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RESEARCH PAPER

# Presynaptic adenosine $A_{2A}$ receptors dampen cannabinoid $CB_1$ receptor-mediated inhibition of corticostriatal glutamatergic transmission

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## BACKGROUND AND PURPOSE

Both cannabinoid $CB_1$ and adenosine $A_{2A}$ receptors ($CB_1$ receptors and $A_{2A}$ receptors) control synaptic transmission at corticostriatal synapses, with great therapeutic importance for neurological and psychiatric disorders. A postsynaptic $CB_1$–$A_{2A}$ receptor interaction has already been elucidated, but the presynaptic $A_{2A}$ receptor-mediated control of presynaptic neuromodulation by $CB_1$ receptors remains to be defined. Because the corticostriatal terminals provide the major input to the basal ganglia, understanding the interactive nature of converging neuromodulation on them will provide us with novel powerful tools to understand the physiology of corticostriatal synaptic transmission and interpret changes associated with pathological conditions.

## EXPERIMENTAL APPROACH

Pharmacological manipulation of $CB_1$ and $A_{2A}$ receptors was carried out in brain nerve terminals isolated from rats and mice, using flow synaptometry, immunoprecipitation, radioligand binding, ATP and glutamate release measurement. Whole-cell patch-clamp recordings were made in horizontal corticostriatal slices.

## KEY RESULTS

Flow synaptometry showed that $A_{2A}$ receptors were extensively co-localized with $CB_1$ receptor-immunopositive corticostriatal terminals and $A_{2A}$ receptors co-immunoprecipitated $CB_1$ receptors in these purified terminals. $A_{2A}$ receptor activation decreased $CB_1$ receptor radioligand binding and decreased the $CB_1$ receptor-mediated inhibition of high-$K^+$-evoked glutamate release in corticostriatal terminals. Accordingly, $A_{2A}$ receptor activation prevented $CB_1$ receptor-mediated paired-pulse facilitation and attenuated the $CB_1$ receptor-mediated inhibition of synaptic transmission in glutamatergic synapses of corticostriatal slices.

## CONCLUSIONS AND IMPLICATIONS

Activation of presynaptic $A_{2A}$ receptors dampened $CB_1$ receptor-mediated inhibition of corticostriatal terminals. This constitutes a thus far unrecognized mechanism to modulate the potent $CB_1$ receptor-mediated presynaptic inhibition, allowing frequency-dependent enhancement of synaptic efficacy at corticostriatal synapses.

### Abbreviations

3Rs, replacement, refinement and reduction of animals in research; ADA, adenosine deaminase; MSN(s), medium spiny neuron(s); OFA, Oncins France Strain A; PPR, paired-pulse ratio; vGlut1, vesicular glutamate transporter 1

## Tables of Links

| TARGETS |
|---|
| **GPCRs**[a] |
| Adenosine $A_1$ receptors |
| Adenosine $A_{2A}$ receptors |
| Cannabinoid $CB_1$ receptors |
| Dopamine $D_2$ receptors |
| **Enzymes**[b] |
| ADA, adenosine deaminase |
| Diacylglycerol lipase |
| Ecto 5′-nucleotidase, NT5E |
| Glutamate decarboxylase |
| **Transporter**[c] |
| vGluT1, vesicular glutamate transporter 1, SLC17A7 |

| LIGANDS |
|---|
| [3H]SR141716A |
| Adenosine |
| AM251 |
| Aminooxyacetic acid |
| ATP |
| Brain-derived neurotrophic factor |
| CGS21680 |
| Fibroblast growth factor |
| Glial cell-derived neurotrophic factor |
| Glutamate |
| WIN55212-2 |

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c]Alexander *et al.*, 2013a,b,c).

## Introduction

The corticostriatal pathway is a massive projection linking virtually the entire neocortex with the striatum – the latter being considered as the major input site of the basal ganglia (Goldman-Rakic and Selemon, 1986; Bolam *et al.*, 2000). The principal neurons of the striatum – medium spiny neurons (MSNs) – integrate synaptic information from functionally diverse cortical regions to process signals controlling goal-directed behaviours and habits (Graybiel, 1995; Yin and Knowlton, 2006). As a gateway to trigger the recruitment of striatal circuits, alterations in the strength of the synaptic connections between the cortex and striatum play a critical role in these adaptive behavioural changes (Di Filippo *et al.*, 2009).

GPCRs, such as the cannabinoid $CB_1$ receptor, are key determinants of synaptic efficacy changes in corticostriatal synapses (Lovinger, 2010). Accordingly, the manipulation of the endocannabinoid system has a profound impact on striatal-dependent behavioural responses (El Manira and Kyriakatos, 2010; Katona and Freund, 2012). Another major controller of striatal function is the adenosine $A_{2A}$ receptor (Schiffmann *et al.*, 2007). These $A_{2A}$ receptors are abundantly located in the dendritic spines of MSNs (Svenningsson *et al.*, 1999) and are also present presynaptically, controlling glutamate release (Ciruela *et al.*, 2006; Quiroz *et al.*, 2009) and corticostriatal plasticity (D'Alcantara *et al.*, 2001; Flajolet *et al.*, 2008). Interestingly, $A_{2A}$ receptors seem to mainly act as a fine-tuning system, adapting the efficiency of different other modulator systems (Sebastião and Ribeiro, 2000; Ferré *et al.*, 2011). The activation of striatal $A_{2A}$ receptors results in $G_s$-mediated accumulation of cAMP ($G_{olf}$ in the MSNs), in contrast to the stimulation of the other abundant striatal adenosine receptor, the inhibitory $G_{i/o}$-coupled adenosine $A_1$ receptors (Dunwiddie and Masino, 2001). In physiological conditions, low-frequency (0.1 < Hz) neuronal activity is accompanied with a modest generation of adenosine, most likely from the metabolism of ATP of astrocytic origin, which exerts tonic inhibition of neighbouring excitatory synapses via $A_1$ receptors (Cunha, 2008). This dominant form of paracrine adenosinergic neuromodulation probably serves to decrease the noise of the system at resting state (Cunha, 2008). In contrast, under high-frequency discharge of the nerve terminals, the ecto-5′-nucleotidase-mediated degradation of ATP, co-released from synaptic vesicles (Sperlágh and Vizi, 1996), will build up synaptic adenosine levels that are sufficient for autocrine $A_{2A}$ receptor activation (Cunha, 2008; Augusto *et al.*, 2013). Pathological conditions such as ischaemia can also increase extracellular adenosine levels via outward transport, which are enough to stimulate both $A_1$ and $A_{2A}$ receptors (Gomes *et al.*, 2011).

$A_{2A}$ receptors have been reported to tightly regulate the endocannabinoid neuromodulation system in the striatum,

as shown by the $A_{2A}$–$CB_1$ receptor interactions in the control of motor dysfunction (Ferré *et al.*, 2010; Lerner *et al.*, 2010; Tozzi *et al.*, 2012) and addiction (Soria *et al.*, 2004; Yao *et al.*, 2006; Rossi *et al.*, 2010; Justinová *et al.*, 2011). This is reinforced by the reported heteromerization of $A_{2A}$ receptors with $CB_1$ receptors that was demonstrated in heterologous expression systems and in the striatum (Carriba *et al.*, 2007). However, this $A_{2A}$–$CB_1$ receptor interaction is mostly interpreted as resulting from a postsynaptic interaction (Yao *et al.*, 2006; Rossi *et al.*, 2010; Cerri *et al.*, 2014; Pinna *et al.*, 2014), whereas the predominant localization of $CB_1$ receptors is presynaptic in the striatum (Köfalvi *et al.*, 2005; Uchigashima *et al.*, 2007). A possible presynaptic interaction between $A_{2A}$ and $CB_1$ receptors has also been proposed to control the motor-depressant and addictive effects of cannabinoids (Ferré *et al.*, 2010; Martíre *et al.*, 2011; Justinová *et al.*, 2014), but detailed experimental evidence is lacking. Here, we set out to further expand our previous observations (Martíre *et al.*, 2011) now using selective presynaptic techniques and combining refined immunological, radioligand binding and functional assays to directly investigate $A_{2A}$–$CB_1$ receptor interactions in glutamatergic nerve terminals of corticostriatal synapses.

