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RESEARCH PAPER

Functional interaction between pre-synaptic α6β2-containing nicotinic and adenosine A_{2A} receptors in the control of dopamine release in the rat striatum

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BACKGROUND AND PURPOSE

Pre-synaptic nicotinic ACh receptors (nAChRs) and adenosine A_{2A} receptors (A_{2A}Rs) are involved in the control of dopamine release and are putative therapeutic targets in Parkinson's disease and addiction. Since A_{2A}Rs have been reported to interact with nAChRs, here we aimed at mapping the possible functional interaction between A_{2A}Rs and nAChRs in rat striatal dopaminergic terminals.

EXPERIMENTAL APPROACH

We pharmacologically characterized the release of dopamine and defined the localization of nAChR subunits in rat striatal nerve terminals *in vitro* and carried out locomotor behavioural sensitization in rats *in vivo*.

KEY RESULTS

In striatal nerve terminals, the selective A_{2A}R agonist CGS21680 inhibited, while the A_{2A}R antagonist ZM241385 potentiated the nicotine-stimulated [^{3}H]dopamine ([^{3}H]DA) release. Upon blockade of the α6 subunit-containing nAChRs, the remaining nicotine-stimulated [^{3}H]DA release was no longer modulated by A_{2A}R ligands. In the locomotor sensitization experiments, nicotine enhanced the locomotor activity on day 7 of repeated nicotine injection, an effect that no longer persisted after 1 week of drug withdrawal. Notably, ZM241385-injected rats developed locomotor sensitization to nicotine already on day 2, which remained persistent upon nicotine withdrawal.

CONCLUSIONS AND IMPLICATIONS

These results provide the first evidence for a functional interaction between nicotinic and adenosine A_{2A}R in striatal dopaminergic terminals, with likely therapeutic consequences for smoking, Parkinson's disease and other dopaminergic disorders.

Abbreviations

[^{3}H]DA, tritiated dopamine; 3Rs, Replacement, Refinement and Reduction of Animals in Research; A_{2A}R, adenosine A_{2A} receptors; A_{2B}R, adenosine A_{2B} receptors; ARRIVE, Animals in Research: Reporting In Vivo Experiments; CGS, CGS21680; DA, dopamine; DHβE, dihydro-β-erythroidine; DMSO, dimethylsulfoxide; MRS, MRS1754; nAChR, nicotinic ACh receptor; ZM, ZM241385; α-BTX, α-bungarotoxin; α-CTX, α-conotoxin PIA

Introduction

The striatum is a major relay nucleus between the neocortex and the basal ganglia. It receives extensive cortical and thalamic inputs, which – after being integrated at the striatal level – are processed by the basal ganglia output nuclei and subsequently sent back to thalamic and cortical areas (Graybiel, 1991). This processing occurs through an orchestrated interaction among several neuromodulators at the pre- and post-synaptic levels (Girault, 2012), where the dopaminergic inputs from the substantia nigra play a prominent role (Gerfen and Surmeier, 2011). Accordingly, the dopaminergic system is crucial in different functions processed through striatal circuits, such as locomotor activity, habit formation or associative and mnemonic functions (Wickens *et al.*, 2007; Dagher and Robbins, 2009; Lovinger, 2010; Cools, 2011). Also, the manipulation of different neuromodulators, including ACh and adenosine, can affect the striatal dopaminergic system and, hence, is a potential strategy to manage striatal-related brain diseases associated with dopaminergic dysfunction such as addiction and Parkinson's disease (Schiffmann *et al.*, 2007; Quik *et al.*, 2011).

In fact, the activation of pre-synaptic ionotropic nicotinic ACh receptors (nAChRs – the nomenclature of receptors follows Alexander *et al.*, 2011) by ACh or by the widely abused alkaloid nicotine can trigger dopamine (DA) release in the striatum, thereby modulating locomotion, drug addiction or memory processes (Calabresi and Di Filippo, 2008; Livingstone and Wonnacott, 2009; Drenan *et al.*, 2010; Gotti *et al.*, 2010; Quik *et al.*, 2011; Threlfell *et al.*, 2012). In parallel, the chronic consumption of caffeine, an adenosine receptor antagonist (Fredholm *et al.*, 1999), or the administration of adenosine A_{2A} receptor (A_{2A}R) ligands can counteract different neuropsychiatric conditions involving the dopaminergic system in the basal ganglia, such as motor disorders, psychoses or addiction (Fredholm *et al.*, 2005; Ferré *et al.*, 2007; Cunha *et al.*, 2008), and functional A_{2A}Rs are located in striatal dopaminergic terminals controlling the release of DA (Chowdhury and Fillenz, 1991; Gomes *et al.*, 2006; 2009). Notably, nicotine use is often accompanied with an increased caffeine intake, and the two psychoactive substances may reinforce each other's action (Swanson *et al.*, 1994). Furthermore, both caffeine and nicotine are pointed out as neuroprotectant in Parkinson's disease (Ross and Petrovitch, 2001). Since chronic caffeine consumption mainly acts through A_{2A}Rs (Ferré, 2008; Cunha and Agostinho, 2010), we now tested if there is a functional interaction between pre-synaptic A_{2A}Rs and nAChRs controlling the function of striatal dopaminergic nerve terminals. Here, we show that A_{2A}Rs activation diminishes the nicotine-stimulated release of DA in isolated nerve terminals, which translates into an ability of the A_{2A}Rs to control nicotine-induced locomotor sensitization *in vivo*.

Methods

Subjects

All studies were conducted in accordance with the principles and procedures outlined as 'Replacement, Refinement and Reduction of Animals in Research' (3Rs) in the guidelines of the European Union (86/609/EEC), Federation for Laboratory Animal Science Associations and the National Centre for the 3Rs [the Animals in Research: Reporting In Vivo Experiments (ARRIVE); Kilkenny *et al.*, (2010)] and were approved by the Animal Care Committee of the Center for Neuroscience and Cell Biology of Coimbra. We also applied the principles of the ARRIVE guideline for the design and the execution of the *in vitro* pharmacological experiments (see below) as well as for data management and interpretation, according to McGrath *et al.*, (2010).

The 75 animals used in this work were male Wistar rats (10–14 weeks old) obtained from Charles River (Barcelona, Spain). Importantly, different tissues from these animals were used in other on-going projects at our research centres. The animals were housed under controlled temperature (23 ± 2°C), subject to a fixed 12 h light/dark cycle, with free access to food and water. All efforts were made to reduce the number of animals used and to minimize their stress and discomfort. The animals used to perform the *in vitro* studies were deeply anaesthetized with 2-bromo-2-chloro-1,1,1-trifluoroethane (halothane; no reaction to handling or tail pinch, while still breathing) before decapitation with a guillotine.

Preparation of synaptosomes

Purified nerve terminals, termed synaptosomes (Whittaker *et al.*, 1964), represent an excellent tool to study pre-synaptic processes free of polysynaptic and glial influences (Raiteri and Raiteri, 2000).

