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ORIGINAL ARTICLE

# Striatal adenosine–cannabinoid receptor interactions in rats over-expressing adenosine $A_{2A}$ receptors

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**Abstract**

Adenosine $A_{2A}$ receptors ($A_{2A}$Rs) and cannabinoid $CB_1$ receptors ($CB_1$Rs) are highly expressed in the striatum, where they functionally interact and form $A_{2A}/CB_1$ heteroreceptor complexes. We investigated the effects of $CB_1$R stimulation in a transgenic rat strain over-expressing $A_{2A}$Rs under the control of the neural-specific enolase promoter ($NSEA_{2A}$ rats) and in age-matched wild-type (WT) animals. The effects of the $CB_1$R agonist WIN 55,212-2 (WIN) were significantly lower in $NSEA_{2A}$ rats than in WT animals, as demonstrated by i) electrophysiological recordings of synaptic transmission in corticostriatal slices; ii) the measurement of glutamate outflow from striatal synaptosomes and iii) *in vivo* experiments on locomotor activity. Moreover, while the effects of WIN were modulated by both $A_{2A}$R agonist (CGS 21680) and antagonists (ZM 241385, KW-6002 and SCH-442416) in WT animals, the $A_{2A}$R antagonists failed to influence WIN-mediated effects in $NSEA_{2A}$ rats. The present results demonstrate that in rats with genetic neuronal over-expression of $A_{2A}$Rs, the effects mediated by $CB_1$R activation in the striatum are significantly reduced, suggesting a change in the stoichiometry of $A_{2A}$ and $CB_1$ receptors and providing a strategy to dissect the involvement of $A_{2A}$R forming or not forming heteromers in the modulation of striatal functions. These findings add additional evidence for the existence of an interaction between striatal $A_{2A}$Rs and $CB_1$Rs, playing a fundamental role in the regulation of striatal functions.

**Keywords:** WIN 55212-2, adenosine $A_{2A}$ receptor, cannabinoid $CB_1$ receptor, locomotor activity, striatum, synaptic transmission.

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Adenosine is an endogenous nucleoside ubiquitously present throughout the body where it interacts with different G-protein–coupled receptors (adenosine $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$ receptor subtypes) to exert multiple physiological effects (Stone *et al.* 2009). In the brain, it acts as a neuromodulator and controls synaptic transmission and neuron excitability. Mainly through the activation of $A_1$ and $A_{2A}$ receptor subtypes, adenosine regulates neurotransmitter release by acting at the pre-synaptic level and modulates the action of the neurotransmitters at the post-synaptic site (Fredholm *et al.* 2001). In addition, adenosine receptors, and in particular $A_{2A}$ receptors ($A_{2A}$Rs), can influence the neuronal activity by facilitating or inhibiting receptors of other neurotransmitters or neuromodulators in different brain areas (Sebastião and Ribeiro 2009). A first demonstration that $A_{2A}$Rs could interact with other receptors in the striatum

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*Abbreviations used*: $A_{2A}$R, adenosine $A_{2A}$ receptors; ACSF, artificial cerebrospinal fluid; $CB_1$Rs, cannabinoid $CB_1$ receptors; FP, field potential; $NSEA_{2A}$, $A_{2A}$Rs under the control of neural-specific enolase promoter.

 

came from the study by Ferré *et al.* (1991) showing that $A_{2A}R$ activation decreases the affinity of $D_2$ receptors for dopamine in rat striatal membranes; such an $A_{2A}/D_2$ receptor interaction has been then extensively studied and proved to be the basis of the behavioural effects of $A_{2A}R$ ligands and of the use of $A_{2A}R$ antagonists as anti-Parkinson drugs (Fuxe *et al.* 2007, 2015; Armentero *et al.* 2011). Besides $D_2$ receptors, $A_{2A}Rs$ interact with other receptors such as group I metabotropic glutamate receptors (mGlu5 subtype) and $A_1$ receptors (Nishi *et al.* 2003; Ciruela *et al.* 2006).

In recent years, an increasing interest has emerged on the interaction between $A_{2A}Rs$ and cannabinoid $CB_1$ receptors ($CB_1R$) (for reviews, see Sebastião and Ribeiro 2009; Ferré *et al.*, 2010; Tebano *et al.* 2012).

$CB_1R$ is a G-protein–coupled receptor (GPCR) that is targeted by anandamide and 2-arachidonoylglycerol (2-AG), the two major endocannabinoids in the brain, and by delta-9-tetrahydrocannabinol, the main addictive ingredient of marijuana (Di Marzo *et al.* 2004; Battista *et al.* 2012). One of the best studied functions of $CB_1Rs$ is the control of neurotransmitter release. In the striatum, the activation of $CB_1Rs$ is the basis of depolarization-induced suppression of inhibition or excitation, in which post-synaptic depolarization induced the release of 2-AG that acts as a retrograde signal on $CB_1Rs$ to reduce pre-synaptic GABA or glutamate release (Uchigashima *et al.* 2007). In *in vitro* preparations, exogenous stimulation of $CB_1Rs$ results as well in the reduction of neurotransmitter release (Gerdeman and Lovinger 2001; Chiodi *et al.* 2012) and *in vivo* in the so-called tetrad syndrome, i.e. motor depression, hypothermia, catalepsy and analgesia (Monory *et al.* 2007).