## Methods

### Animals

All animal care and experimental procedures were 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 (Kilkenny *et al*, 2010) and were approved by the Animal Care Committee of the Center for Neuroscience and Cell Biology of Coimbra and by the Centre for Interdisciplinary Research in Biology in College de France. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al*, 2010; McGrath *et al.*, 2010). A total of 70 animals were used in the experiments described here.

Animals were housed under a 12 h light on/off cycle with *ad libitum* access to food and water. Forty-nine male Wistar rats (180–240 g, 8–10-week-old) were purchased from Charles River (Barcelona, Spain) and six Oncins France Strain A (OFA) rats (16–22 postnatal days) from Charles River (L'Arbresle, France). Five pairs of $A_{2A}$ receptor and $CB_1$ receptor null-mutant (knockout, KO) male mice on CD-1 background (Ledent *et al.*, 1997; 1999) and their wild-type (WT) littermates (35–45 g, 8–12-week-old) were also used and were genotyped by tail snips.

### Synaptosomal preparations

Experimental procedures were carried out as previously described by Ferreira *et al.*, (2009). Briefly, the animals were decapitated under halothane anaesthesia, and their brains were quickly removed into ice-cold 0.32 M sucrose solution containing 10 mM HEPES, 1 mM EDTA and 1/500 v/v protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA), pH 7.4. The pair of striata was rapidly dissected and homogenized instantly with a glass potter and a teflon homogenizer, and centrifuged at 3000× *g* for 5 min. The supernatant was collected and centrifuged at 13 000× *g* for 10 min to obtain the P2 crude synaptosomal fraction. For immunolabelling and flow cytometry analysis, the P2 fraction was further purified on a discontinuous Percoll gradient (3, 10 and 23%), as described in Köfalvi *et al.* (2005). The purified synaptosomes were kept at –80°C until use.

### Immunolabelling and flow cytometric analysis of purified nerve terminals

Immunochemical labelling was performed according to a method for staining of intracellular antigens (Schmid *et al.*, 1991; Gylys *et al.*, 2000), with little modification. Briefly, purified nerve terminals were fixed in 1 mL of 0.25% paraformaldehyde in PBS (135 mM NaCl, 1.3 mM KCl, 3.2 mM $NaH_2PO_4$ and 0.5 mM $KH_2PO_4$) for 1 h at 4°C and then centrifuged at 3000×*g* for 3 min at 4°C. For permeabilization, the pellets were incubated in PBS with 0.2% Tween-20 for 15 min at 37°C and then centrifuged at 3000× *g* for 3 min. The pellets were then resuspended in PBS for immunolabelling. Primary and secondary antibodies (Supporting Information Table S1) were diluted in PBS containing 2% normal goat serum (Vector Laboratories, Burlingame, CA, USA). For validation/titration of the primary antibodies, see Supporting Information Fig. S1. Incubation volume was 100 µL and incubation time was 30 min at 4°C for both the primary and the secondary antibodies. Each incubation was followed by three washes in PBS with 0.2% Tween-20 and centrifugation at 3000× *g* for 3 min. The samples were resuspended in filtered PBS for flow-synaptometric analysis.

Analysis was performed using a FACSCalibur flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA – equipped with a 488 nm argon-ion laser). Sample flow was set at 350 events per second; 50 000 ungated events were collected for analysis. A threshold was set on forward light scatter to exclude debris. To correct for spectral overlap during multicolour flow cytometry experiments, colour compensation was performed. Offline data analysis was performed using BD Cell Quest Pro software (Becton, Dickinson and Company). For detailed description see Supporting Information Fig. S1.

### Receptor binding

Synaptosomal membranes were prepared as previously described (Rebola *et al.*, 2005) upon resuspensions of P2 synaptosomes in 2 mL of ice-cold assay solution [50 mM Tris HCl, 3 mM $MgCl_2$, 1 µM $CaCl_2$, 2 mM EDTA and protease inhibitor cocktail (Sigma-Aldrich), pH 7.4]. Single point $CB_1$ receptor binding experiment with 3.82 ± 0.29 nM ($n$ = 7) of the $CB_1$ receptor antagonist/inverse agonist, [$^3$H]SR141716A, was carried out as before (Ferreira *et al.*, 2012), with 30 min pre-incubation in the presence of adenosine deaminase (ADA, 2 U·mL$^{-1}$) and of the diacylglycerol lipase inhibitor, OMDM188 (300 nM, a kind gift of Dr Vincenzo Di Marzo). Non-specific binding was determined using the $CB_1$ receptor antagonist/inverse agonist AM251 (1 µM). Each of the seven independent assays was carried out on synaptic membranes derived from two rats, altogether 14 rats, and assayed in quadruplicate (28 filters per condition). The tritium content of each sample was counted using a Tricarb 2900TR β-counter (Perkin Elmer, Lisbon, Portugal). The specific binding was expressed as amount of ligand specifically bound per milligram of protein.

## Immunoprecipitation

Immunoprecipitation assays were carried out in both crude and Percoll-purified rat striatal synaptosomal fractions (pooled from three rats to obtain enough material), as previously described (Marques *et al*., 2013). Briefly, protein extracts were incubated with 50% protein G-Sepharose bead slurry (GE Healthcare, Little Chalfont, UK) for 3 h at 4°C to eliminate non-specific binding. After incubation, the pre-cleared supernatants containing 1 mg protein were incubated overnight with rotation at 4°C with a mouse anti-$A_{2A}$ receptor antiserum (Merck-Millipore, Darmstadt, Germany) pre-coupled covalently to protein G-sepharose (GE Healthcare), in the presence of 1% BSA (Sigma-Aldrich) and protease inhibitors (Roche Diagnostics, Amadora, Portugal). The beads were washed three times with isolation buffer containing 150 mM KCl, 20 mM MOPS and 1% Triton X-100 (pH 7.4) and resuspended in six-times diluted SDS-PAGE sample buffer [0.35 M Tris, 30% glycerol, 10% SDS, 0.6 M dithiothreitol, 0.012% bromophenol blue (pH 6.8)]. Bound proteins eluted from the immune complexes were denatured by heating to 95°C for 5 min and then separated by electrophoresis on SDS-PAGE gels. Proteins were then electrotransferred onto nitrocellulose membranes (Amersham, Little Chalfont, UK) and probed with rabbit anti-$CB_1$ receptor (Supporting Information Table S1) and mouse anti-$A_{2A}$ receptor (Supporting Information Table S1) antibodies, diluted in Tris-buffered saline supplemented with Tween-20 (0.1% v/v) and BSA (5% m·$v^{-1}$). Immunoreactivity was visualized using HRP-conjugated goat anti-rabbit or anti-mouse secondary antibodies (Thermo Scientific Pierce, Rockford, IL, USA) with a subsequent incubation with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific Pierce) and the images were acquired using Versadoc3000 apparatus and analysed with ImageLab software (Bio-Rad, Amadora, Portugal). A negative control containing the same amount of mouse $IgG_{2A}$ instead of the mouse anti-$A_{2A}$ receptor antibody was run in parallel for each experiment.