Partially purified synaptosomes (P2 fraction) for release experiments were obtained as previously described (Ferreira *et al.*, 2009). Briefly, the caudate-putamen region without the nucleus accumbens (hereafter simply: striatum) were quickly dissected out into 2 mL ice-cold sucrose solution (0.32 M, containing 5 mM HEPES, pH 7.4). After homogenization with a Teflon homogenizer, and centrifugation at 5000× *g* for 5 min, the supernatant was collected and centrifuged at 13 000× *g* for 10 min to obtain the P2 synaptosomal fraction.

Synaptosomes purified by a 45% Percoll gradient for Western blotting were obtained as previously described (Rebola *et al.*, 2005). Briefly, the two striata from one animal were homogenized in an ice-cold sucrose-HEPES medium containing 0.32 M sucrose, 1 mM EDTA, 0.1% BSA and 10 mM HEPES (pH 7.4). The homogenate was spun at 3000× *g* for 10 min at 4°C and the supernatant was spun again at 14 000× *g* for 12 min. The pellet (P2 fraction) was resuspended in 1 mL of Percoll 45% (v/v) made up in Krebs–HEPES–Ringer (KHR) medium (in mM: NaCl 140, EDTA 1, KCl 5, glucose 5 and HEPES 10, pH 7.4) and spun again at 14 000× *g* for 2 min. The synaptosomes (top layer) were then removed and washed once with KHR medium at 14 000× *g* for 2 min. The synaptosomal pellet obtained was solubilized in 5% SDS supplemented with 100 μM PMSF, 2 mM DTT and a protease inhibitor cocktail. The protein concentration was then determined using the bicinchoninic acid (BCA) protein assay reagent and the samples added to a 1/6 volume of 6 × SDS-PAGE sample buffer [30% (v/v) glycerol, 0.6 M dithiothreitol (DTT), 10% (w/v) SDS and 375 mM Tris–HCl, and 0.012% bromophenol blue, pH 6.8] and the volume adjusted with milliQ water to normalize for a maximum of 2 $\mu g \cdot \mu L^{-1}$.

Western blot

The samples were denaturated by boiling at 95°C for 5 min and separated by SDS-PAGE electrophoresis using 10% polyacrylamide resolving gels and 4% polyacrylamide concentrating (stacking) gels under reducing conditions at 80–120 mV. Pre-stained precision protein standards (Bio-Rad, Amadora, Portugal) were run simultaneously with the samples to help identify the proteins of interest. The proteins in the gel were then electrophoretically transferred (1A current, for 1.5 h at 4°C with constant agitation) to previously activated PVDF membranes (0.45 µm). After blocking for 1 h at room temperature with 5% essential fatty acid-free BSA in Tris-buffered saline (Tris 20 mM, NaCl 140 mM, pH 7.6) containing 0.1% Tween 20 (TBS-T) to prevent nonspecific binding, the membranes were incubated overnight at 4°C with the primary antibody diluted in TBS-T with 1% BSA. After three washing periods of 15 min with TBS-T, the membranes were incubated with the appropriate alkaline phosphatase-tagged secondary antibody diluted in TBS-T containing 1% BSA for 2 h at room temperature. After three 15 min washes with TBS-T, the membranes were incubated with enhanced chemifluorescence (ECF) substrate and visualized in a VersaDoc 3000 imaging system with the assistance of Quantity One software (both from Bio-Rad). The membranes were then reprobed and tested for β-actin immunoreactivity to confirm that similar amounts of protein were applied to the gels.

Tritiated dopamine ([^{3}H]DA) release from rat striatal synaptosomes

The experiments were carried out as previously reported (Ferreira *et al.*, 2009; Martíre *et al.*, 2011). The P2 synaptosomal fraction was diluted to 0.5 mL with Krebs–HEPES solution (in mM: NaCl 113, KCl 3, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, glucose 10, HEPES 15, pH 7.4, 37°C) containing the monoamine oxidase B inhibitor, pargyline (10 µM). Synaptosomes were then incubated for 10 min in the presence of tritiated dopamine ([^{3}H]DA; ~60 Ci × mmol^{-1}, final concentration, 150 nM). A 16-microvolume chamber superfusion set-up was filled with the pre-loaded synaptosomes, which were trapped by layers of Whatman GF/C filters and superfused continuously at a rate of 0.8 mL·min^{-1} at 37°C until the end of the experiment. This system allows testing seven pharmacological treatments in duplicate (each averaged as n = 1) to reduce the number of animals utilized, in accordance with the ARRIVE guidelines. After a 10 min washout period, nine 2 min samples were collected for liquid scintillation assay.

The radioactivity content of each sample and of the filters with the trapped synaptosomes was counted by a Tricarb β-counter (PerkinElmer, Waltham, MA, USA). Disintegrations per minute values were expressed as fractional release (FR%), that is, the percent of actual content in the effluent as a function of the total synaptosomal content of radioactivity.

After collecting four 2 min samples as a baseline, nicotine and adenosine receptor ligands, alone or in combination, were applied through the superfusion solution. nAChR antagonists were present since the beginning of the 10 min washout period, until the end of the experiment. For the calculation of treatment effects, please consult the simulated examples presented in the Supporting Information Figure S1.

Adenosine release from rat striatal synaptosomes

Adenosine release was assayed both in batch-like conditions as well as upon superfusion of synaptosomes. In batch-like conditions, striatal synaptosomes (~1.2 mg protein × mL^{-1}) were incubated at 37°C for 15 min in the presence of the adenosine deaminase inhibitor, erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA; 20 µM). Half of the synaptosomal samples were challenged with nicotine (1 µM) for 8 min at 37°C and the other half served as control. The mixtures were then centrifuged, at 14000× g for 10 min at 4°C and the supernatant was used for HPLC analysis, in duplicate. In superfusion conditions, the synaptosomes were superfused for 15 min in a manner similar to the [^{3}H]DA release assay. The synaptosomes were then exposed for 5 min to EHNA (20 µM) or EHNA combined with nicotine (1 µM) and the effluents were collected for HPLC analysis.

The separation and quantification of adenosine and its metabolites was carried out by HPLC, as previously described (Cunha and Sebastião, 1993), employing a LiChroCart-RT 125-4 C-18 reverse-phase column (particle size, 5 µm) combined with a UV detector set to 254 nm. The mobile phase consisted of KH_2PO_4 (100 mM) and methanol (85/15 v/v%) at pH 6.50 with the flow rate of 1 mL × min^{-1} and a loop volume of 50 µL. The identification and quantification of adenosine and its metabolites was achieved by calculating the peak areas then converted to concentration values (expressed as µmol × mg protein^{-1}) by calibration with known standards ranging from 0.1 to 10 µM.

Locomotor behavioural analysis and drug administration

The nicotine-induced locomotor sensitization was assessed based on previous reports (Werling *et al.*, 2009; Wellman *et al.*, 2011). The open-field test was performed in a sound-attenuated room with low-intensity light maintained constant during the testing period (Prut and Belzung, 2003). During the tests, the experimenter stayed in a room adjacent to the one where the test was performed. To remove the odour traces left by the previous animal, the floor and walls of the equipment were cleaned with 10% ethanol before testing the next animal. Locomotor behaviour was monitored in a square open-field arena, with 100 × 100 cm and 60 cm height, made of dark grey polyvinyl chloride plastic. The locomotor activity was evaluated by measuring the total distance travelled over a period of 30 min. Data were analysed using the Any-maze video tracking software (Stoelting, IL, USA).

