 5 days of stable baseline. During this period of stable baseline, the mean total daily intake (μg) was calculated against the self-administered unit doses. The accepted level of significance was $P < 0.05$. All statistical analyses were done with software Prism, version 5 (GraphPad).

Results

In vitro *characterization*

Binding to nAChR subtypes. The binding affinities (K_i) of CC4, varenicline, Cyt and nicotine for native rat α6β2-, α4β2- and α7̃nAChR subtypes, and the transfected human α3β4-nAChR subtype, were determined with competition-binding experiments (Table 1 and Figure 2). Varenicline had the highest affinity for all of the subtypes, followed by Cyt, nicotine and CC4. Although CC4 had the lowest affinity for all of the subtypes, it exhibited the highest selectivity ratios between the α3β4- and α4β2̃nAChR sites (185-fold between α3β4̃ and α6β2̃nAChR sites (400-fold) and between α7̃nAChR and α4β2̃nAChR sites (500-fold) and α7-and α6β2̃nAChR sites (1083-fold). These differences in affinity indicate greater selectivity of CC4 for β2-nAChR subtypes than that shown by the other compounds tested. Very little difference in selectivity between α4β2- and α6β2*-nAChR sites was noted for any of these compounds.

CC4 is a partial agonist of the nAChR subtypes: electrophysiological recordings of transfected subtypes. The effects of CC4 on heterologously expressed human nAChR subtypes measured electrophysiologically are shown in Figure 3. For each nAChR subtype, all of the data were normalized to those obtained upon superfusion of the same cell with 1 mM ACh and are summarized in Table 2. The efficacy of CC4 was very low for all of the subtypes and always lower than that of Cyt. In particular, and in line with previous reports (Luetje and Patrick, 1991; Chavez-Noriega *et al.*, 1997; Carbonnelle *et al.*, 2003), Cyt was a partial agonist of the α4β2-nAChR subtype.

Recently, it has been reported that varenicline acts as a potent agonist of the 5-HT_3 receptors (Lummis *et al.*, 2011). As a further control, we tested both CC4 and varenicline on human 5-HT_3A and 5-HT_3AB receptors. In agreement with those results, we found that 200 μM varenicline activated responses of both 5-HT_3A (91 ± 17) and 5-HT_3AB (89 ± 3%) comparable with those elicited by 200 μM 5-hydroxytryptamine. In contrast, CC4 concentrations of 10, 50 and 200 μM ($n = 6$) CC4 did not elicit any detectable currents on cells expressing the 5-HT_3A and 5-HT_3AB receptors.

CC4 is a partial agonist of the α4β2- and α6β2*-nAChR subtypes for nAChR-mediated [^{3}H]-DA release from striatal slices and synaptosomes.* Agonist potencies and affinities in the release of [^{3}H]-DA were evaluated in striatal slices and

Table 1

Affinity (K_i, nM) of nicotinic agonists for native immunoimmobilized α6β2* subtype and membrane-bound α4β2, α3β4 and α7 subtypes

nAChR subtype	CC4			Varenicline			Cytisine			Nicotine		
	Ki (%CV)	Ratio/ α4β2	Ratio/ α6β2	K_i (%CV)	Ratio/ α4β2	Ratio/ α6β2	K_i (%CV)	Ratio/ α4β2	Ratio/ α6β2	K_i (%CV)	Ratio/ α4β2	Ratio/ α6β2
α4β2 rat	26 (15)	–	2.1	0.91 (16)	–	0.75	2.1 (23)	–	1	10 (21)	–	1.1
α6β2 rat	12 (21)	0.5	–	1.2 (20)	1.3	–	2.1 (18)	1	–	9.5 (29)	1.0	–
α3β4 human	4 800 (25)	185	400	22 (23)	24	14	285 (23)	136	136	256 (27)	26	27
α7 rat	13 000 (13)	500	1083	37 (20)	41	31	228 (15)	109	109	234 (29)	23	25

The K_i values were derived from the results of at least three separate saturation and competition-binding experiments. The curves were fitted using a non-linear least squares analysis programme and the *F*-test. The numbers in brackets refer to the percentage coefficient of variation. The binding and competition on the immunoimmobilized striatal α6β2* subtype and membrane bound cortical α4β2 and transfected α3β4 subtypes was measured using as [^{3}H]epibatidine; and the binding and competition of the hippocampal α7 subtype was measured using [^{125}I]-abungarotoxin. The ratios are between the K_i values determined for each compound on the α4β2, α6β2, α3β4 or α7 subtypes and the rat α4β2 or α6β2 K_i values.

Table 2

Effects of CC4 and cytisine on transfected human nicotinic receptor subtypes

	α4β2	α3β4	α7	αβεδ
CC4				
$I/I_{MAX\ ACh}$ (%)	2.7 ± 0.2	4.8 ± 3.3	19 ± 6	15 ± 8
EC_{50} (μM)	2.2 ± 0.3	27.6 ± 0.1	n.d.	n.d.
n_H	0.8 ± 0.2	1.51 ± 0.01	n.d.	n.d.
n	8	9	6	6
Cytisine				
$I/I_{MAX\ ACh}$ (%)	9.4 ± 0.6	76 ± 4	83 ± 15	18 ± 5
EC_{50} (μM)	11.6 ± 0.8	19.0 ± 0.9	97.9 ± 0.6	n.d.
n_H	0.9 ± 0.4	1.6 ± 0.3	0.8 ± 0.2	n.d.
n	11	11	8	6

Dose–response curves were constructed by sequentially applying different concentrations of agonists, and normalizing the current amplitudes to the value obtained by using 1 mM ACh on the same cell. For the quantitative estimations of agonist actions, dose–response relationship was fitted when possible, by the equation:
$I = I_{max}\ [[C]^{nH}/(EC_{50}{}^{nH} + [C]^{nH})]$, where I is the peak current amplitude induced by the agonist at concentration [C]; I_{max} is the maximum response of the cell; nH the Hill coefficient; and EC_{50} the concentration at which a half maximum response is induced.