## [$^{14}$C]glutamate release

Experiments were carried out with slight modifications to previous publications (Köfalvi *et al*., 2005), which are: the synaptosomes were loaded with [$^{14}$C]-U-glutamate (20 μM) for 10 min and the superfused synaptosomes, trapped in the 16 microvolume chamber release system, were stimulated with 30 mM KCl twice for 1 min ($S_1$, $S_2$), with a 10 min interval. All Krebs-HEPES solutions used for this assay contained the glutamate decarboxylase inhibitor aminooxyacetic acid (100 μM) to prevent [$^{14}$C]glutamate degradation. For detailed description, see Köfalvi *et al*. (2005) and Ferreira *et al*. (2009). The validation of the $A_1$ receptor, $A_{2A}$ receptor as well as $CB_1$ receptor -mediated neuromodulation in Wistar rat and CD-1 mouse striatal synaptosomes is summarized in Supporting Information Table S2.

## ATP release assay from striatal synaptosomes

ATP quantification was carried out in 96-well plates, using a Perkin Elmer Victor$^3$ multi-label plate reader in luminometer mode. The ATP assay mix (Sigma-Aldrich) used by us allows quantitative bioluminescent determination of very low ATP levels ranging from $2 \times 10^{-12}$ to $8 \times 10^{-5}$ M, according to Navizet *et al*. (2011). Solutions used were (i) basal saline medium (in mM): 115 NaCl, 3 KCl, 1.2 $KH_2PO_4$, 25 HEPES, 10 glucose, 1.2 $MgSO_4$, 1 $CaCl_2$, pH = 7.4; and (ii) potassium saline medium (in mM): 118 KCl, 1.2 $KH_2PO_4$, 25 HEPES, 10 glucose, 1.2 $MgSO_4$, 1 $CaCl_2$, pH = 7.4. A 150 μL aliquot of basal saline medium with compounds to be tested or their vehicle, DMSO (0.1% v/v), 15 μL of ATP assay mix and 35 μL synaptosomal suspension (~1 mg·$mL^{-1}$) provided the 200 μL final reaction volume. This mixture was incubated at 25°C for 3 min in an Eppendorf tube to ensure functional recovery of the sample and then it was transferred into a well of the plate at 25°C, inside the reader. Afterwards, a kinetic protocol was initiated with the duration of 140 s. During the first 60 s, a stable baseline was recorded corresponding to the basal extrasynaptic ATP level. Subsequently, synaptosomes were stimulated with KCl (30 mM) or were challenged only with the same amount of NaCl serving as osmotic control. Average readings in the presence of high NaCl were subtracted from KCl-stimulated average readings.

## Electrophysiology

Whole-cell patch-clamp recordings from MSNs were made in horizontal brain slices from OFA rats (Figure 5A) (postnatal days 15–22), as previously described (Fino *et al*., 2005). Briefly, the artificial CSF contained (in mM): 125 NaCl, 2.5 KCl, 25 glucose, 25 $NaHCO_3$, 1.25 $NaH_2PO_4$, 2 $CaCl_2$, 1 $MgCl_2$ and 10 μM pyruvic acid bubbled with 95% $O_2$ and 5% $CO_2$; the borosilicate glass pipettes (6–8 MΩ) contained (in mM): 105 potassium gluconate, 30 KCl, 10 HEPES, 10 phosphocreatine, 4 ATP-Mg, 0.3 GTP-Na, 0.3 EGTA (adjusted to pH 7.35 with KOH). All the experiments were carried in the presence of 50 μM picrotoxin (Sigma-Aldrich). Electrical stimulation was performed with a bipolar electrode (Phymep, Paris, France), placed at the layer 5 of the somatosensory cerebral cortex, by applying a monophasic and constant current (duration: 100–150 μs) (ISO-Flex stimulator controlled by a Master-8, A.M.P.I., Jerusalem, Israel). All recordings were performed at 32°C using a temperature control system (Bath-controller V, Luigs & Neumann, Ratingen, Germany). Individual neurons were identified using infrared differential interference contrast microscopy with CCD camera (Hamamatsu C2400-07; Hamamatsu, Japan). Signals were amplified using an EPC9-2 amplifier (HEKA Elektronik, Lambrecht, Germany). The range of access resistance was 80–200 MΩ. The liquid junction potential was calculated and corrected. Voltage-clamp recordings were filtered at 5 kHz and sampled at 10 kHz using the program Pulse-8.53 (HEKA Elektronik). The series resistance was compensated at 75–80% and variation of series resistance above 20% led to the rejection of the experiment. Offline analysis was performed using Igor-Pro 6.0.3 (Wavemetrics, Lake Oswego, OR, USA).

## Data analysis

Raw effect data from [$^{14}$C]glutamate and ATP release assays and from electrophysiology were normalized to the appropriate control of the same experiment. These normalized data were tested for normality by the Kolmogorov–Smirnov normality test. Statistical significance was calculated by one-sample *t*-test against the hypothetical value of 100 (as 100%, i.e. vehicle control). Pairs of treatment or condition groups were compared with Student's paired *t*-test, while the anti-

body titration curves were compared with two-way ANOVA between the WT and the KO mice. $P < 0.05$ was accepted as significant difference.

