Title: Presynaptic $A_{2A}$ adenosine receptors dampen $CB_{1}$ cannabinoid receptor-mediated inhibition of corticostriatal glutamatergic transmission

Running Title: $A_{2A}$R-$CB_{1}$R interaction in corticostriatal terminals

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BACKGROUND AND PURPOSE: Both CB₁ cannabinoid and A₂A adenosine receptors (CB₁Rs and A₂ARs) control synaptic transmission at corticostriatal synapses, with great therapeutic importance for neurological and psychiatric disorders. A post-synaptic CB₁R-A₂AR interaction has already been unraveled, but the presynaptic A₂AR-mediated control of presynaptic neuromodulation by CB₁Rs remains to be defined. Since the corticostriatal terminals provide the major input of 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: Here we employ selective presynaptic tools to study the putative presynaptic interaction between the two neuromodulator systems. Pharmacological manipulation of CB₁R and A₂AR was carried out in isolated nerve terminals used for flow synaptometry, immunoprecipitation, radioligand binding, ATP and glutamate release measurement, as well as in whole-cell patch-clamp recordings in horizontal corticostriatal slices.

RESULTS: Flow synaptometry showed that A₂AR are extensively co-localized with CB₁R-immunopositive corticostriatal terminals, and A₂AR co-immunoprecipitated CB₁R in these purified terminals. A₂AR activation decreased CB₁R radioligand binding and decreased the CB₁R-mediated inhibition of high-K⁺-evoked glutamate release in corticostriatal terminals. Accordingly,
A2AR activation prevented CB1R-mediated paired-pulse facilitation and attenuated the CB1R-mediated inhibition of synaptic transmission in glutamatergic synapses of corticostriatal slices.

**CONCLUSIONS AND IMPLICATIONS:** These results show that presynaptic A2AR dampens CB1R-mediated inhibition of corticostriatal terminals. This constitutes a thus far unrecognized mechanism to shut-down the potent CB1R-mediated presynaptic inhibition, enabling a frequency-dependent enhancement of synaptic efficacy at corticostriatal synapses.

**Abbreviations**

3Rs, Replacement, Refinement and Reduction of Animals in Research; A1R(s), A1 adenosine receptors; A2AR(s), A2A adenosine receptor(s); ADA, adenosine deaminase; ARRIVE, Animals in Research: Reporting In Vivo Experiments; ATP, adenosine triphosphate; BDNF, brain-derived neurotrophic factor; CB1R(s), CB1 cannabinoid receptor(s); CCD, charge-coupled device; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetic acid; EPSC(s), excitatory postsynaptic potential(s); FACS, fluorescence-activated cell sorting; FELASA, Federation for Laboratory Animal Science Associations; FGF, fibroblast growth factor; FR%, fractional release percent; [3H]GABA, tritiated γ-aminobutyric acid; GDNF, glial cell-derived neurotrophic factor; GTP, guanosine triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IgG2A, immunoglobulin G2A; L-DOPA, L-3,4-dihydroxyphenylalanine; MSN(s), medium spiny neuron(s); OFA, Oncins France Strain A; PBS, phosphate-buffered saline; PPR, paired-pulse ratio; SDS, sodium dodecyl-sulphate; SDS-PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; Triton-X 100, polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether; vGlut1, vesicular glutamate transporter 1;
INTRODUCTION

The corticostriatal pathway is a massive projection linking virtually the entire neocortex with the striatum – the latter being considered as the major input station 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 behaviors and habits (Graybiel et al., 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 behavioral changes (Di Filippo et al., 2009).

G protein-coupled receptors such as the CB$_1$ cannabinoid receptor (CB$_1$R) are key determinants of synaptic efficacy changes in cortico-striatal synapses (Lovinger, 2010). Accordingly, the manipulation of the endocannabinoid system has a profound impact on striatal-dependent behavioral responses (El Manira and Kyriakatos, 2010; Katona and Freund 2012). Another major controller of striatal function is the A$_{2A}$ adenosine receptor (A$_{2A}$R) (Schiffmann et al., 2007). These A$_{2A}$Rs 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 cortico-striatal plasticity (D’Alcantara et al., 2001; Flajolet et al., 2008). Interestingly, A$_{2A}$Rs 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$_{2A}$Rs 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 A$_1$ adenosine receptors (A$_1$Rs, Dunwiddie and Masino, 2001). In physiological conditions, low-frequency (0.1 < Hz) neuronal activity is accompanied with a modest generation
of adenosine, likely from the metabolism of ATP of astrocytic origin, which exerts tonic inhibition of neighbouring excitatory synapses via \(A_1\)Rs (Cunha, 2008). This dominant form of paracrine adenosinergic neuromodulation probably serves as 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 \(A_{2A}\)R activation (Cunha, 2008; Augusto et al., 2013). Pathological conditions such as ischemia can also increase extracellular adenosine levels via outward transport, which are enough to stimulate both \(A_1\)Rs and \(A_{2A}\)Rs (Gomes et al., 2011).