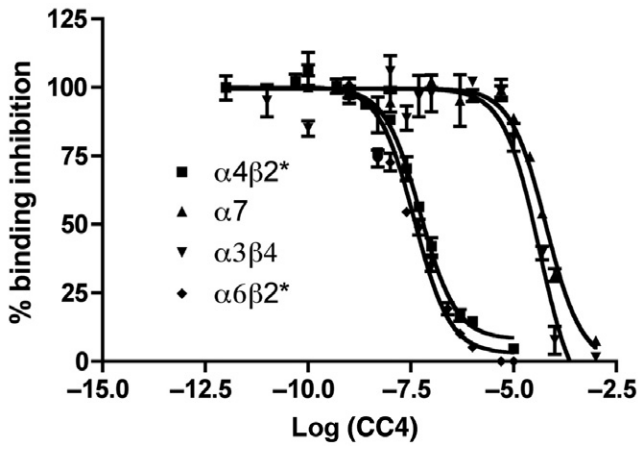

Figure 2

Inhibition of [^{3}H]-epibatidine binding to the α4β2, α6β2 and α3β4 subtypes and of [^{125}I]-αBungarotoxin binding to the α7 subtype by increasing concentration of CC4 samples were incubated for 5 min, then [^{3}H]-epibatidine (0.1 nM for α4β2 and α6β2 or 0.25 nM for α3β4) or [^{125}I]-αBungarotoxin (1 nM) was added and samples were incubated overnight at 4°C. In each experiment, each dilution of the drug was tested in triplicate. All data are expressed as percent-specific binding using 10 nM epibatidine to determine non-specific [^{3}H]-epibatidine binding and 1 μM unlabeled αBungarotoxin to determine non-specific [^{125}I]-αBungarotoxin binding.

Table 3

Functional potency, efficacy and MII sensitivity of agonist-induced [^{3}H]-DA release from striatal slices

	EC_{50} (nM)	% efficacy in comparison with to 10 μM nicotine
CC4	302 (193–472)	42.6
CC4 + MII	256 (150–360)	28.1
Varenicline	56 (35–90)	46.9
Varenicline + MII	45 (17–119)	29
Cytisine	51.5 (28–96)	40.2
Cytisine + MII	47 (12–109)	22.6
Nicotine	87 (70–108)	100
Nicotine + MII	64 (38–109)	49

The values in parentheses indicate ranges. The EC_{50} and efficacy values were determined by means of non-linear regression analysis of the dose–response activation curves of CC4, varenicline, cytisine and nicotine, in the absence and presence of 100 nM αconotoxin MII (MII) (n = 3–4). The activity in the absence of MII represents the activity of both the α4β2* and α6β2* subtypes whereas the activity in the presence of MII only represents the activity of the α4β2* subtype. The efficacy data were normalized to the response obtained using 10 μM nicotine.

synaptosomes. In striatal slices, nicotine (0.01–100 μM) elicited concentration-dependent release of [^{3}H]-DA, with an EC_{50} value of 87 nM (95% confidence interval 70–108 nM) (Figure 4A). The release was blocked by the nicotinic antagonists DhβE (IC_{50} = 2.2 μM) and MEC (IC_{50} = 0.23 μM). CC4, Cyt and varenicline had similar partial agonist efficacy (in relation to the effect of 10 μM nicotine) of, respectively, 42.6% (CC4), 46.9% (varenicline) and 40.2% (Cyt) (Figure 4A). The EC_{50} values shown in Table 3 are very similar for Cyt (51.5 nM) and varenicline (56 nM) but, in line with its lower affinity, the EC_{50} of CC4 was much higher (302 nM) (Figure 4A). Release experiments in the presence of 100 nM αconotoxin MII (MII), a toxin selective for the α3/α6β2* subtypes (Salminen *et al.*, 2004) made it possible to determine

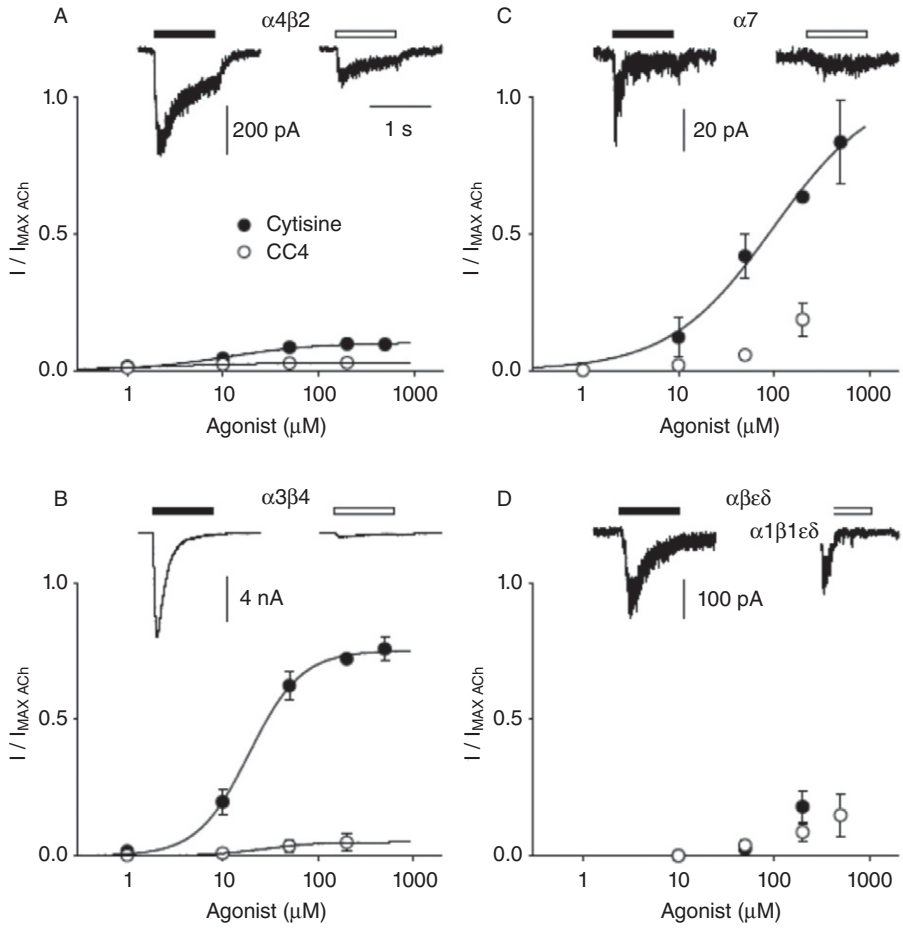

Figure 3

Effects of CYT and CC4 on transfected human subtypes. Agonist effect properties of CC4 and CYT on human α4β2 (A), α3β4 (B), α7 (C), and $(\alpha1)_2\beta1\varepsilon\delta$ nAChRs (D) transiently transfected into the GH4C1 rat anterior pituitary cell line. The upper part of each panel shows typical traces obtained for each subtype by 250 μM CYT (●) and 250 μM CC4 (○). The activation responses are normalized to the maximal response to 1 mM ACh.

the contribution of the α4β2*- and α6β2*-nAChR subtypes to agonist–induced [^{3}H]-DA release (as this activity is not mediated by α3β2*). The activity of the α6β2*-nAChR subtype is estimated as the difference between the activity in the absence of MII (representing the activity of both the α4β2*- and α6β2* -nAChR subtypes) and the activity in the presence of MII (representing the activity of only the α4β2*-nAChR subtype). As shown in Table 3, 50% of nicotine-stimulated [^{3}H]-DA release was inhibited by MII, indicating that under these conditions, [^{3}H]-DA release mediated by α4β2*- and α6β2*-nAChR subtypes is approximately equal. Maximal CC4-induced [^{3}H]-DA release was completely inhibited by DhβE (IC_{50} 1.6 μM) and partially (65%) by MII (IC_{50} 2.8 nM) (Figure 4B). MII also inhibited a greater fraction of [^{3}H]-DA release elicited by the other partial agonists, Cyt and varenicline. This pattern of relative sensitivity to MII inhibition indicates that all three partial agonists are relatively less efficacious at α6β2*-nAChR than at α4β2*-nAChR.