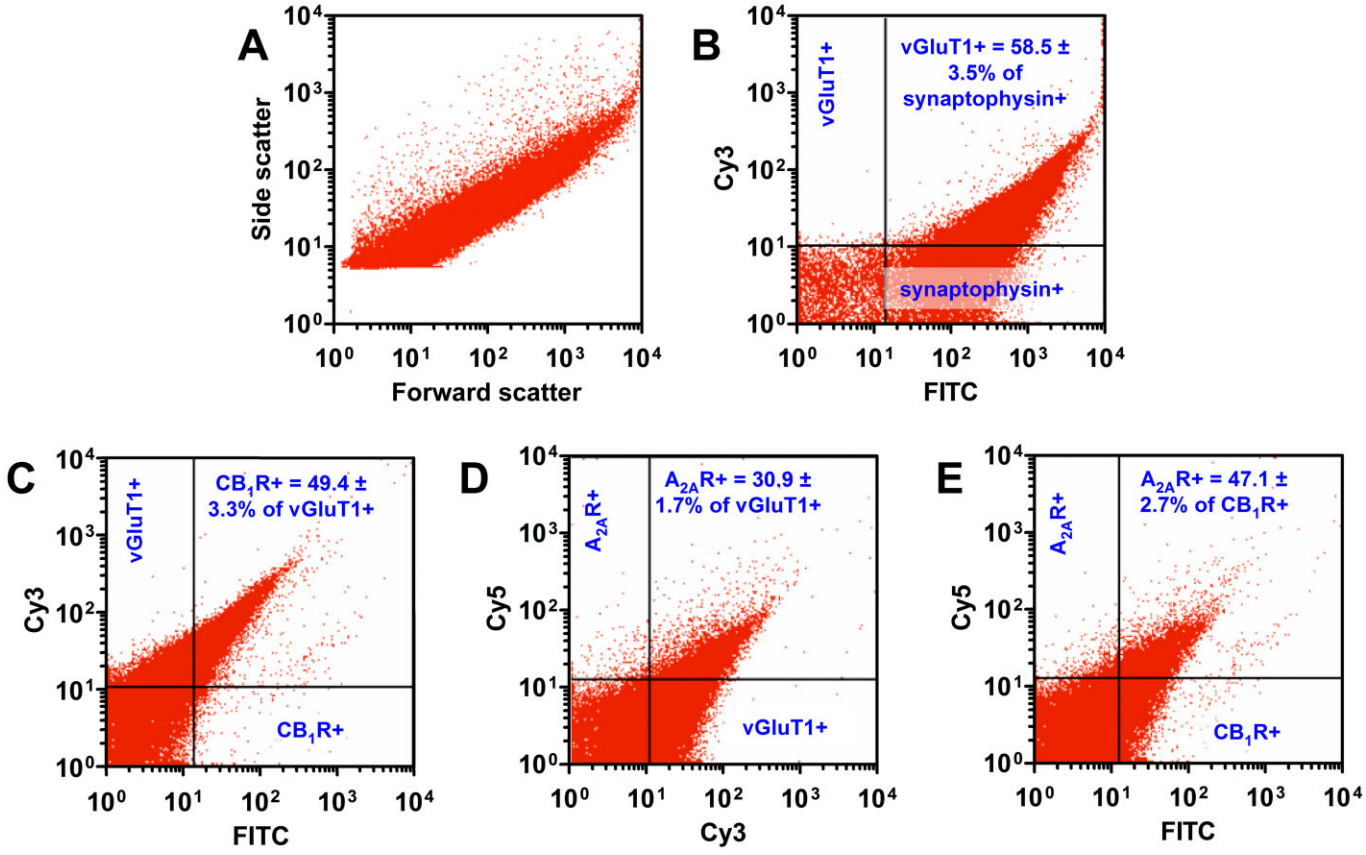
### *Materials*

1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(1-piperidyl)pyrazole-3-carboxamide (AM251), (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone (WIN55212-2) and (6aR,10aR)-6a,7,10,10a-tetrahydro-3-[5-(1H-imidazol-1-yl)-1,1-dimethylpentyl]-6,6,9-trimethyl-6H-dibenzo[b,d]pyran-1-ol (O-2545) were purchased from Abcam Biochemicals, Cambridge, UK; 3-[4-[2-[[6-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino]ethyl]phenyl]-propanoic acid (CGS21680) was purchased from Tocris Bioscience, Bristol, UK; (DMSO, MOPS, aminooxyacetic acid, halothane, HEPES, Percoll, ADA, BSA and sucrose were purchased from Sigma-Aldrich. [$^3$H]SR141716A (specific activity: 60 Ci·mmol$^{-1}$; 1 mCi·mL$^{-1}$) and [$^{14}$C]-U-glutamate (specific activity: 200 mCi·mmol$^{-1}$; 0.1 mCi·mL$^{-1}$) were purchased from American Radiolabeled Chemicals (St Louis, MO, USA). All other reagents were purchased from Merck Biosciences (Darmstadt, Germany).

## Results

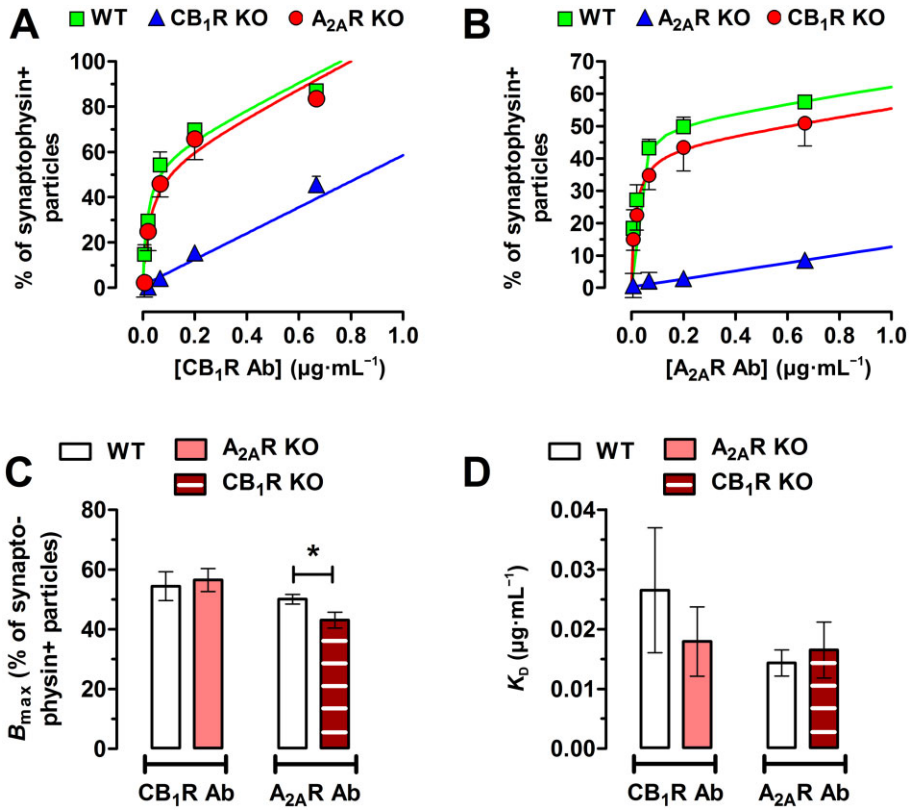
### *$A_{2A}$ and $CB_1$ receptors co-localize in corticostriatal glutamatergic nerve terminals*

A basic piece of evidence in a study of the functional cross-talk between $CB_1$ and $A_{2A}$ receptors in corticostriatal glutamatergic terminals is to demonstrate their simultaneous co-localization at these sites. Thus, we analysed striatal synaptosomes via flow synaptometry (Figure 1A): we double-labelled them for synaptophysin and the vesicular glutamate transporter vGluT1 (Figure 1B) or triple-labelled for vGluT1, $CB_1$ and $A_{2A}$ receptors (Figure 1C–E). Concerning the double labelling, 85.2 ± 2.5% ($n$ = 3) of the analysed particles were positive for synaptophysin. Of these particles, 58.5 ± 3.5% were positive for vGluT1 (Figure 1B). Triple labelling allowed to precisely quantify that 49.4 ± 3.3% of the vGluT1-positive terminals also stained for $CB_1$ receptors ($n$ = 3) (Figure 1C)




## Figure 1

Flow-synaptometric analysis of immunolabelled Percoll-gradient purified striatal synaptosomes. (A) Representative flow synaptometry plot of striatal synaptosomes for size (forward scatter is proportional to the particle diameter) and for complexity/granularity (side scatter). (B) Representative plot and statistics of synaptosomes double-labelled for synaptophysin (a marker of synaptosomes) and vGluT1 (a marker of corticostriatal terminals). (C and D) Representative plots and statistics of synaptosomes labelled for vGluT1/$CB_1$ receptor (C) and vGluT1/$A_{2A}$ receptor (D) respectively (the two graphs are derived from the same triple-labelled sample). (E) Representative plot and statistics of vGluT1-positive synaptosomes expressing $CB_1$ and $A_{2A}$ receptors. Note that most of the nerve terminals staining positive for $A_{2A}$ receptors ($A_{2A}$R) were also positive for $CB_1$ receptors ($CB_1$R).




## Figure 2

(A) Total binding isotherms of the anti-$CB_1$ receptor antibody in the corticostriatal terminals of the WT versus the $A_{2A}$ receptor KO mice ($A_{2A}$R KO), while the $CB_1$ receptor KO mice ($CB_1$ KO) display antibody binding of non-specific nature only. (B) Total binding isotherms of the anti-$A_{2A}$ receptor antibody in the corticostriatal terminals of the WT versus the $CB_1$ receptor KO mice, while the $A_{2A}$ receptor KO mice display antibody binding of non-specific nature only. (C) Bar graphs representing the mean values of the maximum binding sites ($B_{max}$) of the anti-$CB_1$ and anti-$A_{2A}$ receptor antibodies. *$P < 0.05$. (D) Bar graphs representing the mean changes of the $K_D$ of the anti-$CB_1$ and anti-$A_{2A}$ receptor antibodies. Bars represent the mean ± SEM of five individual experiments.

and 30.9 ± 1.7% bore $A_{2A}$ receptors ($n = 3$) (Figure 1D). Additionally, 47.1 ± 2.7% of the $CB_1$ receptor-positive terminals were also positive for $A_{2A}$ receptors ($n = 3$) (Figure 1E). In other words, as supported by Figure 1E, 75.1 ± 3.6% ($n = 3$) of the $A_{2A}$ receptor-positive terminals were also positive for $CB_1$ receptors in vGlut1-positive nerve terminals.