\(A_{2A}\)R have been reported to tightly regulate the endocannabinoid neuromodulation system in the striatum, as heralded by the documented \(A_{2A}\)R-CB1R 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; Rosi et al., 2010; Justinová et al., 2011). This is re-enforced by the reported heteromerization of \(A_{2A}\)R with CB1R that was demonstrated in heterologous expression systems and in the striatum (Carriba et al., 2007). However, this \(A_{2A}\)R-CB1R 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 CB1R is presynaptic in the striatum (Köfalvi et al., 2005; Uchigashima et al., 2007). A possible presynaptic interaction between \(A_{2A}\)R and CB1R has also been proposed to control the motor-depressant and addictive effects of cannabinoids (Ferré et al., 2010; Martire et al., 2011; Justinová et al., 2014), yet detailed experimental evidence is lacking. Here we set our aims to further expand our previous observations (Matíre et al., 2011) now with selective presynaptic techniques, combining refined immunological, radioligand binding and functional assays to directly investigate \(A_{2A}\)R-CB1R interaction in glutamatergic nerve terminals of cortico-striatal synapses.
METHODS

Subjects

All studies were conducted in accordance with the principles and procedures outlined as "3Rs" in the guidelines of EU (86/609/EEC), FELASA, and the National Centre for the 3Rs (the ARRIVE; 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.

Animals were housed with 12 h light on/off cycles and 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 6 OFA (Oncins France Strain A) rats (16-22 post-natal days) from Charles–River (L’Arbresle, France). Five pairs of A2AR and CB1R null-mutant (knockout) male mice on CD-1 background (Ledent et al., 1997, 1999) and their wild-type 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 (Ferreira et al., 2009). Briefly, the animals were decapitated under halothane anesthesia, and their brains were quickly removed into ice-cold 0.32 M sucrose solution containing 5 mM HEPES, 1 mM EDTA, and 1/500 v/v protease inhibitor cocktail Sigma-Aldrich (Saint Louis, MO, USA), pH 7.4, homogenized instantly and centrifuged at 3,000 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 immunolabeling and flow cytometry analysis, the P2 fraction was further purified in
discontinuous Percoll gradient (3, 10 and 23%), as described in Köfalvi et al. (2005). The purified synaptosomes were kept at -80 ºC until use.