The nicotine solution was prepared fresh each day by dissolving nicotine bitartrate in an isotonic saline solution (0.9% NaCl) neutralized to pH 7.2. ZM241385 (ZM) was dissolved in a saline solution with 5% dimethylsulfoxide (DMSO). Nicotine (0.5 mg·kg^{-1}, as nicotine-tartrate salt) or saline were injected subcutaneously in a volume of 1 mL·kg^{-1} of body weight immediately before the test period, whereas ZM (1 mg·kg^{-1}) or vehicle solution were administered intraperitoneally 30 min before the test period. The dose of each drug was chosen based on previous studies in rats showing the induction of a robust and long-lasting locomotor sensitization by nicotine (Schoffelmeer *et al.*, 2002; Le Foll *et al.*,

2003; Kayir *et al.*, 2009) and the efficient antagonism of A_{2A}Rs by ZM (Poucher *et al.*, 1996; Prediger and Takahashi, 2005; Montandon *et al.*, 2008) at the current doses used.

For this assay, the rats were randomly divided into four experimental groups. On the first 2 days, all the rats were adapted to the open-field arena for 30 min each day (habituation). For the next 8 consecutive days, all rats received two daily injections before the test period. ZM or its vehicle (0.9% saline plus 5% DMSO) was injected 30 min before the test, while nicotine or its vehicle (0.9% saline) was injected 30 min later, immediately before the behavioural test. Rats were grouped based on the following injection scheme: vehicle–vehicle (n = 3), vehicle–nicotine (n = 4), ZM–vehicle (n = 4) and ZM–nicotine (n = 5).

Following the 8 day test period, the rats from all groups remained drug-free for 7 consecutive days. On drug-free day 8, all rats were injected with nicotine (0.5 mg·kg^{-1}) and immediately placed in the open-field arena (challenge day).

Materials and chemicals

PMSF, DL-DTT, protease inhibitor cocktail (leupeptin, pepstatin A, chymostatin and antipain), halothane, pargyline, BSA, (-)-nicotine hydrogen tartrate salt and Whatman GF/C filters were obtained from Sigma (Sintra, Portugal). SDS and the Quantity one software were from Bio-Rad. PVDF membranes, pre-stained precision protein standards and ECF were purchased from Amersham Biosciences (Amadora, Portugal). BCA protein assay reagents were from Thermo Scientific (Pierce Biotechnology, Rockford, IL, USA). 3,4-[ring-2,5,6-^{3}H]dihydroxyphenylethylamine ([^{3}H]DA) was from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). α-bungarotoxin (α-BTX), α-conotoxin-PIA (α-CTX), dihydro-β-erythroidine (DHβE), 2-[*p*-(2-carboxyethyl)phenethylamino]-5'-*N*-ethylcarboxamido adenosine [CGS21680 (CGS)], EHNA and N-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]-acetamide [MRS1754 (MRS)] were from Tocris Bioscience (Bristol, UK); and 4-(2-[7-amino-2-(2-furyl)]triazolo[2,3-a][1,3,5]triazin-5-ylamino] ethyl)phenol (ZM) was from Abcam Biochemicals (Cambridge, UK). Any-maze was from Stoelting. Non-water soluble materials were dissolved in DMSO and further diluted in H_2O, aliquoted and kept at –20°C until use.

The antibodies used were as follows: rat anti-nicotinic α7 receptor (1:3000) and rabbit anti-nicotinic α4 receptor (1:3000; Abcam Biochemicals); rabbit anti-nicotinic α6 receptor (1:3000) and rabbit anti-nicotinic β2 receptor (1:15000; Merck Millipore, Darmstadt, Germany); mouse anti-β-actin (Sigma); alkaline phosphatase-labelled (AP) goat anti-rabbit (1:20000) or anti-mouse (1:20000) antibodies (GE Healthcare, Lisbon, Portugal); and AP chicken anti-rat (1:3000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Data presentation

All data are expressed as means ± SEM of the indicated number of independent observations (n). Raw effect data from release and sensitization experiments were normalized to the respective vehicle control except when noted. Normalized data were tested for normality by the Kolmogorov–Smirnov normality tests and statistical significance was calculated by one-sample t-test against a hypothetical value of 100 or 0 (when normalized). If more than two groups were compared, one-way ANOVA with Dunett's *post hoc* test was performed. Data from paired experiments were compared with the pairwise version of the above mentioned tests, and a value of $P < 0.05$ was accepted as a significant difference.

Results

Nicotine stimulates the release of [^{3}H]DA from rat striatal synaptosomes in an adenosine A_{2A} receptor-dependent manner

Nicotine (1, 30, 300 nM and 3 μM) stimulated the release of [^{3}H]DA in a concentration-dependent manner [EC_{50} = 68.0 ± 17.2 nM; maximal effect (E_{max}) = 6.80 ± 0.67 FR%; n = 10–21 rats, in duplicate; Figure 1A and B]. Next, we evaluated the effect of A_{2A}R ligands on the release of DA; the A_{2A}R agonist CGS (30 nM) also stimulated the release of [^{3}H]DA (0.63 ± 0.25 FR%, n = 10, $P < 0.05$, t = 2.503, d.f. = 9; Figure 2), while the A_{2A}R antagonist ZM (100 nM) was without effect (0.16 ±

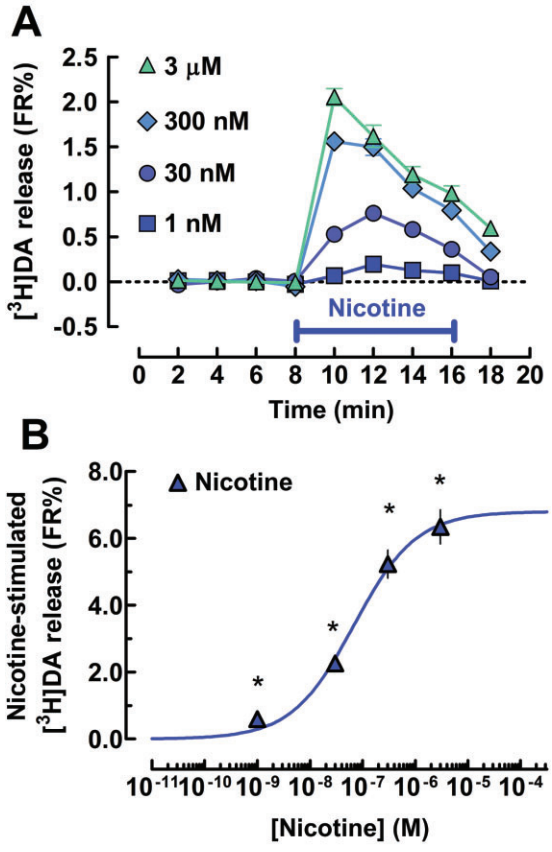

Figure 1

Nicotine stimulates [^{3}H]dopamine ([^{3}H]DA) release from rat striatal synaptosomes. (A) Time course of the averaged release of [^{3}H]DA. Synaptosomes were treated with various concentrations of nicotine (1, 30, 300 nM or 3 μM) for 8 min, as indicated by the horizontal bar. (B) Concentration-response curve for nicotine to trigger the release of [^{3}H]DA. *$P < 0.001$ versus non-stimulated control; n = 10–21 independent observations in duplicate.