Interestingly, the selectivity analysis of the $CB_1$ receptor (Figure 2A) and the $A_{2A}$ receptor (Figure 2B) antibodies in the $CB_1$ receptor and $A_{2A}$ receptor KO mice and their WT littermates (Supporting Information Figure S1) suggested that $CB_1$ receptors help the localization of $A_{2A}$ receptors to the presynapse. We found that the titration (saturation binding) curve of the anti-$A_{2A}$ receptor antibody showed a decrease of the number of binding sites in vGluT1-positive terminals of $CB_1$ receptor KO mice, compared with WT mice ($n = 5$; $P < 0.05$; Figure 2C), which was not accompanied by any change in antibody affinities to its respective receptor (Figure 2D).

### *$A_{2A}$ receptors co-immunoprecipitate with $CB_1$ receptors in striatal nerve terminals*

The extensive co-localization of $CB_1$ and $A_{2A}$ receptors in nerve terminals hints at a possible physical interaction of $A_{2A}$ and $CB_1$ receptors, as reported in cultured cells and in striatal homogenates (Carriba *et al.*, 2007). To test this hypothesis, we performed immunoprecipitation with an anti-$A_{2A}$ receptor antibody in both crude (the so-called P2 fraction) and in Percoll-purified striatal nerve terminals. Subsequent immunoblot analyses of the composition of the immunoprecipitates demonstrated $CB_1$ receptors at the expected ~51 kDa MW, with particular enrichment in the $A_{2A}$ receptor-immunoprecipitated fractions, compared with the initial (non-immunoprecipitated) homogenate (Figure 3).

The strong co-localization and co-immunoprecipitation data prompted us to test if $A_{2A}$ receptor activation affected $CB_1$ receptor binding. Experiments were carried out under conditions of minimal levels of endogenous adenosine and 2-arachidonoyl-glycerol levels, after treatment with ADA and OMDM188 (see Methods section). A single-point receptor binding assay showed that CGS21680 (30 nM) decreased the binding of the radiolabelled $CB_1$ receptor-selective ligand [$^3$H]SR141716A to synaptosomal membranes from 3.23 ± 0.17 to 2.68 ± 0.18 pmol·mg$^{-1}$ of protein ($n = 7$ in quadruplicates, $P < 0.05$) (figure not shown).

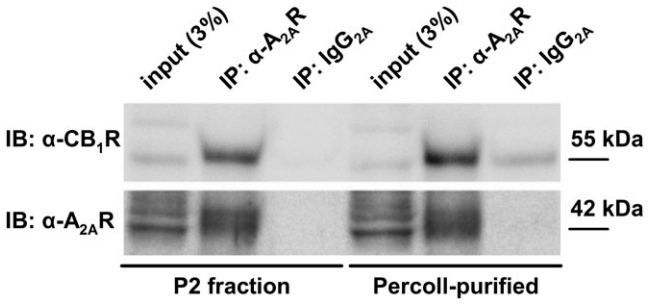



**Figure 3**

Co-immunoprecipitation (IP) of $A_{2A}$ and $CB_1$ receptors in crude (P2 fraction) and Percoll-purified striatal synaptosomes. $CB_1$ receptors were readily detected and enriched in complexes immunoprecipitated with the anti-$A_{2A}$ receptor antibody, but not with mouse $IgG_{2A}$, either in P2 or Percoll-purified fractions of rat striatal synaptosomes. The label 'input (3%)' refers to the protein load corresponding to 3% of the protein quantity used for the IP.

## *$A_{2A}$ receptor activation decreases the potency of $CB_1$ receptor agonists in striatal glutamatergic terminals*

Repetitive ($S_1$ and $S_2$) stimulation with high $K^+$ (30 mM for 1 min) triggered the release of similar amounts of [$^{14}$C]glutamate (Figure 4A) in a $Ca^{2+}$-dependent manner: the first stimulation-evoked release ($S_1$) was 54.1 ± 5.8% smaller in $Ca^{2+}$-free condition (10 mM $MgCl_2$ combined with 100 nM $CaCl_2$; $n$ = 6, $P$ < 0.001) when compared with that under normal condition. The $CB_1$ receptor agonists, WIN55212-2 (0.1–3 μM; $n \geq 6$) or O-2545 (300 nM; $n$ = 5), added 4 min before the second stimulus ($S_2$), decreased the $S_2/S_1$ ratio (i.e. the second stimulus-evoked release) between 10.8 ± 3.8 to 45.4 ± 4.5%, depending on the concentration used ($P$ < 0.05) (Figure 4A and B). The effect of WIN55212-2 was prevented by the selective $CB_1$ receptor antagonist/inverse agonist AM251 (1 μM; $n$ = 6) present during the pre-perfusion period (Figure 4B). In contrast, neither the $A_{2A}$ receptor agonist, CGS21680 (30 nM) (Figure 4B), nor two $A_{2A}$ receptor antagonists had significant effect on the release of [$^{14}$C]glutamate evoked by either 15 or 30 mM $K^+$ either in the rat or in the CD-1 mouse striatal synaptosomes (see Supporting Information Table S2). This indicates that a possible occlusion of the effects of CGS21680, by either an excessive $K^+$ stimulus or by endogenous activation of $A_{2A}$ receptors did not occur, in these conditions.

However, CGS21680, when co-applied with WIN55212-2 ($n \geq 6$) or O-2545 ($n$ = 5), prevented the inhibition of the 30 mM $K^+$-evoked release of [$^{14}$C]glutamate by nanomolar concentrations of these $CB_1$ receptor agonists (Figure 4B) [$P$ > 0.05 for CGS21680+WIN55212-2 (0.1 and 0.3 μM) vs. DMSO control]. CGS21680 also significantly attenuated the inhibition by WIN55212-2 at 3 μM ($n$ = 6) (Figure 4B) [$P$ < 0.05 for CGS21680+WIN55212-2 (3 μM) vs. WIN55212-2 (3 μM) alone].

## *$CB_1$ receptor activation attenuates ATP release in nerve terminals of the rat striatum*

Besides being a source of phasic adenosine levels which activate the $A_{2A}$ receptors (Augusto *et al.* 2013), ATP is also a co-transmitter to glutamate (Burnstock, 2013). Based on the earlier data on [$^{14}$C]glutamate release, we expected $CB_1$ receptor activation to also suppress the release of ATP from striatal nerve terminals. Indeed, we observed that WIN55212-2 (1 μM) inhibited the high-$K^+$-evoked release of ATP by 24.4 ± 6.7% ($n$ = 6, $P$ < 0.05) (Figure 4C and D), which was again prevented by the $CB_1$ receptor antagonist, AM251 (1 μM), which *per se* did not alter the evoked release of ATP ($P$ > 0.05) (Figure 4D).

## *$A_{2A}$ receptor activation inhibits $CB_1$ receptor-mediated depression of glutamatergic transmission in the dorsolateral striatum*

It is well known that $CB_1$ receptors depress corticostriatal glutamatergic transmission (Gerdeman and Lovinger, 2001). Accordingly, the $CB_1$ receptor agonist WIN55212-2 (500 nM) reduced the amplitude of evoked monosynaptic EPSCs by 17.5 ± 5.0% after 5 min and by 37.9 ± 12.5% after 16 min ($n$ = 8, $P$ < 0.05) (Figure 5B-D). The $CB_1$ receptor antagonist AM251 (500 nM) prevented this WIN55212-2-induced depression of synaptic transmission while having no effect alone ($n$ = 5) (Figure 5D).