When increasing concentrations of CC4 were co-applied with a 10-μM concentration of nicotine, [^{3}H]-DA release was reduced by 54% with an IC_{50} value of 16.2 μM. The residual 46% activity observed with high concentrations of CC4 was not significantly different from the maximal effect induced by CC4 alone (46.2%) (Figure 4D).

The effects of CC4 on [^{3}H]-DA release was further studied using striatal synaptosomes obtained from WT mice as previously described (Salminen *et al.*, 2004), and the results are shown in Figure 4C. As was noted with striatal slices, in synaptosomes, CC4 was also a partial agonist of the MII-sensitive (α6β2*-nAChR) component with an efficacy that was 62% that of nicotine, and an EC_{50} of 1.2 ± 0.3 μM. Similarly, in the case of the α4β2*-nAChR subtype, the efficacy of CC4 was 34% that of nicotine, and its EC_{50} was 0.4 ± 0.2 μM. In comparison, the efficacy of Cyt on the synaptosomal α6β2* and α4β2*nAChR subtypes was 71 and 37% of nicotine, with EC_{50} values of 0.03 and 0.47 μM, respectively (Salminen *et al.*, 2004). Corresponding EC_{50} values for nicotine were similar to CC4 (0.77 and 1.6 μM, respectively) (Salminen *et al.*, 2004).

CC4 at low concentrations desensitizes α4β2 nAChRs. The properties of CC4 were also investigated by measuring α4β2*-nAChR mediated $^{86}Rb^+$ efflux in crude mouse thalamic synaptosomes (Figure 4H). Similar to the results for activation of