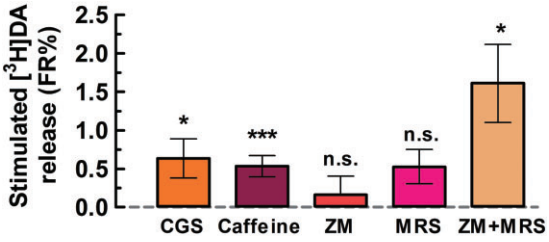

Figure 2

The adenosine $A_{2A}R$ agonist CGS (30 nM), but neither the $A_{2A}R$ antagonist ZM (100 nM) nor the $A_{2B}R$ antagonist MRS (200 nM), stimulates [^{3}H]DA release from rat striatal synaptosomes. The non-selective adenosine receptor antagonist, caffeine (50 μM), also stimulates [^{3}H]DA release, which may be explained by the observation that the simultaneous blockade of $A_{2A}Rs$ and $A_{2B}Rs$ by ZM and MRS also facilitated [^{3}H]DA release. Data are mean ± SEM of 7–38 experiments performed in duplicate. *P < 0.05, ***P < 0.01 versus 0 FR% (i.e. no change in baseline); n.s., not significant.

0.24 FR%, n = 18, P > 0.5, t = 0.6823, d.f. = 17; Figure 2). Since ZM can also antagonize adenosine A_{2B} receptors ($A_{2B}Rs$) with lower potency than $A_{2A}Rs$, we also tested the $A_{2B}R$ antagonist MRS (200 nM), which was found without effect *per se* (0.53 ± 0.22 FR%, n = 7, P > 0.05, t = 0.2383, d.f. = 6; Figure 2). Interestingly, the combined application of ZM and MRS stimulated the release of [^{3}H]DA (1.61 ± 0.51 FR%, n = 9, P < 0.05, t = 3.165, d.f. = 8; Figure 2), suggesting a cooperative interaction between $A_{2A}Rs$ and $A_{2B}Rs$ similar to that observed in splenocytes (Moriyama and Sitkovsky, 2010). Additionally, the non-selective adenosine receptor antagonist, caffeine (50 μM), also stimulated the release of [^{3}H]DA (0.53 ± 0.13 FR%, n = 38, P < 0.0001, t = 3.903, d.f. = 37; Figure 2).

In experiments combining $A_{2A}R$ ligands with nicotine, we observed that CGS (30 nM) inhibited the effect of nicotine on [^{3}H]DA release at the two lowest concentrations of nicotine (1 and 30 nM), while it had no significant impact on the effect of higher nicotine concentrations (300 nM and 3 μM; Figure 3A and B). In the presence of CGS, the effect of nicotine (1 nM) was abolished (–0.54 ± 0.28 FR%, n = 8, P < 0.05 vs. CGS alone), while the effect of nicotine at 30 nM (2.53 ± 0.21 FR%, n = 18) was reduced by 53.1 ± 11.6% (P < 0.01, t = 4.457, d.f. = 6) to 1.47 ± 0.43 FR%, n = 7. The effect of CGS was concentration-dependent (Figure 3B), displaying an IC_{50} of 11.0 ± 6.3 nM and an I_{max} of 53.8 ± 7.4% for the inhibition of 30 nM nicotine-induced DA release (n = 9–11; curve not shown).

In agreement with the involvement of $A_{2A}Rs$ to inhibit the action of nicotine, the $A_{2A}R$ antagonist ZM (100 nM) facilitated the action of different concentrations of nicotine: at 30 nM by 63.4 ± 10.0% (to 3.66 ± 0.22 FR%, n = 7, P < 0.05, t = 3.169, d.f. = 6); at 300 nM by 26.2 ± 7.6% (to 6.33 ± 0.44 FR%, n = 8, P < 0.01, t = 3.623, d.f. = 7); and at 3 μM by 26.9 ± 5.3% (to 8.55 ± 0.58 FR%, n = 6, P < 0.01, t = 4.835, d.f. = 5; Figure 4A and B). Because of its relevance as the most widely consumed psychoactive drug worldwide (Fredholm *et al.*, 1999), we next tested the impact of caffeine on nicotine-induced DA release. The acute administration of caffeine (10 μM, data not shown and 50 μM) failed to facilitate the action of nicotine (30 and 300 nM) and actually diminished that of 3 μM nicotine (to

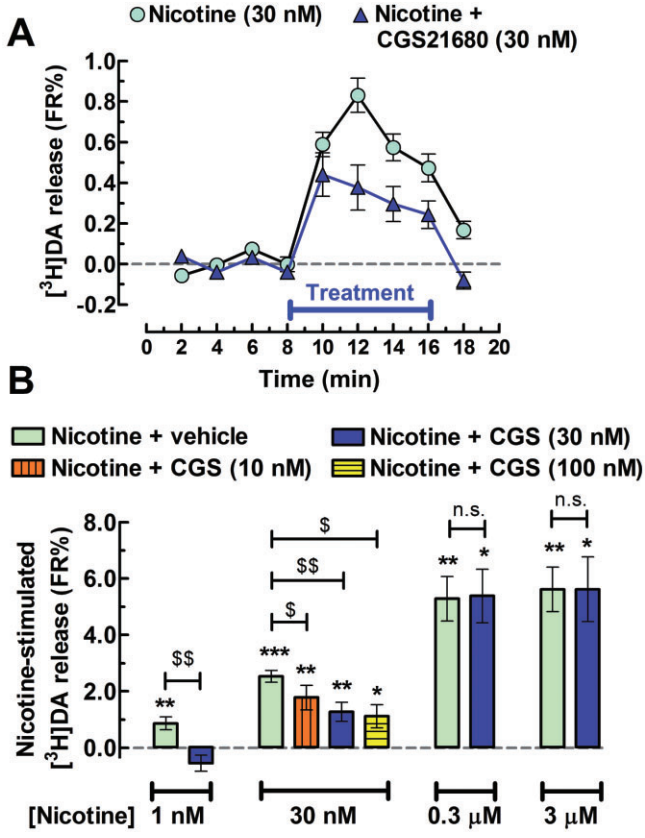

Figure 3

Adenosine $A_{2A}R$ activation inhibits the nicotine-induced [^{3}H]DA release from rat striatal synaptosomes. (A) Time course and (B) bar graph displaying the averaged release of [^{3}H]DA induced by various concentrations of nicotine alone or in the presence of $A_{2A}R$ agonist, CGS (10–100 nM). The co-administration of CGS and nicotine occurred as indicated by the horizontal bar in (A). Data are mean ± SEM of 6–18 experiments performed in duplicate. *P < 0.05, **P < 0.01 and ***P < 0.001 versus 0 FR% (i.e. no change in baseline); $^{\$}P$ < 0.05 and $^{\$\$}P$ < 0.01 between nicotine alone (green bar) and nicotine with CGS; n.s., not significant.