Interactions between $A_{2A}Rs$ and $CB_1Rs$ have been postulated since the study by Andersson *et al.* (2005), demonstrating that genetic inactivation of $A_{2A}Rs$ reduced the phosphorylation of DARPP-32 at Thr34 and the motor depression produced by the $CB_1R$ agonist CP55,940. This result has been corroborated by the study by Carriba *et al.* (2007), which demonstrated that a low dose of an $A_{2A}R$ antagonist, devoid of any motor effect by itself, counteracted the motor depressant effects produced by the intrastriatal administration of a cannabinoid $CB_1R$ agonist. In addition, they showed for the first time that $CB_1Rs$ and $A_{2A}Rs$ physically interact to form heteromeric complexes in co-transfected HEK293T cells and in rat striatum. Other studies made clear that $A_{2A}Rs$ exert a facilitatory role on $CB_1Rs$ and that a basal level of $A_{2A}Rs$ activation is required for CB-mediated effects to appear (Yao *et al.* 2006; Tebano *et al.* 2009 Justinová *et al.* 2011). However, substantial evidence suggests that $A_{2A}Rs$ may also negatively control $CB_1R$-dependent effects: (i) in the striatum, chronic $A_{2A}R$ blockade by caffeine increases the pre-synaptic effects of $CB_1R$ stimulation at the GABAergic synapses (Rossi *et al.* 2009); (ii) the blockade or activation of $A_{2A}Rs$ increases or decreases, respectively, $CB_1R$-dependent long-term depression in the dorsal striatum (Lerner *et al.*, 2010; Lerner and Kreitzer 2012); (iii) $A_{2A}R$ activation inhibits $CB_1R$-mediated depression of synaptic transmission and $CB_1R$-mediated inhibition of 4-aminopyridine-evoked glutamate release (Martire *et al.* 2011); (iv) $A_{2A}R$ activation decreases $CB_1R$ radioligand binding and decreased the $CB_1R$-mediated inhibition of high $K^+$-evoked glutamate release in corticostriatal terminals (Ferreira *et al.* 2015). Overall these findings demonstrate a strong functional interaction between striatal $A_{2A}$ and $CB_1$ receptors, but reveal—at the same time—that the mechanisms of this interaction are still not clear.

The aim of the present study is to further explore the above interaction by investigating $CB_1R$-mediated effects in a transgenic rat strain over-expressing $A_{2A}Rs$ under the control of the neural-specific enolase promoter (Giménez-Llort *et al.* 2007). These rats might represent a valuable model to study the interaction between $A_{2A}$ and $CB_1$ receptors, given the recent study by Ferreira *et al.* (2015) demonstrating the existence of $A_{2A}$-$CB_1$ heteromers in striatal glutamatergic terminals. Interestingly, furthermore, some new advancements in the area of GPCR oligomerization lead to the concept that GPCR heteromers are constituted mainly by heteromers of homodimers (see Ferré *et al.* 2014 for a review). In this view, up-regulation of $A_{2A}Rs$ might results in a change in the stoichiometry of $A_{2A}$ and $CB_1$ receptors in the striatum and in the proportion of $A_{2A}Rs$ forming or not forming heteromers with $CB_1Rs$, thus allowing to dissect out the relative contribution of different $A_{2A}R$ oligomers in the regulation of striatal functions.

With this in mind, we studied the effects of the $CB_1R$ agonist WIN 55,212-2 on synaptic transmission, motor behaviour and glutamate release and found that these effects are blunted in the presence of a constitutive up-regulation of $A_{2A}Rs$.

## Materials and methods

### Animals

A colony of transgenic rats over-expressing $A_{2A}Rs$ in the central nervous system under the control of the neural-specific enolase promoter ($NSEA_{2A}$) was established in the animal facility of the Istituto Superiore di Sanità. Transgenic rats were generated, as previously described (Giménez-Llort *et al.* 2007), by microinjection of a DNA construct into the male pronucleus of Sprague–Dawley rat zygotes with established methods (Popova *et al.* 2002). The construct contained a full-length human $A_{2A}$ cDNA cloned into an expression vector 3′ of the 1.8 kb rat NSE promoter and 5′ of an intron/polyadenylation cassette of SV40 virus. The animals were kept under standardized temperature, humidity and lighting conditions with free access to water and food. All procedures met the European guidelines for the care and use of laboratory animals (2010/63/UE) and those of the Italian Ministry of Health (Decreto Legislativo 116/92 and Decreto Legislativo 26/2014). Animals were used between 3 and 4 months of age.

### Genotyping of rats

Transgenic rats were identified by PCR (30 cycles, 54°C annealing temperature) on their genomic DNA isolated from tail biopsies by the use of the following transgene-specific primers: SV40ipa5: 5′-G AAGGAACCTTACTTCTGTGG-3′ and SV40ipa3: 5′-TCTTGTA TAGCAGTGCAG C-3′.

### Western blotting

Striatal tissues from $NSEA_{2A}$ rats and age-matched controls were homogenized on ice in Tris-HCl (50 mM, pH 7.4) with a protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO, USA) and centrifuged at 750 *g* to remove debris. The supernatant was centrifuged at 109 000 *g* for 45 min at 4°C. The pellet was washed, resuspended in the same buffer and used for protein determination. Twenty or fifty micrograms of protein was separated by 8% sodium dodecyl sulphate–polyacrylamide electrophoresis gels and transferred to polyvinylidene difluoride membranes by electroblotting overnight at 4°C. To avoid non-specific immunodetection, membranes were incubated for 1 h in Tween-20-Tris-buffered saline (50 mM Tris–HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.4) containing 5% non-fat milk. Blots were incubated overnight at 4°C with a rabbit anti-$CB_1$ antibody (1 : 500 dilution, PA1-745; Affinity Bioreagents, Florence, Italy) and with a mouse anti-β-tubulin or anti-β-actin (1 : 20 000 dilution; Sigma-Aldrich). After incubation with secondary antibodies (Chemicon, Temecula, CA, USA), immunoreactive bands were revealed by enhanced chemiluminescent substrate onto X-ray films. Densitometric analysis was conducted using the open-source image-processing software ImageJ64 (http://imagej.nih.gov/ij/) onto six different experiments. $CB_1$R immunoreactive bands were normalized with respect to β-tubulin.

### Electrophysiology

Corticostriatal slices were prepared as previously described (Chiodi *et al.* 2012). Briefly, rats were decapitated under ether anaesthesia, and coronal slices (300 μm) were cut with a vibratome and incubated for 1 h in artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 3.5 KCl, 1.2 $NaH_2PO_4$, 1.2 $MgCl_2$, 2 $CaCl_2$, 25 $NaHCO_3$ and 11 glucose (pH 7.3) saturated with 95% $O_2$ and 5% $CO_2$. Single slices were transferred to a submerged recording chamber and superfused with ACSF at 32–33°C at a rate of 2.7–3 mL/min. All drugs were applied by bath perfusion with ACSF containing the final concentration of the drug. Appropriate stock solutions of drugs were made and diluted with ACSF just before application.