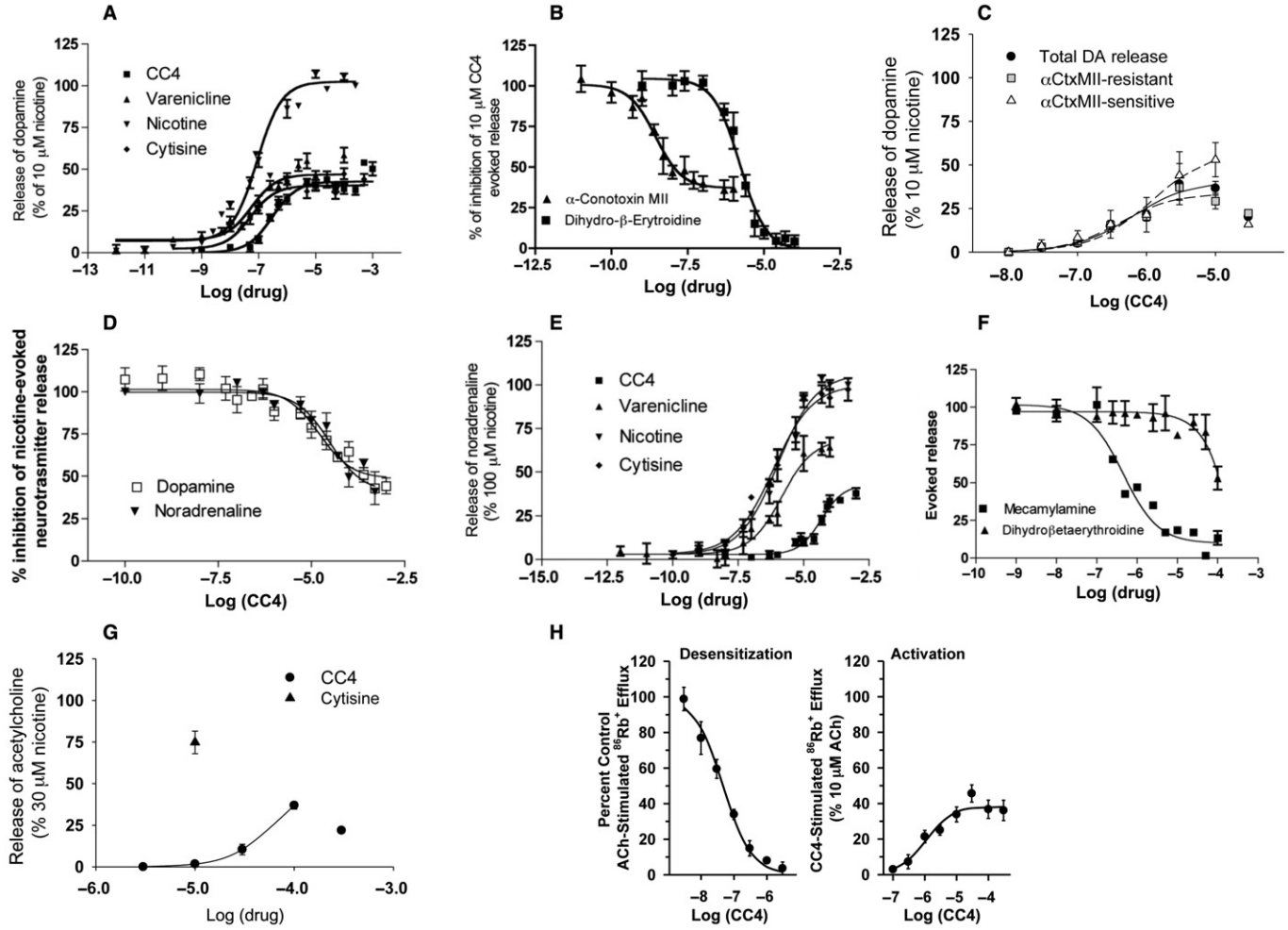

Figure 4

Effect of CC4 on neurotransmitter release from brain slices and synaptosomes. (A) Concentration–response curves of agonist-induced [^{3}H]-DA release from rat striatal slices. The slices were preloaded with 100 nM [^{3}H]-DA and exposed to the indicated concentrations of CC4, varenicline, CYT and nicotine. The results were normalized and expressed as percentages of 10 μM nicotine-induced release. The data points represent the mean values ± SEM of three to four separate experiments performed in triplicate. (B) Nicotinic antagonist inhibition of CC4-induced [^{3}H]-DA release from striatal slices. CC4 (10 μM) was incubated in the absence (not shown) and presence of the indicated concentrations of MII (▲) and DhβE (■). The values were normalized to the release obtained using 10 μM CC4 alone (100% release above baseline). Mean values ± SEM of four independent determinations performed in triplicate. (C) Concentration–response curves of agonist-induced [^{3}H]-DA release from mouse striatal synaptosomes. The synaptosomes were preloaded with 100 nM [^{3}H]-DA and exposed to the indicated concentration of CC4 in the presence (□) or absence (●) of MII. The results were normalized to baseline release. Release induced by 10 μM nicotine is also shown. The data points represent the mean SEM of three to six separate synaptosome preparations. (D) Inhibition of 10 μM nicotine-induced [^{3}H]-DA release from rat striatal slices and 100 μM nicotine-induced [^{3}H]-NA release from rat hippocampal slices in the presence of increasing concentration of CC4. (E) Concentration–response curves of agonist-induced [^{3}H]-NA release from rat hippocampal slices. The slices were preloaded with 100 nM [^{3}H]-NA and exposed to the indicated concentrations of CC4, varenicline, CYT and nicotine. The results were normalized and expressed as a percentage of 100 μM nicotine-induced release. The data points represent the mean ± SEM of three to four separate experiments performed in triplicate. (F) Nicotinic antagonist inhibition of nicotine-induced [^{3}H]-NA release from rat hippocampal slices. Nicotine (100 μM) was incubated in the absence (not shown) and presence of the indicated concentrations of MEC (■) and DhβE (▲). The values were normalized to the release obtained using 100 μM nicotine alone (100% release above baseline). Mean values ± SEM of three independent determinations performed in triplicate. (G) Concentration–response curves for agonist-induced [^{3}H]-ACh release. Synaptosomes prepared from IPn were preloaded with 100 nM [^{3}H]choline and exposed to the indicated concentrations of CC4. Results are normalized to baseline release. Data points represent mean ± SEM of four separate synaptosome preparations. (H) Concentration–response curves for CC4 desensitization of $^{86}Rb^+$ efflux stimulated by 10 μM ACh (left) and CC4 activation of $^{86}Rb^+$ efflux. Data in both panels are mean ± SEM from six mice. All data are normalized to the response elicited by exposure to 10 μM ACh.

other α4β2*-nAChR, CC4 was a partial agonist for the stimulation of $^{86}Rb^+$ efflux. Maximal efflux elicited by CC4 was 39.2 ± 2.3% of that elicited by 10 μM ACh. The apparent EC_{50} for CC4-stimulated $^{86}Rb^+$ efflux was 1.1 ± 0.4 μM. In addition, the ability of CC4 to desensitize ACh-stimulated $^{86}Rb^+$ efflux was determined by exposing thalamic synaptosomes to one of several CC4 concentrations for 8 min prior to stimulation with 10 μM ACh. CC4 treatment elicited a concentration-dependent decrease with an apparent DC_{50} value of 46.4 ± 8.1 nM. Complete inhibition of ACh-stimulated $^{86}Rb^+$ efflux occurred following treatment with 3 μM CC4.

CC4 acts as a low-potency partial agonist of the native α3β4 subtype. The release of [^{3}H]-NA from rat hippocampus is mediated by the α3β4-nAChR subtype (Clarke and Reuben, 1996; Azam and McIntosh, 2006). Nicotine elicited a saturable, concentration-dependent release of [^{3}H]-NA from hippocampal slices with an EC_{50} = 1.05 μM.) This [^{3}H]-NA release was blocked by the nicotinic antagonists MEC (IC_{50} = 0.57 μM) and DhβE IC_{50} = 30 μM) (Figure 4F). Cyt and varenicline also elicited [^{3}H]-NA release with, respectively, EC_{50} values of 0.71 and 1.48 μM and efficacies 94 and 69% of that of 100 μM nicotine, respectively (Figure 4E). CC4 induced also [^{3}H]-NA release with an EC_{50} = 47.8 μM and an efficacy that was 42.9% that of nicotine (Figure 4E).

We also tested the agonist/antagonist effects of CC4 on hippocampal [^{3}H]-NA release by co-applying increasing concentrations of CC4 with 100 μM nicotine. We found that, when combined with nicotine, CC4 reduced [^{3}H]-NA release by 59% with an IC_{50} value of 30 μM, 41% of the residual activity was not significantly different from the maximal effect induced by CC4 alone (42.9%) (Figure 4D).

Previously published data have shown that the release of [^{3}H]-ACh from IPn synaptosomes is mediated by the α3β4 *-nAChR subtype as it is not affected by null mutation of the β2 α2, α4, α5, α6 or α7 subunits, but is dependent on the β4 subunit, and partially on the β3 subunit (Grady *et al.*, 2001; 2009). This assay is not affected by the α6β2*-nAChR selective antagonist MII, but is decreased by treatment with the α3β4-nAChR selective antagonist α-conotoxin Au1B (Grady *et al.*, 2001). When tested on IPn synaptosomes, CC4 induced [^{3}H]-ACh release in a concentration-dependent manner, showing a partial agonist profile with a maximal response that is 36% that of nicotine, 49% that of Cyt and an EC_{50} of 36 μM (Figure 4G).

Behavioural effects of CC4

Nicotine-induced DA release in the mesolimbic pathway is necessary for nicotine self-administration [reviewed in (De Biasi and Dani, 2011)]. Because the *in vitro* data indicate CC4 modulates DA release in the striatum, we evaluated the effects of CC4 *in vivo*. Two models that assess the reinforcing properties of compounds were used: CPP and i.c.v. self-administration. The effects of CC4 were compared with those of nicotine and Cyt.

CC4 evokes a slight CPP, but attenuates nicotine-induced CPP. Rats showed an equivalent average amount of time spent in each of the two outer conditioning compartments during the conditioning phase (464 ± 26 s in the circles and 487 ± 27 s in the striped compartments, $P = 0.47$, not significant). CPP induced by different doses of nicotine, CC4 or Cyt is shown in Figure 5A. One-way ANOVA revealed a significant between subject treatment effect when comparing the time in the drug-paired compartment during the pre- and post-conditioning period [F (3,36) = 4.11, $P < 0.001$ for nicotine; F (5,54) = 4.51, $P < 0.001$ for CC4; F (4,45) = 8.65, $P < 0.0001$ for Cyt]. *Post hoc* analysis showed that nicotine and CC4 increased the time spent in the drug-paired compartment on the post-conditioning day at a dose of 0.1 mg·kg^{-1}, whereas, Cyt did so at doses of 0.1 and 0.5 mg·kg^{-1}. The highest doses of the three compounds were ineffective.