**Immunolabeling and flow cytometric analysis of purified nerve terminals**

Immunohistochemical labeling 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 phosphate-buffered saline (PBS: 135 mM NaCl, 1.3 mM KCl, 3.2 mM NaH$_2$PO$_4$ and 0.5 mM KH$_2$PO$_4$) for 1 hour at 4 ºC and then centrifuged at 3000 g for 3 min at 4 ºC. For permeabilization, the pellets were incubated in PBS with 0.2% Tween-20 for 15 min at 37 ºC and then centrifuged at 3000 g for 3 min. The pellets were then resuspended in PBS for immunolabeling. Primary and secondary antibodies (Supporting Information Table S1) were diluted in PBS containing 2% normal goat serum (Vector Laboratories, CA, USA). For validation/titration of the primary antibodies see Supporting Information Figure S1. Incubation volume was 100 uL and incubation time was 30 min at 4 ºC for both the primary and the secondary antibodies. Each incubation was followed by 3 times washing 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, 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 multicolor flow cytometry experiments, color compensation was performed. Offline data analysis was performed using BD Cell Quest Pro software (Becton, Dickinson and Company, USA). For detailed description see Supporting Information Figure 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 MgCl₂, 1 µM CaCl₂, 2 mM EDTA and protease inhibitor cocktail (Sigma), pH 7.4]. Single point CB₁R binding experiment with 3.82±0.29 nM (n=7) of the CB₁R antagonist/inverse agonist, [³H]SR141716A was carried out as before (Ferreira et al., 2012), with 30 min preincubation in the presence of adenosine deaminase (ADA, 2 U/mL) and of the diacylglycerol lipase inhibitor, OMDM188 (300 nM, a kind gift of Dr. Vincenzo Di Marzo). Non-specific binding was determined by using the CB₁R antagonist/inverse agonist, AM251 (1 µM). Each of the 7 independent assays were carried out on synaptic membranes derived from 2 rats, altogether 14 rats, and assayed in quadruplicate (28 filters/condition). The tritium content of each sample was counted using a Tricarb 2900TR β-counter (PerkinElmer). 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 3 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, UK) for 3 hours at 4 ºC to eliminate non-specific binding. After incubation, the precleared supernatants containing 1 mg protein were incubated overnight with rotation at 4 ºC with a mouse anti-A₂₅R antiserum (Millipore) pre-coupled covalently to protein G-sepharose (GE Healthcare), in the presence of 1% bovine serum albumin (Sigma) and protease inhibitors (Roche Diagnostics). The beads were washed 3 times with isolation buffer containing 150 mM KCl, 20 mM 3-(N-morpholino)propanesulfonic acid and 1% Triton X-100.
(pH 7.4) and resuspended in 6× 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 denaturated 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) and probed with rabbit anti-CB1R (Table S1) and mouse anti-A2AR (Table S1) diluted in Tris-buffered saline supplemented with Tween 20 (0.1% v/v) and bovine serumalbumine (5% m/v). Immunoreactivity was visualized using horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies (Pierce) with a subsequent incubation with SuperSignal West Pico Chemiluminescent Substrate (Pierce), and the images were acquired using Versadoc3000 apparatus and analysed with ImageLab software (BioRad). A negative control containing the same amount of mouse IgG2A instead of the mouse anti-A2AR antibody was run in parallel for each experiment.

[^14C]glutamate release

Experiments were carried out with slight modifications to previous publications (Köfalvi et al., 2005), which are: the synaptosomes were loaded with[^14C]-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 fir 1 min (S1, S2), with a 10-min interval. All Krebs-HEPES solutions used for this assay contained the habitual glutamate decarboxylase inhibitor aminooxy acetic acid (100 µM) to prevent[^14C]glutamate degradation. For detailed discription, see Köfalvi et al. (2005) and Ferreira et al. (2009). The validation of the A1R, A2AR as well as CB1R-mediated neuromodulation in Wistar rat and CD-1 mouse striatal synaptosomes is summarized in Table S2.
**ATP release assay from striatal synaptosomes**

ATP quantification was carried out in 96-well plates, by the help of a Perkin Elmer Victor\textsuperscript{3} multilabel 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: i) Basal saline medium (in mM): 115 NaCl, 3 KCl, 1.2 KH$_2$PO$_4$, 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$_2$PO$_4$, 25 HEPES, 10 glucose, 1.2 MgSO$_4$, 1 CaCl$_2$, pH = 7.4. A 150 µL aliquot of basal saline medium (+treatment/vehicle), 15 µL of ATP assay mix and 35 µL synaptosomal suspension (~1 mg/mL) provided the 200 µL final reaction volume. This mixture was incubated at 25 °C during 3 min in an Eppendorf tube to ensure functional recovery of the sample, then was transferred into a well of the plate at 25 °C, inside the reader. Afterwards, a kinetic protocol was initiated with the duration 140 sec. During first 60 sec, 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 medium spiny neurons were performed in horizontal brain slices from OFA rats (Figure 5A) (postnatal days 15–22), as previously described (Fino et al. 2005). Briefly, the artificial cerebrospinal fluid (containing in (mM)): 125 NaCl, 2.5 KCl, 25 glucose, 25 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 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 ºC 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 he 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. Off-line analysis was performed using Igor-Pro 6.0.3 (Wavemetrics, Lake Oswego, OR, USA).