Extracellular field potentials (FPs) were recorded in the dorsomedial striatum with a glass microelectrode and evoked at the frequency of 0.05 Hz by stimulating the white matter with a bipolar platinum/iridium concentric electrode (FHC, Bowdoin, ME, USA). Signals were acquired with the DAM-80 AC differential amplifier (WPI Instruments, Sarasota, FL, USA) and analysed with WinLTP software (Anderson and Collingridge 2007). Ten min of stable baseline recording preceded drug application. The effects of the drugs were expressed as percentage variation with respect to basal values, taking as 100% the average of the values obtained over the 5 min immediately before the application of the test compound.

In some experiments, a protocol of paired pulse stimulation was applied, in which the afferent corticostriatal fibres were stimulated twice with an inter-pulse interval of 50 ms. Under control conditions, such a protocol results in a condition of paired pulse facilitation, in which the response elicited by the second stimulus (R2) is greater than that elicited by the first stimulus (R1).

The degree of paired pulse facilitation is quantified by the R2/R1 ratio, and modifications of this ratio are indicative of changes in presynaptic neurotransmitter release (Schulz *et al.* 1994).

### Striatal synaptosome preparation

Lightly anaesthetized animals were killed by decapitation, and the striata were dissected out from the brain and immediately frozen on dry ice and stored at −80°C. On the day of the experiments, the striata were homogenized in ice-cold buffered (pH 7.4) sucrose solution (0.32 M), the homogenate centrifugated (10 min; 2500 *g*, 4°C), the supernatant collected and the synaptosomes isolated by centrifugation (20 min; 9500 *g*, 4°C). The $P_2$ pellet fraction was resuspended in 5 mL of Krebs solution (mM: NaCl 118.5, KCl 4.7, $CaCl_2$ 1.2, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 10; gassed with 95% $O_2$/5% $CO_2$). Synaptosomes were then maintained in Krebs solution (37°C) for 20 min. Thereafter, aliquots of synaptosomal suspensions were distributed on microporous filters (0.5 mL/filter), placed at the bottom of a set of parallel superfusion chambers and perfused with aerated (95% $O_2$/5% $CO_2$) Krebs solution (37°C).

### Spontaneous glutamate efflux

After a 30-min washout period, nine consecutive 5-min fractions were collected. The cannabinoid receptor agonist WIN (1 μM), the $A_{2A}$R agonist CGS 21680 (10 nM) and antagonist ZM 241385 (100 nM) were added, alone or in combination, to the perfusion medium from the fourth sample until the end of the experiment. Control synaptosomes perfused with Krebs solution were assayed in parallel.

### $K^+$-evoked glutamate efflux

After the collection of three basal samples, synaptosomes were depolarized with KCl (15 mM; 90 s). When required, WIN (1 μM), the $A_{2A}$R agonist CGS 21680 (10 nM) and antagonist ZM 241385 (100 nM) were added, alone or in combination, to the perfusion medium concomitantly with the depolarizing stimulus.

### Glutamate measurement and data analysis

In each sample, glutamate levels (nmol/min/g of protein) were measured by HPLC coupled with fluorimetric detection. The effects of treatments on spontaneous glutamate efflux were determined as percent changes from basal values (mean of the first three samples). $K^+$-evoked glutamate efflux was expressed as percent increase over the spontaneous release (mean of the two fractions collected prior to the depolarizing stimulus). The effect of treatment was expressed as percent ratio of the depolarization-evoked neurotransmitter overflow calculated in the presence of the drug versus that obtained under control conditions, always assayed in parallel. Protein was determined according to Bradford (1976).

### Locomotor activity

Spontaneous locomotor activity was measured in automated cages (43 × 43 × 22 cm, Automex II; Columbus Instruments, Columbus, OH, USA), which allows to measure ‘total’ motor activity as well as to discriminate among horizontal, vertical and stereotyped

activity. The animals were individually placed in the activity motor cage in a sound proof room, and the motor activity, expressed as the number of beam breaks, was recorded over 60 min.

### Statistical analysis

Results are expressed as mean values ± standard error of the mean (SEM). Statistical analysis was carried out by Student's *t*-test or Mann–Whitney *U*-test. Group variability and interaction were compared using two-way ANOVA, including genotype and treatment as between-subject factors, followed by Sidack's *post hoc* test for multiple comparisons. Significance was accepted at $p < 0.05$.

## Results

### Animals

Rats of both genders were used. $NSEA_{2A}$ rats showed a significant reduction in the body weight as compared with WT animals (body weight males, in grams: WT = 410.53 ± 11.44, $NSEA_{2A}$ = 341.8 ± 10.02, $p < 0.05$ Student's *t*-test; body weight females, in grams: WT = 242.9 ± 6.91, $NSEA_{2A}$ = 206.3 ± 4.69, $p < 0.05$ Student's *t*-test, Figure S1a). In addition, consistent with the role played by $A_{2A}$Rs in locomotion, $NSEA_{2A}$ rats displayed a reduced spontaneous motor activity with respect to WT rats. This was assessed in the activity metre cage and demonstrated by the reduction in the total number of beams recorded in naïve, non-habituated animals ($NSEA_{2A}$ = 2490 ± 249, WT = 5658 ± 680, $p < 0.05$, Student's *t*-test, Figure S1b). In order to confirm the over-expression of $A_{2A}$Rs, a western blot analysis in the hippocampus, cortex and striatum from $NSEA_{2A}$ and WT rats, was performed. As shown in Figure S1c, $NSEA_{2A}$ rats displayed a clear increase in the expression level of $A_{2A}$Rs.