In the antagonism studies, a significant treatment effect was found when comparing the time in the drug-paired compartment during pre-and post-conditioning periods, in rats given CC4, Cyt or MEC before each drug pairing [F (9,90) = 9.53, $P < 0.0001$, one-way ANOVA] (Figure 5B). *Post hoc* analysis showed that MEC (10 mg·kg^{-1}), CC4 (5 and 10 mg·kg^{-1}) and Cyt (2.5 mg·kg^{-1}) alone, did not change the mean time spent in the drug-paired side between the pre-and post-conditioning periods for saline treated rats. However, when potential antagonists were administered with the maximally effective dose of nicotine (0.1 mg·kg^{-1}), CC4 and Cyt significantly reduced the time spent in the drug-paired compartment during post-conditioning, in comparison with nicotine alone, whereas MEC, significantly, even if slightly, reduced the nicotine effect.

CC4 did not show any effect on motor activity at any dose used (see Supporting Information Figure S1).

CC4 is self-administered i.c.v. During the training phase, the operant responding of the rats trained to press both levers did not change before and after surgery for i.c.v. cannula implantation (data not shown). The intake of water, delivered after each lever pressing, did not vary during the training or testing procedure nor did food intake or body weight (data not shown).

Figure 6A shows the effect of nicotine, CC4 and Cyt on the time course of the self-administration of one representative rat during the training and testing procedures on the last 10 days of each self-administration period. Starting from i.c.v. infusions of 0.03 μg of nicotine, 0.008 μg of CC4 or 0.001 μg of Cyt, there was a progressive increase in number of pressings of the less preferred lever.

The highest concentration of nicotine decreased the number of pressings delivering the drug and increased the number of pressings delivering vehicle.

Figure 6B shows the mean number of pressings delivering vehicle or increasing concentrations of nicotine, CC4 or Cyt in the different groups of rats. The self-administration of different unit doses significantly changed the operant responding of nicotine [F(9,40) = 16.75, $P < 0.0001$], CC4 [F(7,72) = 11.23, $P < 0.0001$] and Cyt [F (13,126) = 22.98, $P < 0.0001$]. *Post hoc* comparison indicated that self-administration of nicotine (0.1 μg·2 μL^{-1}) significantly increased the mean number of drug-associated lever pressings in comparison with the corresponding vehicle. Consequently, the mean daily intake progressively increased as nicotine dose increased from 0.003 to 0.1 μg·2 μL^{-1} [F(3,36) = 18.34, $P < 0.0001$] (Figure 6C). A significant reduction in the number of drug-associated lever pressings was

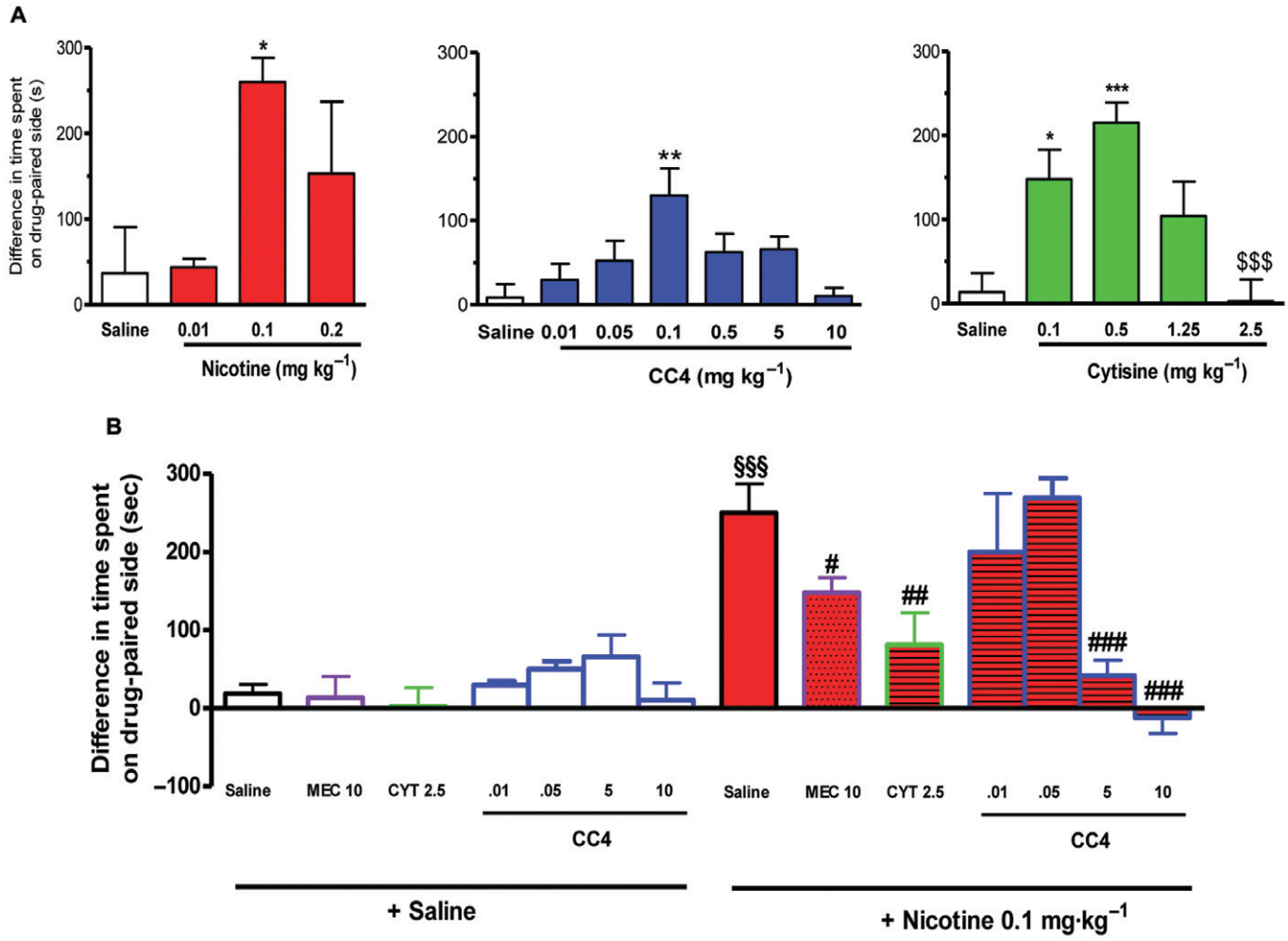

Figure 5

Effect of CC4 on conditioned place preference. Nicotine, CC4 and CYT all elicited conditioned place preference. (A) Dose–response curves for nicotine, CC4 and CYT given i.p. 5 min before the test during each conditioning session. CC4, CYT and MEC antagonized nicotine-elicted CPP. (B) Effect of MEC (10 mg·kg^{-1}), CYT (2.5 mg·kg^{-1}) or CC4 (0.01, 0.05, 5 or 10 mg·kg^{-1}) given 10 min before nicotine (0.1 mg·kg^{-1}), on conditioned place preference. Place preference was evaluated as the time spent in the drug-paired compartment before and after conditioning on the test day, during which no drug or vehicle was injected. Preference was calculated by subtracting the time (mean ± SEM) spent in the drug-paired compartment before drug conditioning from the time spent after drug conditioning. ($n = 10$ rats for each group). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ significantly different from the corresponding saline group during post-conditioning; $^{§§§}P < 0.001$ significantly different from 0.5 mg·kg^{-1} CYT; $^{§§§}P < 0.001$ significantly different from saline, MEC, CC4 (5–10), CYT alone; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ compared with saline + nicotine (ANOVA followed by a *post hoc* Tukey's test).