**Data treatment**

Raw effect data from [14C]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 antibody titration curves were compared with Two-way ANOVA between the WT and the KO mice. A $P < 0.05$ was accepted as significant difference.
**Chemicals**

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-benzoazin-6-yl]-1-naphthenylmethanone (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, UK; 3-[4-[2-[[6-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-y1]purin-2-yl]amino]ethyl]phenyl]-propanoic acid (CGS21680) was purchased from Tocris Bioscience, UK; dimethylsulfoxide (DMSO), 3-(N-morpholino)propanesulfonic acid (MOPS), aminooxyacetic acid, halothane, HEPES, Percoll, adenosine deaminase (ADA), bovine serumalbumine, and sucrose were purchased from Sigma (Saint Louis, MO, USA). \[^3\text{H}]\text{SR141716A}\) and \[^{14}\text{C}]\text{-U-glutamate}\) were purchased from American Radiolabeled Chemicals (Saint Louis, MO, USA). All other reagents were purchased from MerckBiosciences (Darmstadt, Germany).

**RESULTS**

**A\textsubscript{2A}R and CB\textsubscript{1}R colocalize in corticostriatal glutamatergic nerve terminals**

A basic first evidence to probe the functional cross-talk between CB\textsubscript{1}R and A\textsubscript{2A}R in corticostriatal glutamatergic terminals is to demonstrate their simultaneous co-localization at these sites. Thus, we analyzed striatal synaptosomes via flow synaptometry (Figure 1A): we double-labeled them for synaptophysin and vesicular glutamate transporter type 1 (vGluT1) (Figure 1B), or triple-labeled for vGluT1, CB\textsubscript{1}R and A\textsubscript{2A}R (Figure 1C-E). Concerning the double labeling, 85.2±2.5% (n=3) of the analyzed particles were positive for synaptophysin. Of these particles, 58.5±3.5% were positive for vGluT1 (Figure 1B). Triple labeling allowed to precisely
quantify that 49.4±3.3% of the vGluT1-positive terminals were endowed with CB₁R (n=3) (Figure 1C) and 30.9±1.7% beared A₂A_R (n=3) (Figure 1D). Additionally, 47.1±2.7% of the CB₁R-positive terminals were also positive for A₂A_R (n=3) (Figure 1E). In other words, as supported by Figure 1E, 75.1±3.6% (n=3) of A₂A_R+ terminals are also positive for CB₁R in vGluT1+ nerve terminals.

Interestingly, the selectivity analysis of the CB₁R (Figure 2A) and the A₂A_R (Figure 2B) antibodies in the CB₁R and A₂A_R KO mice and their wild-type littermates (Supporting Information Figure S1) suggested that CB₁Rs help the targeting of A₂A_R to the presynapse: in fact, the titration (saturation binding) curve of the anti-A₂A_R antibody revealed a decrease of the number of binding sites in vGluT1-positive terminals of CB₁R KO compared to 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).

**A₂A_R co-immunoprecipitates the CB₁R in striatal nerve terminals**

The extensive co-localization of CB₁R-A₂A_R in nerve terminals hints at a possible physical interaction of A₂A_R and CB₁R, as reported in culture cells and striatal homogenates (Carriba et al., 2007). To test this hypothesis, we performed immunoprecipitation with an anti-A₂A_R 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 allowed detecting CB₁R at the expected ~51 kDa molecular weight, with particular enrichment in the A₂A_R-immunoprecipitated fractions when compared to the initial (non-immunoprecipitated) homogenate (Figure 3).

The strong co-localization and co-immunoprecipitation data prompted us to test if A₂A_R activation affected CB₁R binding. Experiments were carried out under the minimization of
endogenous adenosine and 2-arachidonoyl-glycerol levels with ADA and OMDM188 (see Materials and Methods). A single point receptor binding assay showed that CGS21680 (30 nM) decreased the binding of the radiolabeled CB₁R-selective ligand, [³H]SR141716A to synaptosomal membranes from 3.23±0.17 to 2.68±0.18 pmol/mg of protein (n=7 in quadruplicates, P<0.05) (figure not shown).