### Striatal expression of $CB_1$ receptor

The hypothesis that the over-expression of $A_{2A}$Rs could result in changes in $CB_1$R expression levels has been tested by western blotting experiments. As shown in Fig. 1, striatal $CB_1$R expression was similar in $NSEA_{2A}$ and WT rats (1.1 ± 0.045 vs. 1.22 ± 0.14, respectively, $n = 4$).

### $CB_1$R agonist-induced depression of synaptic transmission and modulation by $A_{2A}$R ligands are altered in $NSEA_{2A}$ rats

We first evaluated basal synaptic transmission in WT and $NSEA_{2A}$ rats by assessing the relationship between the FP amplitude and the stimulus intensities (input–output curves) and by applying a protocol of paired-pulse stimulation to evaluate changes in pre-synaptic neurotransmitter release (see Methods). Input–output curves and paired-pulse stimulation did not differ between the two genotypes suggesting that basal synaptic activity was not altered by the over-expression of $A_{2A}$Rs (data not shown).

Then, the effect of a $CB_1$R agonist on synaptic transmission was investigated. As previously reported, in rat corticostriatal slices, the $CB_1$R agonist WIN 55,212-2 (WIN, 2 μM) induced a marked depression of synaptic transmission (Gerdeman and Lovinger 2001; Pintor *et al.* 2006; Martire *et al.* 2011), and this effect was evident in both WT and $NSEA_{2A}$ rats (Fig. 2a). However, 40 min after the application of the compound, WIN-induced depression of FP was significantly reduced in $NSEA_{2A}$ rats with respect to WT animals (63.78 ± 2.6% of basal, $n = 9$ and 29.13 ± 4.9% of basal, $n = 7$, respectively, $p < 0.05$ Mann–Whitney *U*-test, Fig. 2b).

We next evaluated the modulation of WIN effects by $A_{2A}$R ligands. As shown in Fig. 3(a) and (b) and consistently with previous findings (Martire *et al.* 2011; Ferreira *et al.* 2015), the selective $A_{2A}$R agonist CGS 21680 (100 nM) reduced WIN-induced synaptic depression in WT (60.02 ± 8.84 of basal, 40 min after WIN application, $p < 0.05$ vs. WIN alone, Mann–Whitney *U*-test, $n = 6$) as well as in $NSEA_{2A}$ rats (88.78 ± 3.87 of basal, 40 min after WIN application, $p < 0.05$ vs. WIN alone, Mann–Whitney *U*-test, $n = 7$).

Previous studies indicate that WIN-induced synaptic depression is modulated not only by the $A_{2A}$R agonist but also by the $A_{2A}$R antagonist ZM 241385 (Tebano *et al.*

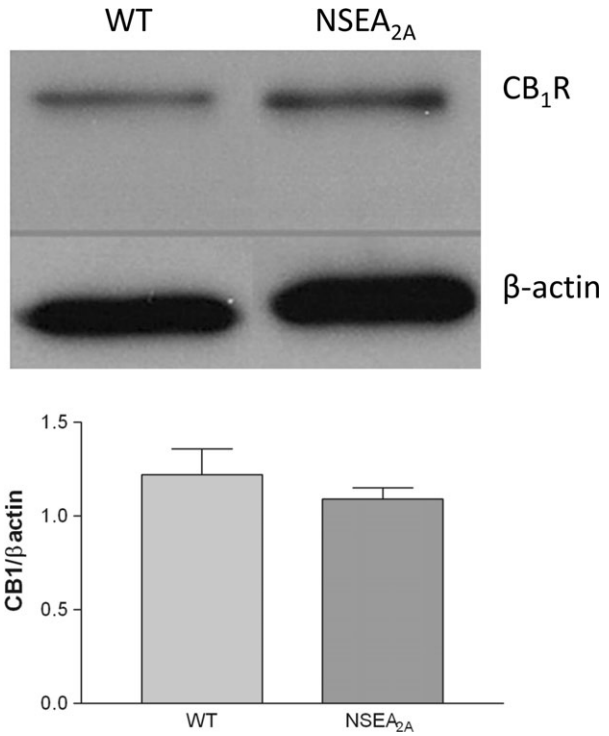


**Fig. 1** Cannabinoid $CB_1$ receptors ($CB_1$Rs) expression in the striatum of neural-specific enolase promoter ($NSEA_{2A}$) and age-matched WT rats. Upper panel: representative western blot experiment. Lower panel: densitometric analysis of the relative abundance of $CB_1$R in the striatum of WT and $NSEA_{2A}$ rats. Each bar is the mean ± SEM of four replications and represents the ratio between the optical density obtained from $CB_1$R and ß-actin.