$A_{2A}$ receptor activation by CGS21680 (30 nM) also prevented the WIN55212-2-induced synaptic depression in the first 5 min ($n$ = 6, $P$ > 0.05) but not at the later time point, although the $CB_1$ receptor-mediated inhibition was smaller in the absence (37.9 ± 12.5%) than in the presence of CGS21680 (29.7 ± 6.1%, $P$ < 0.05) (Figure 5D). Of note, CGS21680 *per se* did not alter basal synaptic transmission ($P$ > 0.05) (Figure 5D).

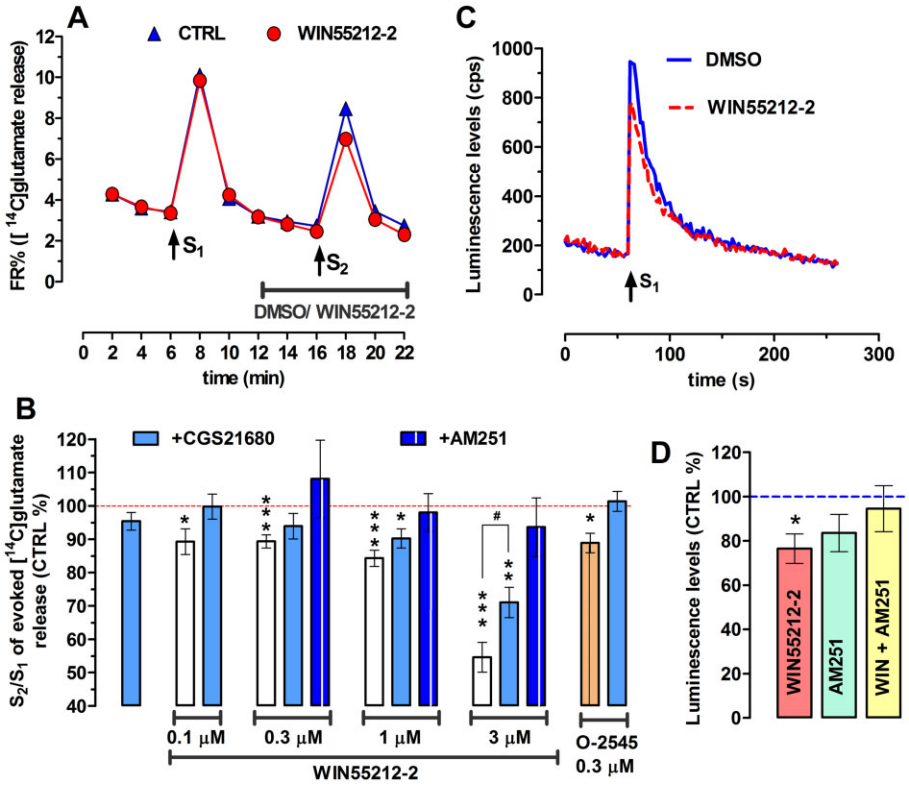
## *$A_{2A}$ receptor activation inhibits $CB_1$ receptor-mediated increase in paired-pulse ratio (PPR)*

While changes in synaptic transmission can be a result of both presynaptic and postsynaptic events, an increase in the PPR reflects presynaptic mechanisms (Schulz *et al.*, 1994; Gerdeman and Lovinger, 2001). Thus, we analysed the monosynaptic EPSC ratios of paired stimuli, delivered with a 25 ms interval (Figure 5A-C,E). The drug-naïve PPR mean value was 0.76 ± 0.03 ($n$ = 22 cells from six rats) (Figure 5B and E). As shown in Figure 5C and E, application of WIN55212-2 (500 nM) for 16 min significantly increased the PPR (by 34.6 ± 17.9%, $n$ = 9, $P$ < 0.05), while a 5 min superfusion period with WIN55212-2 was not enough to cause a significant PPR increase (by 6.4 ± 4.7%, $n$ = 9, $P$ > 0.05). The $CB_1$ receptor antagonist AM251 (500 nM) prevented the WIN55212-2-induced increase in PPR while having no effect alone ($n$ = 5) (Figure 5E).

In accordance with the previous findings, CGS21680 (30 nM, added 10 min before WIN55212-2) prevented WIN55212-2 from increasing the PPR ($n \geq 6$), while *per se* did not alter basal synaptic transmission ($P$ > 0.05) (Figure 5E).

# Discussion

The present study provides direct evidence for the physical and functional interaction of $A_{2A}$ and $CB_1$ receptors in corti-