**A₂A R activation decreases the potency of CB₁ R agonists in striatal glutamatergic terminals**

Repetitive (S₁ and S₂) stimulation with high K⁺ (30 mM for 1 min) triggered the release of similar amounts of [¹⁴C]glutamate (Figure 4A) in a Ca²⁺-dependent manner: the first stimulation-evoked release (S₁) was 54.1±5.8% smaller in Ca²⁺-free condition (10 mM MgCl₂ combined with 100 nM CaCl₂; n=6, P<0.001) when compared to that under normal condition. The CB₁ R agonists, WIN55212-2 (0.1 - 3 µM; n≥6) or O-2545 (300 nM; n=5), added 4 min before the second stimulus (S₂), decreased the S₂/S₁ 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 4B). The effect of WIN55212-2 was prevented by the selective CB₁ R antagonist/inverse agonist AM251 (1 µM; n=6) present since the preperfusion period (Figure 4B). In contrast, neither the A₂A R agonist, CGS21680 (30 nM) (Figure 4B) nor two A₂A R antagonists had significant effect on the release of [¹⁴C]glutamate evoked by either 15 or 30 mM K⁺, and either in the rat or the CD-1 mouse striatal synaptosomes (see Table S2). This indicates that a possible occlusion of CGS21680 effect by either a too strong K⁺ stimulus or by endogenous activation of A₂A Rs was not the case.

However, CGS21680 when co-applied with WIN55212-2 (n≥6) or O-2545 (n=5), prevented the inhibition of the 30 mM K⁺-evoked release of [¹⁴C]glutamate by nanomolar concentrations of these CB₁ R 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).

**CB₁R activation attenuates ATP release in nerve terminals of the rat striatum**

Although ATP is not only the source of adenosine, the endogenous agonist of the A₂A Rs, it is a co-transmitter to glutamate (Burnstock, 2013). In line with the above data on [¹⁴C]glutamate release, we expected CB₁R 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 4D), which was again prevented by the CB₁R antagonist, AM251 (1 µM), which *per se* did not alter the evoked release of ATP (P > 0.05) (Figure 4D).

**A₂A R activation inhibits CB₁R-mediated depression of glutamatergic transmission in the dorsolateral striatum**

It is well known that CB₁R depress corticostriatal glutamatergic transmission (Gerdeman and Lovinger, 2001). Accordingly, the CB₁R 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 CB₁R antagonist AM251 (500 nM) prevented this WIN55212-2-induced depression of synaptic transmission while having no effect alone (n=5) (Figure 5D).

A₂A R 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 CB₁R-
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).

**A2AR activation inhibits CB1R-mediated increase in paired-pulse ratio**

While changes in synaptic transmission can be a result of both pre- and post-synaptic events, an increase in the paired-pulse ratio (PPR) reflects presynaptic mechanisms (Schulz et al. 1994; Gerdeman and Lovinger, 2001). Thus, we analyzed 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 6 rats) (Figure 5B and 5E). As shown in Figure 5C and E, 16 min application of WIN55212-2 (500 nM) led to a significant increase in PPR (by 34.6±17.9%, n=9, P<0.05), while a 5 min superfusion period with WIN55212-2 was still not enough to cause a significant PPR increase (by 6.4±4.7%, n=9, P>0.05). The CB1R 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≥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 A2AR and CB1R in corticostriatal terminals. Indeed, we now provide for the first time direct evidence for the co-localization of both A2AR and CB1R 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 (e.g. Grimsey et al., 2008). Here we carefully titrated our primary antibodies, and validated them in the knockout mice and their wild-type littermates whenever possible. This allowed us to avoid common mistakes, such as false co-localizations or misestimation of the frequency of labeling.

Furthermore, we showed that these A2A and CB1 receptors form presynaptic heteromers in purified striatal nerve terminals, which is a novel information, since 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 signaling, 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, yet, we speculate from the binding data and the fact that the two receptors on their own utilize different pools of G proteins that the interaction involves physical modulation of conformation, rather that occurring at the level of G proteins.

This physical association of A2A and CB1 receptors is suggestive of a tight functional interplay in the control of glutamatergic nerve terminals in the striatum. The functional consequences of this finding were revealed now with a combination of direct presynaptic tools of increasing complexity (radioligand binding in nerve terminal membranes, glutamate release assay in acutely isolated nerve terminals and paired-pulse ratio measurements in isolated monosynaptic contacts in corticostriatal slices), all of which showed that A2A activation significantly attenuates CB1 function. In particular, we documented the ability of A2A to dampen the robust presynaptic CB1-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 underlying A2ARs. However, as the CB1Rs mediating the increase in the paired-pulse ratio are
presynaptic, we can indirectly infer that those A$_2$ARs co-localize presynaptically with these CB$_1$Rs, as also strongly suggested by the neurochemical data.