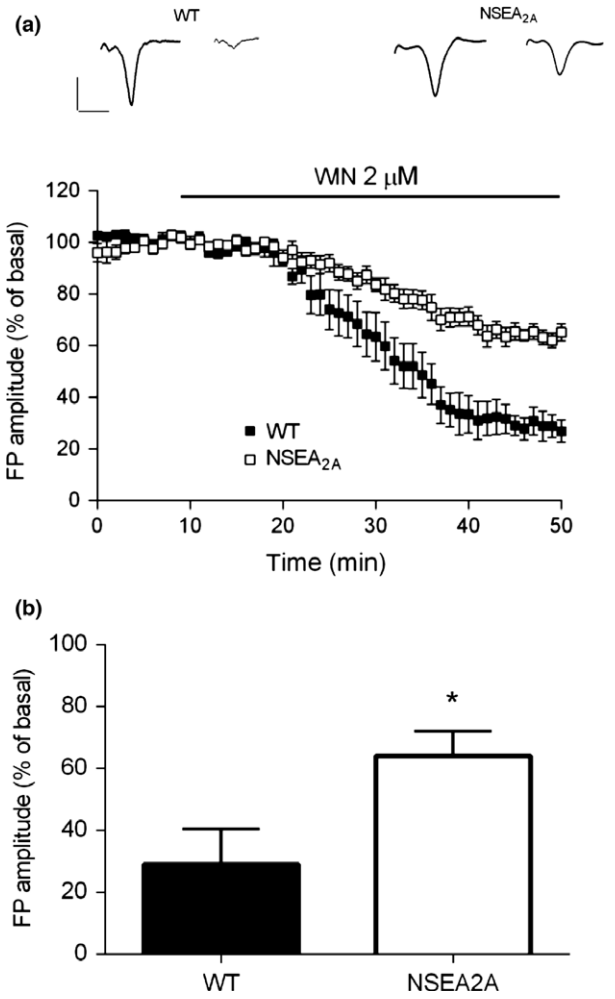


**Fig. 2** Electrophysiological experiments showing the effect of WIN 55,212-2 on synaptic transmission in corticostriatal slices from WT and neural-specific enolase promoter ($NSEA_{2A}$) rats. (a) Slice perfusion with 2 μM WIN 55,212-2 (WIN) induced a marked reduction of field potential (FP) amplitude that was larger in WT than in $NSEA_{2A}$ rats. Each point represents the mean ± SEM of 7–9 slices obtained from at least five different animals. Insets show FPs recorded in basal condition and 40 min after WIN application. Each trace is the average of three successive FPs (artefacts of stimulation have been truncated). The horizontal bars indicate the period of drug application. Calibration bars: 0.5 mV, 5 ms. (b) Bar graph showing the effect of WIN, measured during the last 5 min of drug application (*$p < 0.05$, significantly different from WT, Mann–Whitney *U*-test).

2009). Accordingly, in WT rats, slice perfusion with ZM 241385 (100-500 nM) reduced WIN-induced synaptic depression (60.01 ± 5.36 of basal, 40 min after WIN application, $p < 0.05$ vs. WIN alone, Mann–Whitney *U*-test, $n = 7$, Fig. 3c). However, the same treatment was ineffective in $NSEA_{2A}$ rats (66.27 ± 5.46 of basal, 40 min after WIN application, not significant vs. WIN alone, Mann–Whitney *U*-test, $n = 6$, Fig. 3d). When we evaluated the effects of other selective $A_{2A}$R antagonists, SCH-442416 (1 μM) and KW-6002 (1 μM), on WIN-mediated synaptic depression, again we found that both drugs reduced WIN effects in WT (Fig. 3c) but were unable to influence the effects of WIN in $NSEA_{2A}$ rats (Fig. 3d).

To verify whether corticostriatal slices from $NSEA_{2A}$ rats were sensitive to the pharmacological effects of $A_{2A}$R antagonists, we evaluated the effect of ZM 241385 on 4-aminopyridine (4-AP)-induced paired-pulse inhibition (PPI) of the synaptic response, a condition in which, upon the application of two consecutive stimuli, the second response (R2) is smaller than the first one (R1) and the R2/R1 ratio results <1. As demonstrated by Tebano *et al.* (2004), ZM 241385, by acting at the pre-synaptic level, significantly reduced 4-AP-induced PPI. Here, we found that ZM 241385 reduced 4-AP-induced PPI in the same way in WT and $NSEA_{2A}$ rats (Figure S2). This finding rules out the possibility of a general unresponsiveness of $NSEA_{2A}$ rats to $A_{2A}$R blockade.

### Spontaneous and $K^+$-evoked glutamate efflux from striatal synaptosomes: modulation by $CB_1$ and $A_{2A}$ receptor ligands

$A_{2A}$Rs and $CB_1$Rs are both expressed on striatal glutamatergic terminals where they control neurotransmitter release. Thus, spontaneous and $K^+$-evoked glutamate efflux and their modulation by $A_{2A}$R and $CB_1$R ligands have been evaluated in striatal synaptosomes prepared from WT and $NSEA_{2A}$ rats.

Spontaneous glutamate efflux was similar in WT and $NSEA_{2A}$ rat striatal synaptosomes (189 ± 11 and 193 ± 13 pmol/mg protein/min, respectively; $p > 0.05$, Student's *t*-test, Fig. 4a), and in both cases, it slightly declined over the duration of the experiment. All treatments did not affect spontaneous glutamate efflux from WT and $NSEA_{2A}$ rat striatal synaptosomes (data not shown).

As shown in Fig. 4b, high $K^+$ stimulation significantly increased glutamate efflux from striatal synaptosomes obtained from both genotypes. However, in striatal synaptosomes from $NSEA_{2A}$ rats, the $K^+$-evoked glutamate efflux was higher than that obtained from WT animals (184 ± 3% and 151 ± 2% of spontaneous glutamate levels, respectively, $p < 0.05$, Student's *t*-test).

We then tested the effects of the $A_{2A}$R agonist CGS 21680 (10 nM) and of the antagonist ZM 241385 (100 nM) on $K^+$-evoked glutamate efflux in WT and $NSEA_{2A}$ rats. Two-way ANOVA analysis demonstrated a significant interaction genotype × treatment [$F(2,31) = 10.11$, $p = 0.0004$] and *post hoc* comparisons revealed that CGS 21680 significantly increased $K^+$-evoked glutamate efflux in both genotypes, but its effect was higher in $NSEA_{2A}$ than in WT rats (140 ± 4% and 123 ± 2% of the respective control levels, respectively, $p < 0.05$ Sidak's multiple comparison test, Fig. 4c). The selective $A_{2A}$R antagonist ZM 241385 (100 nM) significantly reduced $K^+$-evoked glutamate efflux in $NSEA_{2A}$ rats