only observed at the unit dose of 0.3 µg·2 µL^{-1} of nicotine. As a result, mean daily nicotine intake was reduced by approximately 50% when the unit dose increased from 0.1 µg·2 µL^{-1} (10.12 ± 1.44 µg·day^{-1}) to 0.3 µg·2 µL^{-1} (5.55 ± 1.00 µg·day^{-1}). The *post hoc* comparison indicated that the self-administration of CC4 (0.3 µg·2 µL^{-1}) significantly increased the mean number of drug-associated lever pressings in comparison with the corresponding vehicle. Thus, mean daily CC4 intake (Figure 6C) progressively increased from 0.003 to 0.3 µg·2 µL^{-1} [$F(2,27) = 234$, $P < 0.0001$]. The *post hoc* analysis also revealed a significant increase in the mean daily intake of 0.3 µg·2 µL^{-1} in comparison with 0.003 and 0.08 µg·2 µL^{-1}. The *post hoc* comparison for Cyt indicated that, except for the unit dose of 0.001 µg/infusion, drug-associated lever pressing was always significantly less than that associated with vehicle lever pressing. However, because of the progressive increase of unit dose, the mean daily intake (Figure 6C) gradually increased from 0.0001 to 0.5 µg·µL^{-1} [$F(5,44) = 7.41$, $P < 0.0001$].

CC4 reduces nicotine i.c.v. self-administration. Figure 7 (left) shows the mean number of lever pressings during nicotine or vehicle self-administration in combination with vehicle, MEC (10 mg·kg^{-1}) or CC4 (5 mg·kg^{-1}), which was significantly different between groups [$F(11,108) = 5.15$, $P < 0.0001$, ANOVA]. The *post hoc* comparisons indicated that pretreatment with MEC or CC4 did not affect the mean number of lever pressings in saline-treated animals in comparison with

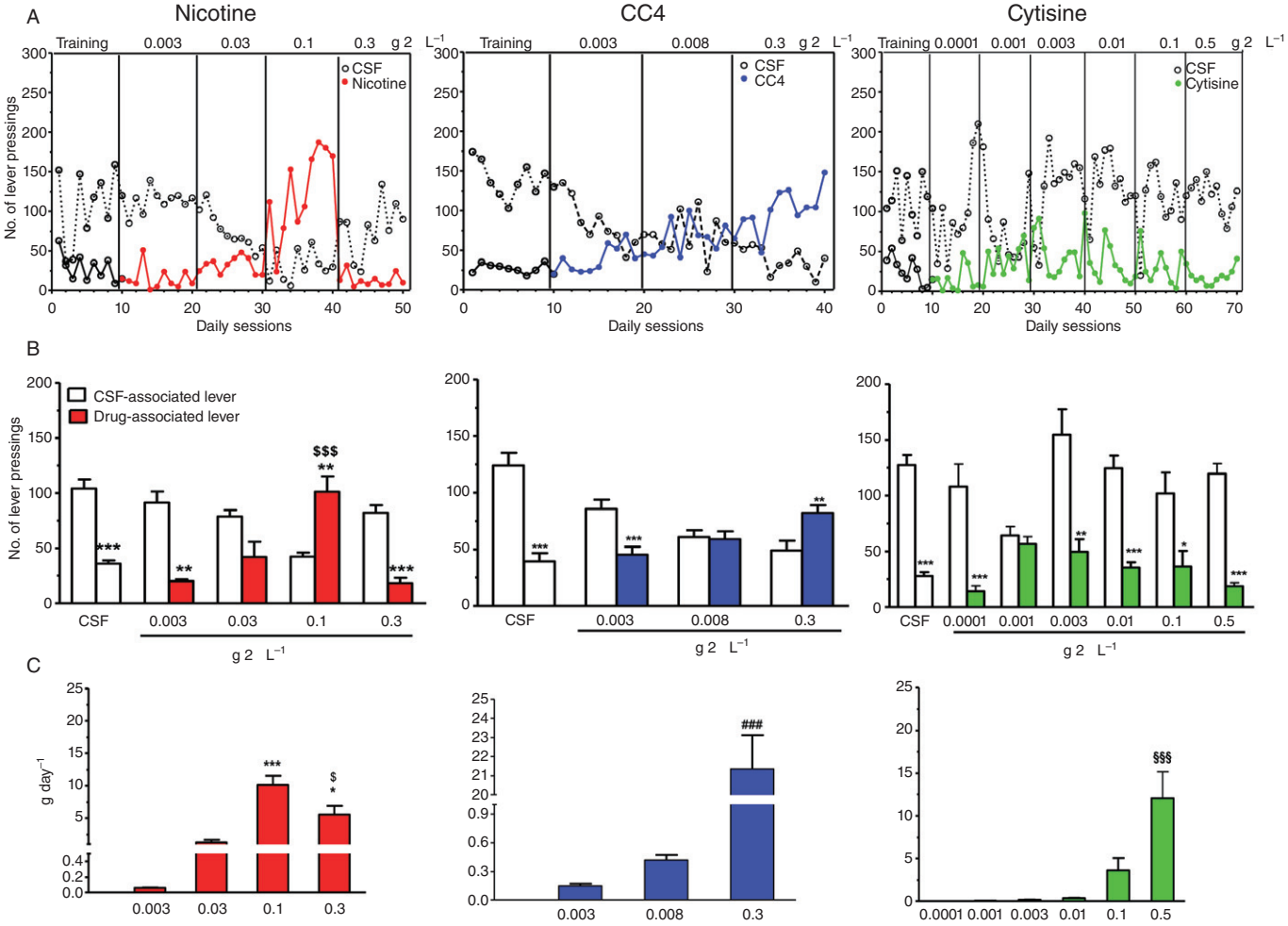

Figure 6

CC4 i.c.v. self-administration. Nicotine, CC4 and CYT were self-administered in a dose-dependent manner. (A) The number of pressings on the preferred and non-preferred lever, in a free-choice situation by one representative rat treated with each compound during a 1-h daily session. CSF was delivered i.c.v. by pressing the lever preferred during training; the compounds were delivered i.c.v. by pressing the lever that was found not preferred during training. (B) The number of pressings on the drug- or vehicle-associated lever during drug delivery. CSF group received CSF by pressing both levers during the same period. The results are mean values (±SEM) of the last five daily sessions after 15 ± 20 days of acquisition, with 10 rats per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus the corresponding CSF group; $^{\$\$\$}P < 0.001$ versus the same lever CSF group (ANOVA followed by a *post hoc* Tukey's test). (C) Daily intake (μg) of self-administered unit doses of each compound. Each value is the mean (±SEM) of the last five daily sessions obtained after 15–20 days of acquisition. *$P < 0.05$, ***$P < 0.001$ compared with 0.003 and 0.03 μg of nicotine per infusion; $^{\$}P < 0.05$ compared with 0.1 μg per infusion of nicotine; $^{\#\#\#}P < 0.001$ compared with 0.003 and 0.008 μg of CC4 per infusion; $^{\S\S\S}P < 0.001$ compared with all of the remaining doses of CYT (ANOVA followed by a *post hoc* Tukey test). $n = 10$ for each group.