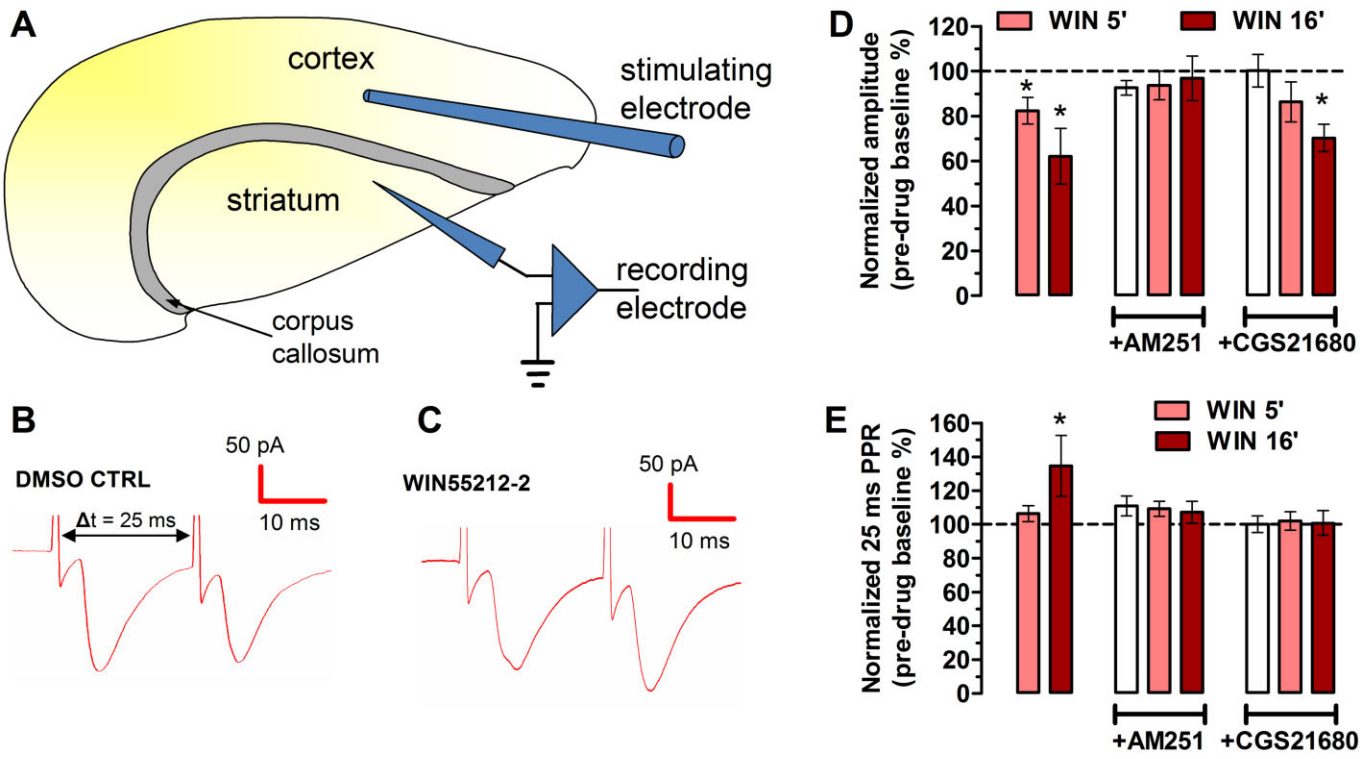

## Figure 4

(A) Time course of [$^{14}$C]glutamate release (as fractional release; FR%) from rat striatal synaptosomes following treatment with WIN55212-2 (1 μM) and the respective control (CTRL). Release was stimulated by high-$K^+$ (30 mM; 2 × 1 min), marked as $S_1$ and $S_2$. WIN55212-2 was added as indicated by the horizontal line. Data are mean ± SEM of $n = 21$ independent observations in duplicate. Missing error bars are inside the symbols. (B) Bar graph representing the effect of $A_{2A}$ receptor activation on the $CB_1$ receptor-induced inhibition of high-$K^+$-evoked release of [$^{14}$C]glutamate. The Y axis represents the effect of the treatment on the $S_2/S_1$ ratio, normalized to the vehicle control. WIN55212-2 *per se* significantly inhibited the release of [$^{14}$C]glutamate in all concentrations (0.1, 0.3, 1 and 3 μM). The inhibitory effect of WIN55212-2 on the $S_2/S_1$ ratio was prevented by the $CB_1$ receptor antagonist AM251 (1 μM, applied during the preperfusion period, i.e. before $S_1$). The selective $A_{2A}$ receptor agonist CGS216880 (30 nM, co-applied with WIN55212-2), which *per se* had no effect on the high-$K^+$-evoked release of [$^{14}$C]glutamate, prevented the action of WIN55212-2 at 0.1 and 0.3 μM and significantly attenuated the action of WIN55212-2 at 3 μM. Similarly, O-2545 (0.3 μM), another $CB_1$ receptor agonist, inhibited the release of [$^{14}$C]glutamate and CGS21680 (30 nM) prevented that action. All bars are mean ± SEM derived from $n \geq 6$ animals. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus DMSO control (red dashed line) and $^{\#}P < 0.05$ versus without CGS21680. (C) Representative time course of high-$K^+$-evoked (32 mM) release of ATP from striatal nerve terminals. Stimulus with KCl is marked as $S_1$. Consistent with ATP being co-released with glutamate, activation of the $CB_1$ receptors by WIN55212-2 (1 μM) also inhibits the KCl-evoked release of ATP. (D) As represented in the bar graphs, the $CB_1$ receptor-mediated inhibition of ATP release ($n = 6$; $^*P < 0.05$) was prevented by the $CB_1$ receptor antagonist, AM251 (1 μM), which *per se* had no effect.

costriatal terminals. Indeed, we now provide for the first time direct evidence for the co-localization of both $A_{2A}$ and $CB_1$ receptors in the same, individually identified corticostriatal glutamatergic nerve terminal. It is important to note that the selectivity of antibodies raises increasing concern in the scientific community (see Grimsey *et al.*, 2008). Here, we carefully titrated our primary antibodies and validated them in the KO mice and their WT littermates whenever possible. This allowed us to avoid common mistakes, such as false co-localizations or mis-estimation of the frequency of labelling.

Furthermore, we showed that these $A_{2A}$ and $CB_1$ receptors form presynaptic heteromers in purified striatal nerve terminals, which is novel information, as this heterodimer was first identified in heterologous expression systems and was reported to be also present in total striatal extracts (Carriba *et al.*, 2007). Heterodimers can interact either at the level of intracellular signalling, or by modulating G protein availability, or simply by physically altering the conformation of the partner receptor (Franco *et al.*, 2006; 2008). Our findings do not directly indicate the molecular nature of interaction but, from the binding data and the fact that the two receptors on their own utilize different pools of G proteins, it is possible that the interaction involves physical modulation of conformation, rather that occurring at the level of G proteins.

This physical association of $A_{2A}$ and $CB_1$ receptors suggests a tight functional interplay in the control of glutamatergic nerve terminals in the striatum. The functional consequences of this finding were demonstrated here with a combination of direct presynaptic tools of increasing complexity (radioligand




## Figure 5

$CB_1$ receptor activation decreases basal synaptic transmission and increases PPR in rat corticostriatal afferents, which is attenuated or prevented by the $A_{2A}$ receptor agonist CGS21680 (30 nM). (A) The whole-cell patch-clamp configuration in horizontal corticostriatal slices from 15–22-day-old rats, with stimulation in the layer V of the adjacent neocortex and recording in the dorsolateral striatum. (B, C) Representative paired-pulse traces (25 ms interpulse interval) in the presence of WIN55212-2 (500 nM) and its vehicle, DMSO. (D) Bar graphs representing the EPSC amplitude values normalized to the pretreatment period after 5 and 16 min of WIN55212-2 perfusion. WIN55212-2 decreased EPSC amplitude at both time points, which was sensitive to AM251, which *per se* had no effect. Ten minutes of pretreatment with CGS21680 attenuated the $CB_1$ receptor-mediated inhibition of EPSC amplitudes. CGS21680 did not produce effects on its own. All bars are mean ± SEM derived from $n \geq 6$ animals. *$P < 0.05$ versus 100% (pretreatment CTRL). (E) Bar graphs representing the normalized PPR (the second response in relation to the first response) at 5 and 16 min after the beginning of WIN55212-2 perfusion. The WIN55212-2-induced increase (*$P < 0.05$) in the PPR was prevented both by AM251 and CGS21680, which had no effects *per se*.

binding in nerve terminal membranes, glutamate release assay in acutely isolated nerve terminals and PPR measurements in isolated monosynaptic contacts in corticostriatal slices), all of which showed that $A_{2A}$ receptor activation significantly attenuated $CB_1$ receptor function. In particular, we showed that activation of $A_{2A}$ receptors decreased the robust presynaptic $CB_1$ receptor-mediated inhibition of corticostriatal glutamate release (Gerdeman and Lovinger, 2001; Köfalvi *et al.*, 2005). This observation *per se* does not directly argue for a presynaptic location of the $A_{2A}$ receptors involved. However, as the $CB_1$ receptors mediating the increase in the PPR are presynaptic, we can indirectly infer that those $A_{2A}$ receptors co-localize presynaptically with these $CB_1$ receptors, as also strongly suggested by the neurochemical data.

The physiological role of this presynaptic $A_{2A}$–$CB_1$ receptor complex is likely to be associated with the well-known high-pass filter phenomenon for corticostriatal activity (Bamford *et al.*, 2004). In fact, it is well established that increased synaptic activity is directly coupled to an increased release of two of the most potent substances acting as presynaptic inhibitory feedback signals, the release of adenosine acting through inhibitory $A_1$ receptors (Fredholm *et al.*, 2005) and endocannabinoids acting through presynaptic $CB_1$ receptors (Lovinger, 2010). The efficiency of these two presynaptic inhibitory systems is best illustrated by the observations that $A_1$ and $CB_1$ receptors are highly abundant GPCRs in the brain. During high-frequency discharge, it is necessary to overcome these efficient presynaptic inhibitory systems to allow the passage of salient information. Therefore, high-pass filters become essential to implement long-term increases of corticostriatal activity with relevant stimuli. The present results add a critical piece of evidence to suggest that $A_{2A}$ receptors participate in this high-pass filtering in response to phasic changes in synaptic adenosine levels (Cunha, 2008). Indeed, it has been previously shown that ATP is co-released with glutamate (Pankratov *et al.*, 2006) in a frequency-dependent manner (Wieraszko *et al.*, 1989; Cunha *et al.*, 1996). Furthermore, Augusto *et al.* (2013) have reported that adenosine, generated from ATP by ecto-5′-nucleotidase, constitutes the particular source that activates striatal $A_{2A}$ receptor. In the case of a low-frequency discharge, the corticostriatal terminals will not produce enough ATP-derived adenosine to activate presynaptic $A_{2A}$ receptor and the extracellular adenosine levels will be enough only to activate the inhibitory $A_1$