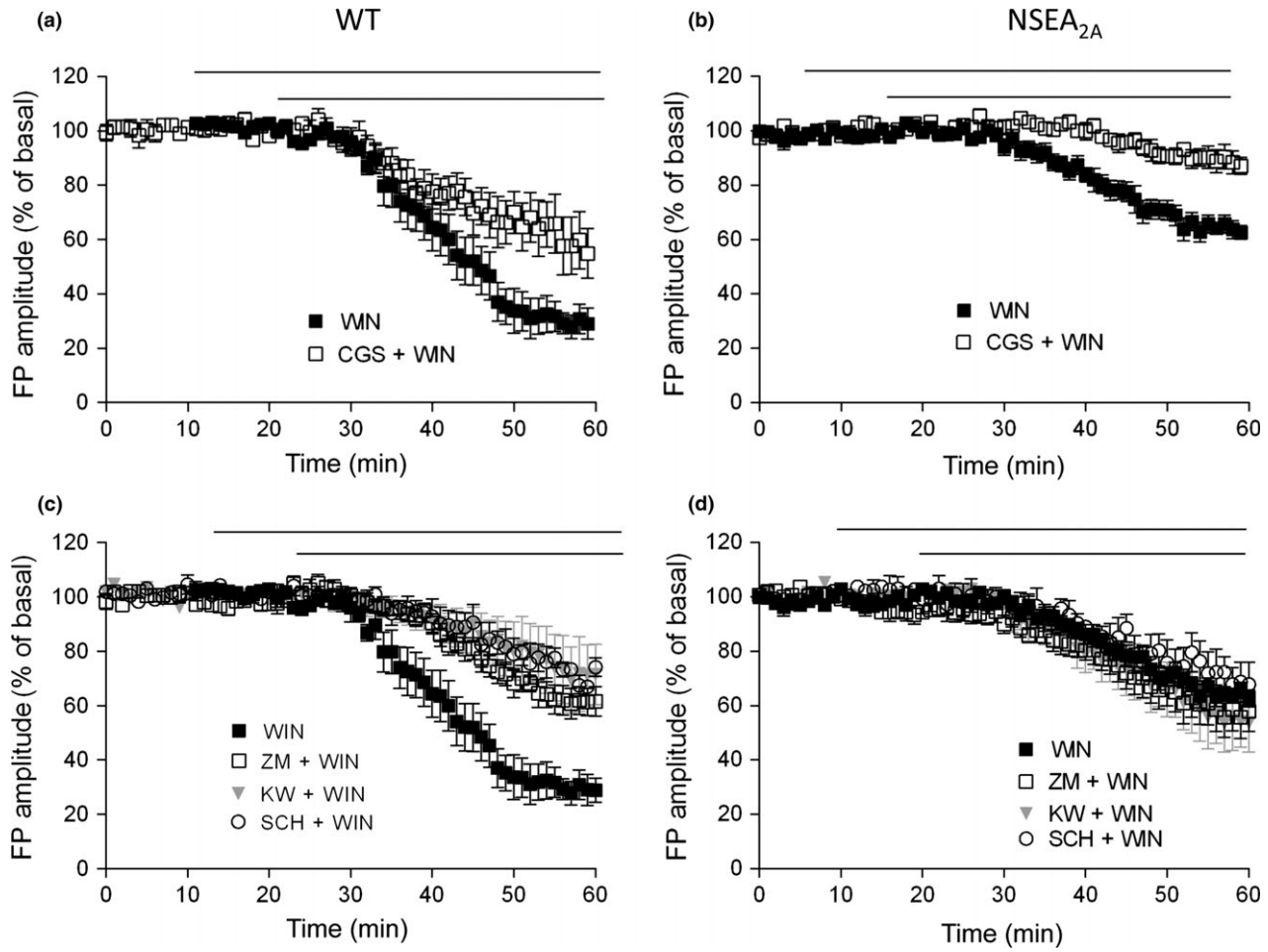


**Fig. 3** Electrophysiological experiments showing the effects of $A_{2A}R$ ligands on WIN 55,212-2-induced synaptic depression in WT and neural-specific enolase promoter ($NSEA_{2A}$) rats. When applied 10 min before and then along with WIN 55,212-2 (WIN), the $A_{2A}R$ agonist CGS 21680 (CGS) reduced the synaptic depression induced by WIN in both WT (a) and $NSEA_{2A}$ rats (b). The $A_{2A}R$ antagonists ZM 241385 (ZM), KW-6002 (KW) and SCH-442416 (SCH), applied 10 min before and then along with WIN, reduced WIN-induced synaptic depression in WT (c) but not in $NSEA_{2A}$ rats (d). Each point represents the mean ± SEM of 3–8 slices obtained from at least three different animals. The horizontal bars indicate the period of drug application.

(86 ± 2% of the control levels, $p < 0.05$, Sidak's multiple comparison test, Fig. 4c) but not in WT animals (95 ± 3% of the control levels, not significant, Fig. 4c).

The effects of the $CB_1$ receptor agonist WIN (1 μM) on $K^+$-evoked glutamate efflux from $NSEA_{2A}$ and WT rat striatal synaptosomes were also tested. Two-way ANOVA analysis demonstrated a significant interaction genotype × treatment [$F(2,28) = 5.82$, $p = 0.0077$] and *post hoc* comparisons revealed that WIN significantly inhibited $K^+$-evoked glutamate efflux from either $NSEA_{2A}$ or WT rat synaptosomes. However, its effect was significantly lower in $NSEA_{2A}$ than in WT rat (84.2 ± 0.86% and 73.16 ± 1.81% of the respective control levels, respectively, $p < 0.05$, Sidak's multiple comparison test).

Finally, ZM 241385 significantly reduced WIN-induced decrease of $K^+$-evoked glutamate efflux from WT (90.83 ± 2.49% of the respective control levels, $p < 0.05$ vs. WIN alone) but not from $NSEA_{2A}$ (85.8 ± 1.24% of the respective control levels, not significant vs. WIN alone) rat synaptosomes (Fig. 4d).