The physiological role of this presynaptic A$_2$AR-CB$_1$R complex is likely 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, namely the release of adenosine acting through inhibitory A$_1$Rs (Fredholm et al., 2005) and endocannabinoids acting through presynaptic CB$_1$Rs (Lovinger, 2010). The efficiency of these two presynaptic inhibitory system is best heralded by the observations that A$_1$Rs and CB$_1$Rs are highly abundant G protein-coupled receptors 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$_2$AR participate in this high-pass filtering in response to phasic changes in synaptic adenosine levels (Cunha, 2008). Indeed, we and others have 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 shown that adenosine, generated from ATP by ecto-5’-nucleotidase, constitutes the particular source that activates striatal A$_2$AR. In the case of a low frequency discharge, the corticostriatal terminals will not produce enough ATP-derived adenosine to activate presynaptic A$_2$AR and the extracellular adenosine levels will be enough only to activate the inhibitory A$_1$R, as previously shown (Ciruela et al., 2006). Moreover, the glutamatergic activity will also produce retrograde inhibitory endocannabinoid signaling (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 afferent; under such conditions, ATP-derived adenosine is now sufficient to activate A$_{2A}$R, which will play a double role of shunting down both presynaptic CB$_1$R inhibition, as now documented, as well as presynaptic A$_1$R inhibition (Ciruela et al., 2006). The engagement of A$_{2A}$R has the additional potential of bolstering the function of different neurotrophins, such as BDNF (Sebastião and Ribeiro, 2009), GDNF (Gomes et al., 2009) as well as FGF (Flajolet et al., 2008), which further assist the implementation of long-term plastic changes in cortico-striatal synapses. Notably, A$_{2A}$R are selectively engaged to control synaptic plasticity rather than basal synaptic transmission in different synapses (Rebola et al., 2008; Costenla et al., 2011), namely in corticostratial synapses (D’Alcantara et al., 2001; Flajolet et al., 2008). This is in agreement with that CGS21680 failed 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 and colleagues (2006) reported a significant increase upon 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 on the high-K$^+$-induced release of [${}^3$H]glutamate in hippocampal synaptosomes (Lopes et al., 2002). We can point out differences in the composition of the assay medium and in the execution of the experiments that may lead to the modification of CGS21680 effect in these assays.

This central hubbing role of A$_{2A}$R as a high pass filter is likely to be further assisted by post-synaptic A$_{2A}$R, which inhibit D$_2$ dopamine receptor-mediated endocannabinoid production in the MSN dendrites (Lerner et al., 2010; Tozzi et al., 2012). Hence, adenosine will exert a
double lock onto endocannabinoid signaling to ensure the rescue of salient corticostriatal activity. Indeed, Bamford and colleagues (2004) noted that post-synaptic D$_2$R-mediated presynaptic inhibition of glutamate release is frequency-dependent, sparing only the most active corticostriatal terminals. This strongly corroborates our hypothesis. Since the principal input, the “drive” of 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$_2$R-stimulated endocannabinoid synthesis in Parkinson’s disease hampers presynaptic CB$_1$R-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$_1$R activity, even after L-DOPA administration. This could be one reason why A$_2$AR blocking strategies are interesting as a palliative strategy adjunct to L-DOPA (Gomes et al., 2011). Another involvement of this newly described presynaptic heterodimer may be in drug addiction. It was recently found by some of these authors that the presynaptic A$_2$AR facilitate the cocaine-induced psychomotor drive in corticostriatal terminals (Shen et al., 2008b, 2013), which once again can be explained by the negative control of A$_2$AR on the CB$_1$R-mediated inhibition of these afferents.

Another example for the importance of this interaction implies that CB$_1$Rs exert neuroprotection both in vitro and in vivo in focal and global ischemia models (Nagayama et al., 1999; Melis et al., 2006). Hence, one can further speculate that ischemic activation of A$_2$ARs would hamper CB$_1$R-mediated neuroprotection. Indeed, A$_2$AR blockade has been shown to be neuroprotective in several models (Gomes et al., 2011).
Altogether, the presynaptic A$_{2A}$R-CB$_1$R complex in corticostriatal terminals emerges as a novel module to optimize 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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**Conflicts of interest**

None.