4.06 ± 0.73 FR%, n = 8, P < 0.01, t = 3.795, d.f. = 7; Figure 4C). Because caffeine (Fredholm *et al.*, 1999) as well as ZM have also been reported to antagonize the $A_{2B}Rs$ of rat and human (Lasley *et al.*, 2007; Li *et al.*, 2007) albeit with a lower potency than $A_{2A}Rs$ (Poucher *et al.*, 1995; Ji and Jacobson, 1999), we next tested the effects of a selective $A_{2B}R$ antagonist, MRS in our assay. MRS (200 nM) antagonized the 30 nM nicotine-stimulated release of [^{3}H]DA by 58.7 ± 11.3% (0.89 ± 0.24 FR%, n = 6, P < 0.05, t = 3.716, d.f. = 5; Figure 4D). When combined with ZM, the two antagonists did not significantly alter the 30 nM nicotine-induced release of DA (to 1.33 ± 0.89 FR%, n = 7, P > 0.05 vs. nicotine alone; Figure 4D). These results indicated that the facilitation of nicotine's action by ZM was not mediated by $A_{2B}Rs$ antagonism, although it appears that $A_{2B}Rs$ may also be involved in the control (qualitatively opposite to the role of $A_{2A}Rs$) of nicotinic receptor function in dopaminergic terminals of the striatum.

The fact that $A_{2A}R$ and $A_{2B}R$ antagonists modify DA release implies the existence of endogenous adenosine presumably

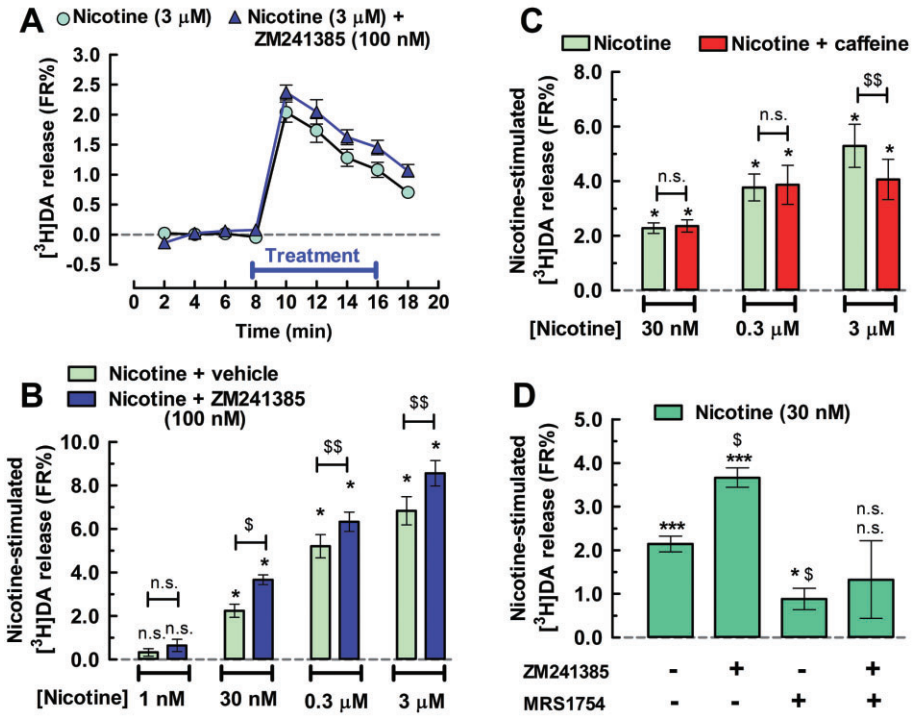

Figure 4

Adenosine A_{2A}R blockade increases the nicotine-induced [^{3}H]DA release from rat striatal synaptosomes. (A) Time course and (B) bar graph displaying the averaged release of [^{3}H]DA induced by nicotine alone or in the presence of the A_{2A}R antagonist, ZM (100 nM). The co-administration of ZM and nicotine occurred as indicated by the horizontal bar in (A). (C) In a similar experimental paradigm, the non-selective adenosine receptor antagonist, caffeine (50 μM), failed to mimic the action of ZM, that is, to facilitate the effect of nicotine. (D) This lack of caffeine effect may be due to the involvement of A_{2B}Rs because the selective A_{2B}R antagonist MRS (200 nM) inhibited the effect of nicotine and prevented the facilitatory action of ZM when the two antagonists were combined. Data are mean ± SEM of 6–13 experiments performed in duplicate. *$P < 0.05$ and ***$P < 0.001$ versus 0 FR% (i.e. no change in baseline); $^{\$}P < 0.05$ and $^{\$\$}P < 0.01$ between the indicated bars in (B) and (C) and when compared to control (nicotine in the absence of any adenosine receptor antagonist, displayed with the leftmost bar) in (D); n.s., not significant.

released from the synaptosomes both tonically and upon nAChR activation. To evaluate this hypothesis, we directly quantified the levels of adenosine in incubated (batch conditions) or superfused synaptosomes. Figure 5 shows that adenosine and its metabolites (inosine and hypoxanthine) were present in concentrations of 6 nmol·mg^{-1} protein in incubated synaptosomes (n = 4), whereas their levels were below the limit of detection in the superfusate (n = 4; figure not shown). Additionally, we found that nicotine (1 μM) failed to modify the extracellular levels of adenosine or its metabolites either in incubated or in superfused synaptosomes (n = 4, $P > 0.05$; Figure 5).

Blockade of the α6-containing nAChRs abrogates the action of A_{2A}R ligands on nicotine-stimulated [^{3}H]DA release

To map the nAChRs subtypes underlying the nicotine-stimulated [^{3}H]DA release from striatal synaptosomes, we surveyed the synaptic membranes with antibodies against different subunits of nAChRs by Western blotting. We found that the subunits α4, α6, α7 and β2 were enriched in the striatal nerve terminals (Figure 6A and B), which is in accordance with previous studies (Kaiser and Wonnacott, 2000; Grady *et al.*, 2002; 2007; Zoli *et al.*, 2002; Meyer *et al.*, 2008;

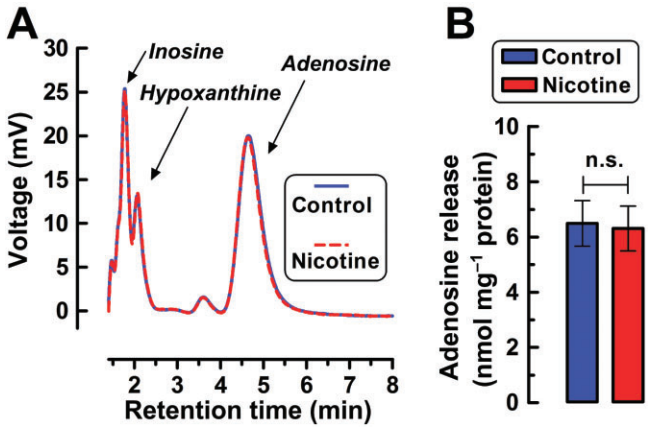

Figure 5

HPLC analysis reveals that striatal synaptosomes release adenosine. (A) Mean chromatograms and (B) mean ± SEM values for the quantified extracellular levels of adenosine and its metabolites in the presence of the adenosine deaminase inhibitor, EHNA (20 μM), upon incubation of synaptosomes (~1.2 mg protein × mL^{-1}) in the absence or in the presence of nicotine (1 μM); similar results were obtained with 30 nM nicotine (not shown). n.s., not significant.