### WIN 55,212-2-induced depression of motor activity is impaired in $NSEA_{2A}$ rats

Having found in *in vitro* experiments a reduced effect of WIN in $NSEA_{2A}$ rats, behavioural experiments were performed to evaluate the *in vivo* response to the $CB_1R$ agonist. Rats of both genotypes were treated i.p. with 5 mg/kg WIN and the motor activity assessed. Two-way ANOVA analysis demonstrated a significant interaction genotype × treatment [$F(1,16) = 5,614$, $p = 0.03$], and *post hoc* comparisons revealed that WIN, injected 15 min prior the test, significantly reduced the motor activity in the WT animals

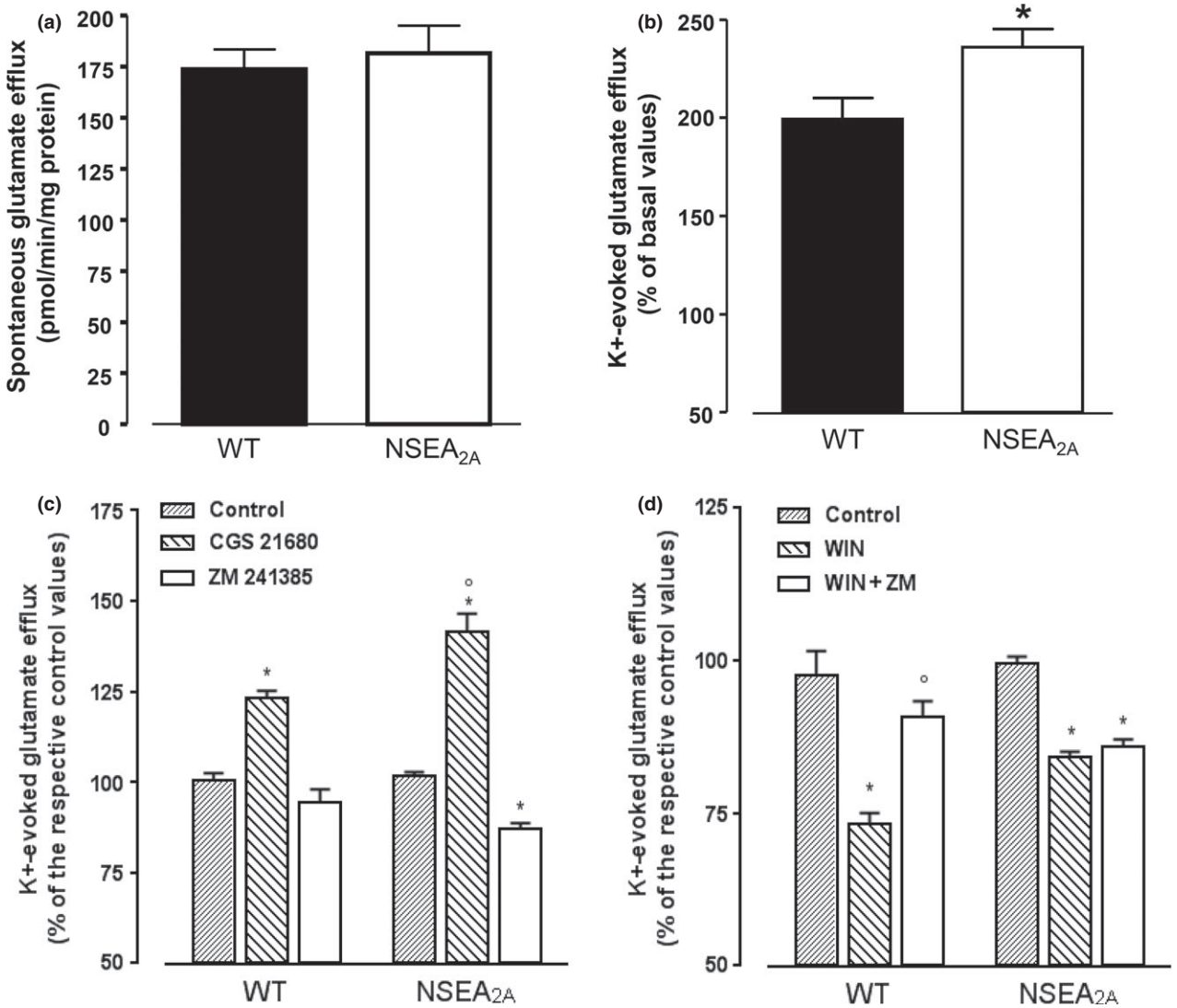


**Fig. 4** Glutamate efflux from striatal synaptosomes and modulation by $CB_1$ and $A_{2A}$ receptor ligands. Spontaneous (a) and $K^+$-evoked (b) glutamate efflux from striatal synaptosomes obtained from WT and neural-specific enolase promoter ($NSEA_{2A}$) rats. Each bar represents the mean ± SEM of 6–7 animals. *$p < 0.05$, significantly different from WT rats (Student's *t*-test). (c): Effects of the $A_{2A}$R agonist CGS 21680 and antagonist ZM 241385 on $K^+$-evoked glutamate efflux from striatal synaptosomes obtained from WT and $NSEA_{2A}$ rats. The $A_{2A}$R ligands were added simultaneously to the depolarizing stimulus and maintained until the end of the collection period. Each bar represents the mean ± SEM of 5–7 animals. *$p < 0.05$, significantly different from the respective control group and °$p < 0.05$ significantly different from the respective WT group according to ANOVA followed Sidack's *post hoc* for multiple comparisons. (d): Effects of the $CB_1$R agonist WIN 55,212-2 (WIN), alone or in combination with the $A_{2A}$R antagonist ZM 241385 (ZM), on $K^+$-evoked glutamate efflux from striatal synaptosomes obtained from WT and $NSEA_{2A}$ rats. The compounds were added simultaneously to the depolarizing stimulus and maintained until the end of the collection period. Each bar represents the mean ± SEM of 5–6 animals. *$p < 0.05$, significantly different from the respective control group and °$p < 0.05$, significantly different from the respective WT group according to ANOVA followed by Sidack's *post hoc* test for multiple comparisons.

($p < 0.05$ Sidack's *post hoc* comparisons between WT-vehicle and WT-WIN), but not in $NSEA_{2A}$ rats (Fig. 5).