vehicle. In contrast, pretreatment with either of the two antagonists significantly reduced the number of lever pressings associated with nicotine alone. Consequently, there was a treatment effect on mean daily intake [$F(2,27) = 26.41$, $P < 0.0001$]. (Figure 7) (right). The *post hoc* comparisons showed a significant decrease in intake in the groups pretreated with MEC or CC4.

Discussion

The findings of this study show that CC4 binds to the α4β2- and α6β2-nAChR subtypes with high affinity and selectivity, and to the α3β4̃ and α7-nAChR subtypes with much lower affinity. CC4 is a partial agonist of all nAChR subtypes, although it is less efficacious on the α3β4̃ and α7̃-nAChR subtypes. CC4 has reinforcing properties in *in vivo* models; however, when co-administered with nicotine, CC4 reduces the reinforcing properties of nicotine.

Our group has previously investigated the pharmacological and functional characteristics of a group of N-substituted Cyt derivatives, and found that they have higher binding affinity for the α4β2– than for the α3β4– and α7-nAChR subtypes. Furthermore, their efficacy on the α3β4̃ and α7-nAChR subtypes is much less than that of Cyt (Carbonnelle *et al.*, 2003). After preliminary studies, we chose CC4 for further investigation because it is highly selective for the α4β2-nAChR subtype and because its lipophilicity is only five

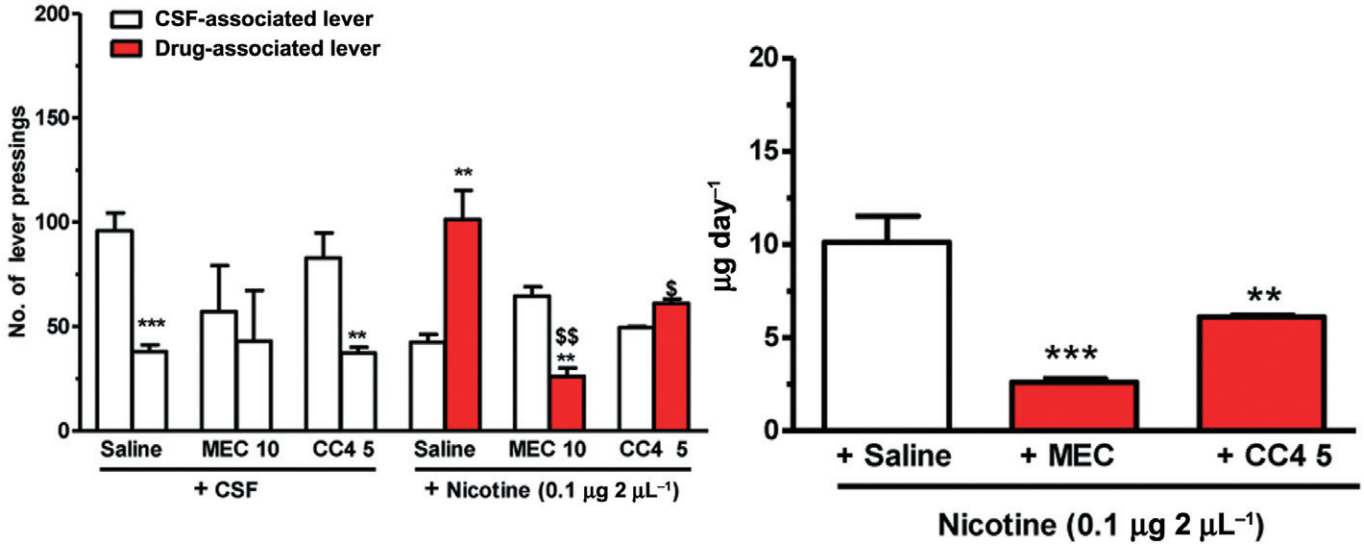

Figure 7

CC4 on nicotine i.c.v. self-administration. Nicotine self-administration is reduced by daily peripheral pretreatment with MEC and CC4. Mean operant responses are shown for a free-choice situation (left) and daily nicotine intake (µg) (right). (Left) Drug-associated lever pressing delivered nicotine (0.1 µg × infusion) and CSF-associated lever pressing delivered CSF (2 µL per infusion) during the last five stable daily sessions of 15 ± 20 days of acquisition ($n = 10$ rats per group). Saline, MEC (10 mg·kg^{-1}) or CC4 (5 mg·kg^{-1}) were given s.c. 30 min before each daily session. $^{**}P < 0.01$, $^{***}P < 0.001$ versus corresponding CSF-associated lever; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ compared versus same lever, nicotine + saline group. (Right) Daily intake (µg) of self-administered nicotine unit dose (0.1 µg × infusion). Each value is the mean (±SEM) of the last five daily sessions obtained after 15–20 days of acquisition. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with nicotine + saline group (ANOVA followed by a *post hoc* Tukey's test.

times less than that of nicotine, but 17 times more than that of Cyt. Because lipophilicity is an important predictor of absorption from the gastrointestinal tract and blood–brain barrier penetration, its favourable lipophilicity indicates that CC4 may be a good candidate for targeting CNS receptors *in vivo* particularly in smoking cessation.

The present study shows that CC4 has similar affinity for native α6β2̃ and α4β2̃nAChR subtypes, and reduced affinity and efficacy for the α3β4- and α7-nAChR subtypes. The affinity of CC4 for β2-nAChR was lower than that of varenicline, Cyt or nicotine. However, as its affinity for the α3β4- and α7-nAChR subtypes was much lower than that of varenicline, Cyt or nicotine, CC4 exhibited a high degree of selectivity for β2-nAChR subtypes.

The affinity of a nicotinic agonist in equilibrium-binding experiments reflects its affinity for desensitized receptors and may not be representative of its functional properties. Consequently, we characterized CC4 with electrophysiological techniques and functional neurotransmitter release assays. The electrophysiological experiments showed that CC4 has a much lower EC_{50} for β2*-nAChRs than for α3β4–nAChR. Moreover, CC4 had a very low intrinsic activity that was always lower than that of Cyt on the human α4β2-, α3β4-, or α7-nAChR and α1β1γδ-nAChR (muscle-type receptors).