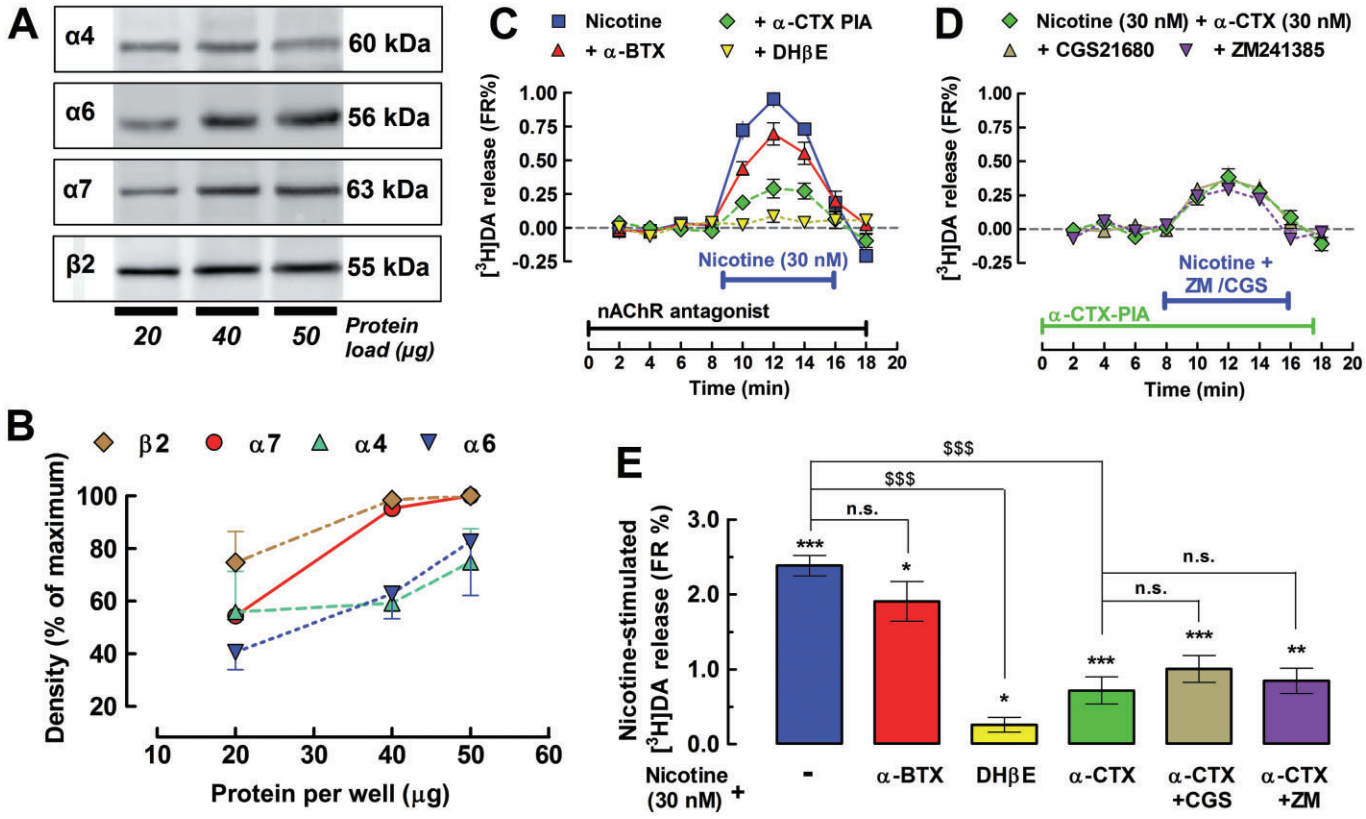

Figure 6

The adenosine A_{2A}R-mediated inhibition of the nicotine-induced [^{3}H]DA release mainly depended on α6β2-containing nicotinic ACh receptors (nAChRs). (A) Western blot analysis revealed specific bands for the different major nAChR subunits in pre-synaptic membrane preparations, with the molecular weight indicated on the right of each excerpt. (B) Quantification of the density of the different subunits obtained from three (α4, α7 and β2) and four (α6) rats under three different protein loads to ensure that the signal is not saturated (maximum = 100% = saturated signal). (C) Time course displaying the averaged [^{3}H]DA release induced by nicotine (30 nM) in the absence or in the presence of either the α6 subunit antagonist α-CTX (30 nM), the α7 subunit antagonist α-BTX (100 nM) or the β2 subunit antagonist DHβE (100 nM). (D) Time course displaying the averaged [^{3}H]DA release, induced by nicotine (30 nM) in the presence of α-CTX (30 nM), when combined with either the A_{2A}R agonist CGS (30 nM) or the A_{2A}R antagonist ZM (100 nM). (E) Bar graph summarizing the sensitivity of 30 nM nicotine-stimulated [^{3}H]DA release under the difference conditions tested in (C) and (D). Data are mean ± SEM of nine experiments performed in duplicate. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus 0 FR% (i.e. no change in baseline); $^{\$\$\$}P < 0.001$ between nicotine alone (blue bar) and nicotine with antagonists of nicotinic acetylcholine receptors; n.s., not significant. Note that neither CGS nor ZM affected the non-α6 subunit-containing nAChR-induced release of [^{3}H]DA, suggesting that it is these α6 subunit-containing nAChRs that are modulated by A_{2A}Rs to control striatal dopamine release.

Livingstone and Wonnacott, 2009). The functional relevance of these subunits was next probed using nAChR antagonists tested against the concentration of nicotine (30 nM) that was found to be sensitive to both CGS and ZM.

As illustrated in Figure 6C and E, α-BTX (100 nM), an antagonist of α7 nAChRs, failed to significantly affect the action of nicotine (30 nM) on [^{3}H]DA release (mean difference, 16.9 ± 15.1%, $P > 0.05$ by repeated measures ANOVA with Dunett's *post hoc* test).