receptor, as previously shown (Ciruela *et al.*, 2006). Moreover, the glutamatergic activity will also produce retrograde inhibitory endocannabinoid signalling (Castillo *et al.*, 2012; Katona and Freund, 2012). By contrast, salient and relevant information that should be encoded as increases of synaptic plasticity are associated with a higher frequency of discharge of corticostriatal afferents. Under such conditions, ATP-derived adenosine would now be enough to activate $A_{2A}$ receptors, which will play a double role of attenuating both presynaptic $CB_1$ receptor inhibition, as shown here, as well as presynaptic $A_1$ receptor inhibition (Ciruela *et al.*, 2006). The engagement of $A_{2A}$ receptors has the additional potential of bolstering the function of different neurotrophins, such as brain-derived neurotrophic factor (Sebastião and Ribeiro, 2009), glial cell-derived neurotrophic factor (Gomes *et al.*, 2009) as well as fibroblast growth factor (Flajolet *et al.*, 2008), which further assist the implementation of long-term plastic changes in corticostriatal synapses. Notably, $A_{2A}$ receptors are selectively engaged to control synaptic plasticity rather than basal synaptic transmission in different synapses (Rebola *et al.*, 2008; Costenla *et al.*, 2011), particularly in corticostriatal synapses (D'Alcantara *et al.*, 2001; Flajolet *et al.*, 2008). This is in agreement with the observed failure of CGS21680 to alter basal synaptic transmission under whole-cell patch-clamp configuration in the dorsolateral (somatosensory) striatum (present study) as well as in extracellular recording in the dorsomedial (associative) striatum (Martíre *et al.*, 2011). Notably, Ciruela *et al.* (2006) reported a significant increase following CGS21680 administration in the high-$K^+$-induced release of glutamate in striatal synaptosomes, which was not observed in the present study, in agreement with a previous study where 4-aminopyridine stimulation was used to provoke [$^{14}$C]glutamate release in striatal synaptosomes (Martíre *et al.*, 2011) and with another report showing the lack of CGS21680 modulation of the high-$K^+$-induced release of [$^3$H]glutamate in hippocampal synaptosomes (Lopes *et al.*, 2002). There are differences in the composition of the assay medium and in the execution of the experiments that may account for the differences in the effects of CGS21680 in these assays.

This central role of $A_{2A}$ receptors as a high-pass filter is likely to be further assisted by postsynaptic $A_{2A}$ receptors, which inhibit dopamine $D_2$ receptor-mediated endocannabinoid production in the MSN dendrites (Lerner *et al.*, 2010; Tozzi *et al.*, 2012). Hence, adenosine will exert a double inhibition of endocannabinoid signalling to ensure the rescue of salient corticostriatal activity. Indeed, Bamford *et al.* (2004) noted that postsynaptic $D_2$ receptor-mediated presynaptic inhibition of glutamate release is frequency-dependent, sparing only the most active corticostriatal terminals. This strongly corroborates our hypothesis.

As the principal input, the 'driver', to the whole basal ganglia is the corticostriatal pathway, the modulation of these afferents by presynaptic receptors will have profound effect on all basal ganglia-related lower- and higher-order brain functions including motor coordination, psychomotor drive, emotions, memory or decision making (Nakano *et al.*, 2000). For instance, it is believed that the lack of $D_2$ receptor-stimulated endocannabinoid synthesis in Parkinson's disease hampers presynaptic $CB_1$ receptor-mediated control of corticostriatal afferents, leading to dyskinesias (Brotchie, 2003; Kreitzer and Malenka, 2007; Shen *et al.*, 2008a). Additionally, we envisage an increased synaptic adenosine production and a further impairment of presynaptic $CB_1$ receptor activity, even after L-DOPA administration. This could be one reason why $A_{2A}$ receptor-blocking strategies are interesting as a palliative strategy in addition to L-DOPA (Gomes *et al.*, 2011). Another involvement of this newly described presynaptic receptor heterodimer may be in drug addiction. It was recently found by some of these authors that the presynaptic $A_{2A}$ receptors facilitate the cocaine-induced psychomotor drive in corticostriatal terminals (Shen *et al.*, 2008b; 2013), which once again can be explained by the negative control by $A_{2A}$ receptors on the $CB_1$ receptor-mediated inhibition of these afferents.

Another example of the importance of this interaction derives from the finding that $CB_1$ receptors exert neuroprotection both *in vitro* and *in vivo* in focal and global ischaemia models (Nagayama *et al.*, 1999; Melis *et al.*, 2006). Hence, one can further speculate that ischaemic activation of $A_{2A}$ receptors would hamper $CB_1$ receptor-mediated neuroprotection. Indeed, $A_{2A}$ receptor blockade has been shown to be neuroprotective in several models (Gomes *et al.*, 2011).

Altogether, the presynaptic $A_{2A}$–$CB_1$ receptor complex in corticostriatal terminals emerges as a novel mode of optimizing corticostriatal information processing. Additionally, the identification of this functional heteromer presynaptically, in the corticostriatal terminals, further strengthens the rationale of simultaneously targeting these two receptors rather than each individually, to achieve more efficient palliative therapies to alleviate striatal pathophysiology in motor and addictive diseases.

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## Author contributions

A. K., S. G. F. and R. A. C. were responsible for the study design. S. G. F., I. N-C. and T. H. performed the flow synaptometry experiments. S. G. F., J. M. M. and R. J. R. were responsible for co-immunoprecipitation. S. G. F. and A. K. were responsible for the $CB_1$ receptor binding assay. S. G. F. and A. K. performed the glutamate release experiments. F. Q. G. and Â. R. T. were responsible for ATP measurement. S. G. F. and L. V. were responsible for electrophysiology. C. L. was responsible for sourcing and genotyping of $A_{2A}$ and $CB_1$ receptor knockout mice and their wild-type littermates. A. K., R. A. C., L. V. and T. H. obtained financial support. S. G. F. was responsible for the first draft.

## 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:

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**Figure S1** Data analysis and validation of the $A_{2A}$ and $CB_1$ receptor antibodies/ titrations. (A and B) Examples of fluorescence histograms (for anti-synaptophysin and anti-$CB_1$ receptor antibodies respectively. Specific signal for single-labelled synaptosomes was calculated by subtracting the percentage of labelling (M2 region) by the secondary antibodies alone (histogram filled with violet colour) from the percentage of labelling with the antibody of interest (histogram in red colour). M1 region represents the unlabelled synaptosomes. (C) Dot plot showing double-labelled synaptosomes for synaptophysin and vGlut1. Double-labelled particles appear in the upper right quadrant. Titration curve of the $A_{2A}$ receptor antibody (D) and $CB_1$ receptor antibody (E) in the wild-type (blue curve with filled circles) and knockout mice (red curve with filled squares). Green boxes indicate the working concentration/ dilution of each antibody used in the rat synaptosomes. (F) Bar graph exhibiting the virtually identical labelling by the two different synaptophysin antibodies ($n$ = 3).
**Table S1** Antibodies used (abbreviations: FS, flow synaptometry; IP, immunoprecipitation).
**Table S2** Comparative pharmacological analysis of $A_1$ and $A_{2A}$ adenosine and $CB_1$ cannabinoid receptors on striatal synaptosomal glutamate release.