**List of Author Contribution**

REFERENCES


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Figure 1 Flow-synaptometric analysis of immunolabeled 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-labeled for synaptophysin (a marker of synaptosomes) and vesicular glutamate transporter 1 (vGluT1; a marker of corticostriatal terminals). (C and D) Representative plots and statistics of synaptosomes labeled for vGluT1/CB1R (C) and vGluT1/A2AR (D), respectively (the two graphs are derived from the same triple-labelled sample). (E) Representative plot and statistics of vGluT1-positive synaptosomes expressing CB1R and A2AR. Note that most of the nerve terminals endowed with A2ARs were also endowed with CB1Rs.

Figure 2 (A) Total binding isotherms of the anti-CB1R antibody in the corticostriatal terminals of the WT (squares) versus the A2AR KO mice (circles), while the CB1R KO mice display antibody binding of non-specific nature only (triangles). (B) Total binding isotherms of the anti-A2AR antibody in the corticostriatal terminals of the WT (squares) versus the CB1R KO mice (circles), while the A2AR KO mice display antibody binding of non-specific nature only (triangles). (C) Bar graphs representing the mean values of the maximum binding sites ($B_{max}$) of the anti-CB1R and anti-A2AR antibodies (*$P<0.05$). (D) Bar graphs representing the mean changes of the dissociation constant ($K_d$) of the anti-CB1R and anti-A2AR antibodies. Bars represent the mean ± SEM of 5 individual experiments.
Figure 3 Co-immunoprecipitation of A$_{2A}$Rs and CB$_1$Rs in crude (P2 fraction) and Percoll-purified striatal synaptosomes. CB$_1$R was readily detected and enriched in complexes immunoprecipitated with the anti-A$_{2A}$R, but not with mouse IgG$_{2A}$, either in P2 or or Percoll-purified fractions of rat striatal synaptosomes.

Figure 4 (A) Fractional release percent (FR%; see Methods) diagram representing the averaged $[^{14}C]$glutamate release curves in rat striatal synaptosomes under treatment with WIN55212-2 (1 µM; circles) and the respective control (triangles). Stimuli with high-K$^+$ (30 mM; 2 × 1 min) are 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. (B) Bar graph representing the effect of the A$_{2A}$R activation on the CB$_1$R-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 (open bars) 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$_1$R antagonist AM251 (1 µM, applied since the preperfusion period, i.e. before S$_1$). The selective A$_{2A}$R 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$_1$R agonist, inhibited the release of $[^{14}C]$glutamate (brown bar), and CGS21680 (30 nM) prevented that action. All bars are mean ± SEM derived from n ≥ 6 animals. *P < 0.05; **P < 0.01; ***P < 0.001 vs. DMSO control (red dashed line), and *P < 0.05 vs without CGS21680. (C) Representative diagram of high-K$^+$ (32 mM) -evoked 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$_1$Rs by WIN55212-2 (1 µM) also inhibits the KCl-evoked release of ATP. (D) As represented in the bar graphs, the CB$_1$R-mediated inhibition of ATP release (n = 6; *$P<0.05$) was prevented by the CB$_1$R antagonist, AM251 (1 µM), which per se had no effect.

**Figure 5** CB$_1$R activation decreases basal synaptic transmission and increases paired-pulse ratio in rat corticostriatal afferents, which is attenuated / prevented by the A$_{2A}$R agonist CGS21680 (30 nM). (A) The whole-cell patch clamp configuration in 15-22 day-old rats' horizontal corticostriatal slices, 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, in a fashion sensitive to AM251, which per se (open bar) had no effect. Ten min pretreatment with CGS21680 attenuated the CB$_1$R-mediated inhibition of EPSC amplitudes. CGS21680 did not produce effect on its own (open bars). All bars are mean ± SEM derived from n ≥ 6 animals. *$P<0.05$ vs. 100% (pretreatment CTRL). (E) Bar graphs representing the normalized paired-pulse ratio (PPR; the second response in relation to the first response) at 5 and 16 minutes after the beginning of WIN55212-2 perfusion. The WIN55212-2-induced increase (*$P<0.05$) in the paired-pulse ratio was prevented both by AM251 and CGS21680, which had no effect per se (open bars).
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