The majority of nicotine binding sites in the brain contains the β2 subunit (Grady *et al.*, 2002; Toyohara and Hashimoto, 2010); accordingly, the β2 subunit-preferring competitive antagonist, DHβE (10 μM), prevented the nicotine-stimulated [^{3}H]DA release by 88.1 ± 5.0% ($n = 5$, $P < 0.001$, by repeated measures ANOVA with Dunett's *post hoc* test; Figure 6C and E). The α6-containing nAChR antagonist, α-CTX (30 nM), largely inhibited the action of nicotine by 69.8 ± 7.5% ($n = 6$, $P < 0.001$, by repeated measures ANOVA with Dunett's *post hoc* test; Figure 6C and E). This suggests that ~70% of the nicotine-stimulated (30 nM) [^{3}H]DA release involves the activation of α6β2-containing receptors. Upon blockade of α6-containing nAChRs (in the presence of α-CTX, 30 nM), the nicotine-induced (30 nM) [^{3}H]DA release was no longer affected by either CGS (30 nM, $n = 9$, $P > 0.05$ vs. α-CTX + nicotine) or ZM (100 nM, $n = 9$, $P > 0.05$ vs. α-CTX + nicotine; Figure 6D and E). Altogether, these data advocate that A_{2A}Rs are selectively coupled to the inhibition of α6β2-containing nAChRs in dopaminergic terminals of the rat striatum.

Nicotine-induced motor sensitization is facilitated by in vivo *A_{2A}R blockade*

The interplay between nAChRs and A_{2A}Rs controlling DA release from striatal nerve terminals prompted us to test the *in vivo* relevance of this A_{2A}Rs-α6β2-containing nAChRs interaction. One simple measure of nicotine action is its ability to

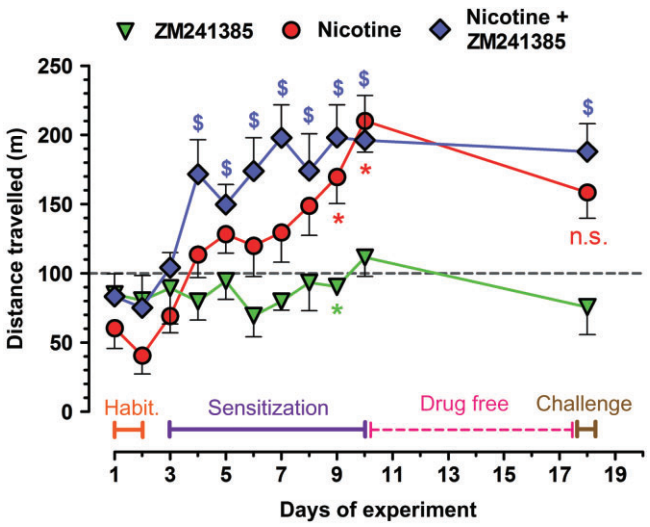

Figure 7

Adenosine A_{2A}R blockade *in vivo* facilitates the sensitization to nicotine-stimulated hyperlocomotion. Sixteen rats were used to create factorial × groups for vehicle–vehicle, ZM–vehicle, vehicle–nicotine and ZM–nicotine injections. The A_{2A}R antagonist ZM (1 mg·kg^{-1}) or its vehicle was injected 30 min before the test, while nicotine (0.5 mg·kg^{-1}) or its vehicle was injected immediately before the test. After the first 2 days of 30 min habituation to the open-field arena each day, the rats received daily injections for 8 days. Significant sensitization to nicotine developed on day 7, which was blunted by the subsequent week of abstinence, as determined by a single injection of nicotine on the day 8 of abstinence (challenge), resulting in no statistically significant difference between the vehicle–vehicle group (to which all data points were normalized, represented by the dashed line) and the nicotine–vehicle group, represented by the red circles at day 18 of the experiment. ZM had no or a minimal hypolocomotor effect throughout the duration of the experiment (green upside-down triangles). However, ZM-injected rats developed sensitization to nicotine already on day 2 (blue diamonds), which was not blunted by the drug-free period, as indicated by a statistically significant difference between the ZM–vehicle group (green upside-down triangles) and the ZM–nicotine group (blue diamonds) at day 18 of the experiment. Data are mean ± SEM of distance travelled during each 30 min of open-field observation. A *$P < 0.05$ representing statistical differences between the nicotine-injected (red circles) or ZM-injected (green triangles) and the vehicle-injected (dashed line) rats; and $^{\$}P < 0.05$ representing statistical differences between the ZM-injected and the ZM + nicotine-injected rats (blue diamonds), as determined with ANOVA of repeated measures and Dunett's *post hoc* analysis.

induce hyperlocomotion (Grottick *et al.*, 2000), which can be rated using the open-field test (Walsh and Cummins, 1976; Prut and Belzung, 2003).

After 2 days of rats' habituation to the open-field arena, one daily injection of nicotine (0.5 mg·kg^{-1}) significantly ($P < 0.05$) enhanced locomotor activity (i.e. sensitization) after 7 days compared to the control rats (i.e. vehicle–vehicle treated; Figure 7). Moreover, the nicotine-induced sensitization was blunted by the subsequent drug-free period (withdrawal; Figure 7), as indicated by similar locomotor activity between groups that are nicotine–vehicle treated and vehicle–vehicle treated ($n = 4$, $P > 0.05$). ZM (1 mg·kg^{-1}) did not alter *per se* the locomotor activity of the animals (Figure 7). Remarkably, ZM-pretreated rats developed a sensitization to nicotine already at day 2, which was no longer blunted by nicotine withdrawal ($n = 5$, $P < 0.05$), that is, locomotor activity remained higher until the challenge day when compared to the ZM–vehicle group (Figure 7).

Discussion and conclusions

The present findings provide pharmacological evidence for adenosine A_{2A}Rs exerting a negative control on the α6β2-containing nAChR-mediated stimulation of DA release from striatal dopaminergic terminals. This observation strengthens the notion that A_{2A}Rs mainly act as fine-tuners of different other neurotransmitters systems (Sebastião and Ribeiro, 2009). In fact, striatal pre-synaptic A_{2A}Rs can negatively control metabotropic receptors such as adenosine A_1Rs (Ciruela *et al.*, 2006), cannabinoid CB_1Rs (Martíre *et al.*, 2011) and glutamate group 5 receptors (Rodrigues *et al.*, 2005), or potentiate catalytic receptors such as glial cell line-derived neurotrophic factor (GDNF) receptors (Gomes *et al.*, 2006; 2009). In other brain areas, A_{2A}Rs also have been shown to control the function of ionotropic receptors such as NMDA (Rebola *et al.*, 2008), AMPA (Dias *et al.*, 2012) or $GABA_A$ receptors (Roseti *et al.*, 2008). Additionally, A_{2A}Rs also control the rate of desensitization of different nAChRs in peripheral preparations including the myenteric plexus (Duarte-Araújo *et al.*, 2004) and the carotid body (Fitzgerald *et al.*, 2009) or in heterologous expression systems (Di Angelantonio *et al.*, 2011). The present report extends this rule to the CNS, in particular to α6β2-containing nAChR in striatal dopaminergic terminals, which we showed to be controlled by A_{2A}Rs.