Given the importance of the mesolimbic dopaminergic system in nicotine dependence, the ability of CC4 to release striatal [^{3}H]-DA was measured in two different *in vitro* preparations of different complexity: rat striatal slices, which preserve the cytoarchitecture and maintain some anatomical relationships that facilitate transmitter cross-talk and mouse striatal synaptosomes, which allow the analysis of isolated DA nerve terminals. Direct comparison of the CC4-evoked responses in the two systems, yielded very similar EC_{50} (0.3 and 0.66 µM) and efficacy values (44 and 43%), thus indicating that CC4-induced striatal [^{3}H]-DA release is due to its partial agonism of α4β2*- and α6β2*-nAChR and also that the effect of CC4 is not species-specific.

In line with its low affinity for the α3β4-nAChR subtype, and low efficacy on heterologously expressed human α3β4̃nAChR subtype, CC4 had partial activity and low potency in releasing [^{3}H]-NA from hippocampal slices and [^{3}H]-ACh from IPn synaptosomes (functions that are mediated by the α3β4-nAChR subtype). In the same assays, varenicline, Cyt and nicotine were much more potent and efficacious than CC4. Overall, the degree of CC4 selectivity for β2-nAChR observed in the binding assays was confirmed by the functional assays.

The subtype selectivity for β2-nAChR are in agreement with our previously published data showing that adding chemical groups of a different class or size to the basic nitrogen of Cyt leads to compounds that lose full agonist activity on the α3β4 and α7 subtypes (Carbonnelle *et al.*, 2003).

It has recently been shown that partial agonists acting on α4β2-nAChRs are useful for smoking cessation presumably because they maintain moderate levels of DA release to counteract withdrawal symptoms while also reducing smoking satisfaction. Varenicline, which has been approved as a therapeutic aid to smoking cessation (Coe *et al.*, 2005), is a partial agonist at α4β2*-nAChRs and a full agonist at α7- and α3β4-nAChRs (Foulds, 2006; Mihalak *et al.*, 2006). Preclinical studies showed that varenicline inhibits nicotine self-administration and nicotine-elicited DA release in the

nucleus accumbens (Rollema *et al.*, 2007b; O'Connor *et al.*, 2010). Clinical trials indicate that it is effective in decreasing smoking relapse in humans (Rollema *et al.*, 2007b; Zierler-Brown and Kyle, 2007; Tutka, 2008).

The availability of a drug such as varenicline, which has proven efficacy in smoking cessation, provides a model compound with which to evaluate the predictive validity of new compounds using the same models. Our *in vitro* studies suggest that CC4 is as efficacious as varenicline in releasing striatal DA. Our *in vivo* studies using CPP and i.c.v self-administration as models of nicotine reinforcement showed that CC4 is reinforcing, albeit to a lesser extent than nicotine. Our data concerning nicotine-induced CPP and self-administration confirm previous findings (Watkins *et al.*, 1999; Biala *et al.*, 2010; Fowler and Kenny, 2011; Kota *et al.*, 2011) showing that mice, rats and squirrel monkeys respond robustly to both tests, and that increasing the dose of nicotine leads to an inverted-U–shaped dose–response curve. We also found that Cyt induced a modest, but significant CPP when administered peripherally to rats, an effect that is in line with its effects when administered into rat ventral tegmental area (Museo and Wise, 1994). Furthermore, the self-administration of Cyt by drug-naive mice is similar to that of nicotine (Rasmussen and Swedberg, 1998). Surprisingly, although a number of studies have shown that Cyt can be useful for smoking cessation (Cahill *et al.*, 2011), there are no published data concerning its antagonistic effects on nicotine-induced motivational effects. We found that Cyt attenuated DA release and blocked nicotine-induced CPP.

The i.c.v. infusion model we used to evaluate self-administration of nicotine, CC4 and Cyt has been successfully used with other addictive drugs (Braida *et al.*, 1998; 2001; 2004; 2008). The advantages of the i.c.v. procedure include its durable preparation, the fact that it allows the simultaneous choice of drug or vehicle, and the limitation of peripheral side effects.

Pretreatment with CC4 significantly reduced i.c.v. nicotine self-administration, thereby reducing nicotine intake. Thus, an antagonistic action of CC4 on the reinforcing properties of nicotine is suggested. In line with the fact that CC4 reduced nicotine-induced DA release when co-administered with nicotine in an *in vitro* system, the observed CC4-induced decrease in operant responding may be interpreted as a decrease in the reinforcing effects of nicotine.

The mechanism of action of CC4 *in vivo* has not been firmly established. Because CC4 is a partial agonist, its effects *in vivo* may be due to a reduced stimulation of nAChRs. In addition, as shown by the rubidium efflux studies, CC4 also desensitizes the nAChR function thereby acting as time-averaged antagonist. Subsequently, as is the case for other Cyt derivatives, CC4 can have a buffering effect on the activity of nicotine, by decreasing the stimulating effects of nicotine and/or by desensitizing nAChRs.

The acute doses of CC4 that we used did not affect spontaneous motor activity. In order to determine whether repeated administration of CC4 elicited locomotor stimulation, we used a procedure previously applied to nicotine to investigate whether CC4 has a sensitizing effect after repeated administration. Our findings showed that, unlike nicotine, CC4 did not elicit any motor sensitization (at least not at the dose used for the behavioural antagonism studies).

One important limiting factor on the efficacy of nicotinic partial agonist therapy for smoking cessation is the possibility of side effects associated with the interaction with other nAChR subtypes or other receptors. In particular, it has been reported that varenicline may cause significant adverse effects, including nausea, because of the activation of 5-HT_3 receptors (Lummis *et al.*, 2011), and an increased risk of serious adverse cardiovascular events (Singh *et al.*, 2011) probably because of its effects on the α3β4*- and α7-nAChR subtypes, which are highly enriched in autonomic ganglia (Wang *et al.*, 2002). As CC4 has no effects on 5-HT_3 receptors and is a selective β2*-nAChRs partial agonist, with very low potency and efficacy on the α3β4- and α7- subtypes, it may maintain varenicline-like effects on smoking cessation but have reduced adverse effects.

In conclusion, the present *in vitro* and *in vivo* findings support the possible development of CC4 or its derivatives as a promising drug for tobacco smoking cessation.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 CC4 does not affect spontaneous locomotion and does not elicit motor sensitization. (A) Increasing concentration of CC4 does not affect locomotion. (B) After repeated treatment with nicotine there is a progressive increase in locomotor response indicating a clear sensitization to nicotine. In contrast no sensitisation to locomotor response was observed after daily treatment with saline or CC4.