The present Western blot data, combined with the pharmacological characterization, indicates an important role of the α6β2-containing nAChR to mediate the DA-releasing action of nicotine. This is in agreement with previous findings that the absence of β2 subunits abrogates the ability of nicotine to trigger DA release from synaptosomes (Grady *et al.*, 2002) and that the α6 subunit has an important role in the regulation of mesolimbic DA release (Calabresi and Di Filippo, 2008; Drenan *et al.*, 2008; Meyer *et al.*, 2008; Quik *et al.*, 2011). Moreover, in striatal terminals of rats, we found nAChR subunits other than the α6 subunit, namely, the α4 and the α7 subunits. This stems from the fact that the majority of the synaptic proteins in striatal synaptic Western blot samples come from glutamatergic and GABAergic terminals, whereas dopaminergic terminals only represent about one-fifth of the total number of nerve terminals (Borycz *et al.*, 2007; Gomes *et al.*, 2009). Thus, whereas α7 nAChRs directly control striatal glutamate release (Kaiser and Wonnacott, 2000; Marchi *et al.*, 2002), this subunit seems to be absent in mesolimbic dopaminergic cells (Zoli *et al.*, 2002). This is not the case for the α4 subunit, which others have reported to be present in dopaminergic terminals in the dorsal striatum (Kaiser and Wonnacott, 2000; Zoli *et al.*, 2002; Exley *et al.*, 2012) and may also play a role in the control of DA release under different experimental conditions (Gotti *et al.*, 2010; Smith *et al.*, 2010; Exley *et al.*, 2012).

This ability of pre-synaptic A_{2A}Rs to control the α6β2-containing nAChR-induced release of DA was extended to an *in vivo* setting, by showing that A_{2A}Rs also controlled the

locomotor sensitization induced by nicotine. This locomotor sensitization to nicotine is known to involve the recruitment of β2-containing nAChRs (Picciotto *et al.*, 1998) and a differential participation of α4-containing and α6-containing, but not α7, nAChRs (Kempsill and Pratt, 2000; Tapper *et al.*, 2004; Gotti *et al.*, 2010; Smith *et al.*, 2010) controlling the release of DA in different regions of the basal ganglia. This differential adaptation of different α4-containing and α6-containing nAChRs upon repeated nicotinic exposure (Tapper *et al.*, 2004; Perry *et al.*, 2007; Perez *et al.*, 2008; Smith *et al.*, 2010) is a likely explanation for the potentiation of nicotinic locomotor sensitization by the tested A_{2A}R antagonist. This is in general agreement with previous studies showing that the non-selective adenosine receptor antagonist, caffeine, bolsters the nicotine-induced increase of locomotor activity (Celik *et al.*, 2006; Cohen *et al.*, 1991). However, it is worth noting that the rewarding properties of nicotine, tested in a place-conditioning paradigm, were decreased in global A_{2A}R knockout mice (Castañé *et al.*, 2006), heralding the hypothesis that different subtypes of nAChRs might be differently controlled by A_{2A}Rs.

Apart from this ability of A_{2A}Rs to control the α6β2-containing nAChR-induced release of DA, the present results also showed that CGS *per se* stimulated the release of DA; this is in agreement with the functional and morphological data identifying the presence of A_{2A}Rs in dopaminergic nerve endings in the striatum (Chowdhury and Fillenz, 1991; Gomes *et al.*, 2006; 2009) and also, with the ability of striatally micro-infused CGS to increase basal DA levels in freely moving rats (Gołembiowska and Zylewska, 1997). This contention for the involvement of A_{2A}Rs was based on the antagonism of the effect of CGS by ZM, which has a 10-fold higher potency to inhibit A_{2A}Rs compared with A_{2B}Rs (Poucher *et al.*, 1995; Ji and Jacobson, 1999). We also probed the possible involvement of A_{2B}Rs and found that the selective A_{2B}R antagonist MRS largely inhibited the effect of nicotine at a concentration (200 nM) threefold lower than its IC_{50} at A_{2A}Rs. Furthermore, the concomitant inhibition of both A_{2A}Rs and A_{2B}Rs extinguished each other's effect. This may mean two subsets of dopaminergic terminals bearing either A_{2A}Rs or A_{2B}Rs, leading to a lack of net change, or alternatively that the two receptors may reside and interact in the same nerve terminals. Intriguingly, neither ZM nor MRS affected DA release p*er se* but when combined they synergistically stimulated DA release. This was also observed when testing the non-selective adenosine receptor antagonist, caffeine, an observation that may be pertinent to the understanding of the addictive profile of caffeine (Svikis *et al.*, 2005). Although the underlying mechanism for how A_{2A}R activation or the simultaneous A_{2A}R/A_{2B}R blockade increases basal DA outflow is unclear, these data provide the first demonstration of a functional interaction between the two A_2R subtypes in the CNS, in a manner similar to that previously reported to occur in splenocytes (Moriyama and Sitkovsky, 2010).

In summary, the present results show that A_{2A}Rs curtail the function of α6β2-containing nAChRs in striatal dopaminergic nerve terminals – an effect that seems relevant for the ability of A_{2A}R antagonists to potentiate the psychomotor effects resulting from a repeated exposure to nicotine. These observations provide a mechanistic insight to explain the frequent correlation in nicotine and caffeine abuse (Swanson *et al.*, 1994). This A_{2A}R–nAChR interaction also paves the way to foster novel therapeutic opportunities to manage motor diseases related with dysfunctional DA signalling, such as Parkinson's disease, where both caffeine and nicotine provide a combined prophylactic benefit (Powers *et al.*, 2008) and where A_{2A}R antagonists are a leading non-dopaminergic therapeutic strategy (Prediger, 2010). Notably, the mechanisms underlying the A_{2A}R-mediated amelioration of Parkinson's disease symptoms are not fully understood and it is also possible that it may involve a rescuing of nAChRs-stimulated phasic DA release (see Threlfell *et al.*, 2012).

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Conflicts of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 A guide for the calculations and statistics of the release experiments. In a single experiment from a rat, various treatments were performed, including a pair of vehicle controls and various pairs of treatments with ligands alone or in combination. To obtain the net effect of each tested drug, the averaged duplicates of the respective controls (not shown) were subtracted from the averaged treatments, leading to the graphs pictured in panels A-D. In this simulated case, the area under the curve (AUC) value for nicotine amounts to 'X' fractional release % (FR%), and the same for CGS21680 gives the value of 'Y' FR%. Additionally, in the same experiment, we combined CGS21680 with nicotine, yielding a total AUC value of 'V' FR% This 'V' value can be perceived as a sum of 'Y' + 'Z' where 'Z' is the modified effect of nicotine after discounting the effect of CGS21680 per se. These raw data were then used for the following comparisons: 'X', 'Y', 'V' were compared to zero FR% (i.e. no effect on dopamine release) and if statistical difference was detected it was marked with *, **, or ***. Furthermore, 'X' was also compared to 'Z' to see whether CGS21680 (or any other adenosinergics alone or in combination) altered the effect of nicotine, and if statistical difference was detected it was labeled with $, $$, or $$$. The respective 'Y' values are displayed in Figure 3. The 'Z' values are displayed in Figures 3, 4 and 6, respectively, while in Figure 7, the same set of symbols are used on locomotor activity instead of dopamine release.