## Discussion

The present results demonstrate that in rats with genetic neuronal over-expression of $A_{2A}$Rs, the effects mediated by $CB_1$R activation are blunted. Specifically, we found that the effects of the $CB_1$R agonist WIN on corticostriatal synaptic transmission, $K^+$-induced glutamate outflow in striatal synaptosomes and locomotor activity are significantly reduced in $NSEA_{2A}$ rats, while the expression level of $CB_1$Rs is unaltered. These results provide a demonstration of a functional interaction between $A_{2A}$Rs and $CB_1$Rs

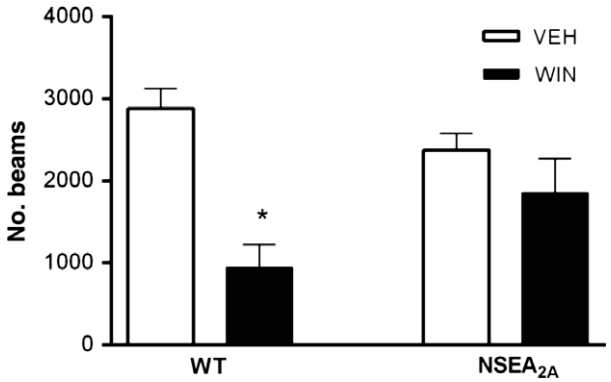


**Fig. 5** Effect of cannabinoid $CB_1$ receptor agonist on spontaneous locomotor activity. Administration of WIN 55,212-2 (WIN, 5 mg/kg i.p.) significantly reduced spontaneous locomotor activity in WT but not in neural-specific enolase promoter ($NSEA_{2A}$) rats. *$p < 0.05$ versus WT-vehicle (VEH), two-way ANOVA followed by Sidack's test for multiple comparisons. Data represent mean ± SEM from 6 to 8 animals.

in an *in vivo* model of a constitutive up-regulation of $A_{2A}$Rs.

In this study, the first observation was that $NSEA_{2A}$ rats showed a reduced body weight compared with WT animals. The finding that neuronal over-expression of $A_{2A}$Rs results in a decrease of body weight could be consistent with previous studies, demonstrating that central administration of adenosine suppresses food intake in rats (Levine and Morley 1983) and reduces feeding induced by opioid receptor agonists (Wager-Srdar *et al.* 1984) along with a more recent article demonstrating that $A_{2A}$R agonists reduce both high-palatability and low-palatability food intake in rats (Micioni Di Bonaventura *et al.* 2012). Our results are also in agreement with a recent article showing a reduction in body weight in transgenic rats over-expressing the human $A_{2A}$R under the control of the CaMKII promoter (Coelho *et al.* 2014). However, given the role played by $CB_1$Rs in the regulation of food intake (Cota *et al.* 2003; D'Addario *et al.* 2014), and the hypofunctionality of $CB_1$Rs described in the current study, an involvement of cannabinoid receptors in the reduction of body weight in $NSEA_{2A}$ rats cannot be excluded.

In corticostriatal slices, we found that WIN-induced depression of synaptic transmission is significantly reduced in $NSEA_{2A}$ as compared with WT rats, suggesting a functional impairment of $CB_1$R signalling in the presence of a constitutive neuronal over-expression of $A_{2A}$Rs. This result is in agreement with previous studies, demonstrating that the activation of $A_{2A}$Rs by the selective $A_{2A}$R agonist CGS 21680 reduced WIN-induced synaptic depression and paired-pulse facilitation in corticostriatal slices (Martire *et al.* 2011; Ferreira *et al.* 2015). Also in the current study, the effect of WIN was prevented by CGS 21680, both in WT and in $NSEA_{2A}$ rats, suggesting that the mechanisms through which the $A_{2A}$R agonist regulates $CB_1$R-mediated effects (probably at the level of the signal transduction pathway, see Tebano *et al.* 2012) may not be altered in $NSEA_{2A}$ rats. The effects of WIN on synaptic transmission can be modulated not only by $A_{2A}$R agonists but also by $A_{2A}$R antagonist, since ZM 241385 reduced WIN-induced synaptic depression in corticostriatal slices (Tebano *et al.* 2009). Interestingly, while ZM 241385 significantly lowered the effect of WIN on synaptic transmission in WT animals, it was ineffective in $NSEA_{2A}$ rats. When we evaluated the effects of two other selective $A_{2A}$R antagonists, SCH-442416 and KW-6002, again we found that they were effective in reducing WIN-mediated effects in WT but not in $A_{2A}$R over-expressing rats. To verify whether corticostriatal slices from $NSEA_{2A}$ rats were sensitive to the pharmacological effects of the $A_{2A}$R antagonist, the effect of ZM 241385 on 4-AP-induced PPI was assessed, and it was found that the antagonist reduced it to the same degree in WT and $NSEA_{2A}$. This finding clearly demonstrates that the lack of effect of $A_{2A}$R antagonists on WIN-induced synaptic depression in $NSEA_{2A}$ does not represent a general hyporesponsiveness of these rats to $A_{2A}$R blockade, but may be specifically linked to $CB_1$R-mediated effects.

In striatal synaptosomes prepared from WT and $NSEA_{2A}$ rats, spontaneous and $K^+$-induced glutamate outflow were evaluated together with their modulation by $A_{2A}$R ligands and the $CB_1$R agonist WIN. It was found that spontaneous glutamate efflux was not different in the two genotypes, in agreement with a recent article which demonstrated that basal tissue glutamate levels were not different in the striatum of $NSEA_{2A}$ animals with respect to control rats (Jastrzębska *et al.* 2014). On the contrary, $K^+$-induced glutamate outflow was (i) significantly increased in $NSEA_{2A}$ with respect to WT rats; (ii) increased by CGS 21680, with a significantly higher effect in $NSEA_{2A}$ than in WT rats; (iii) reduced by ZM 241385 in $NSEA_{2A}$ but not in WT rats; (iv) reduced by WIN, with a significantly lower effect in $NSEA_{2A}$ than in WT. These results demonstrate that the $A_{2A}$R over-expression results in a neuronal gain of function of these receptors at the pre-synaptic level. Under this condition, the effect of WIN in reducing $K^+$-induced glutamate outflow is significantly attenuated in $NSEA_{2A}$ with respect to WT rats. Moreover, while in WT the effect of WIN is prevented by ZM 241385, the $A_{2A}$R antagonist did not prevent $CB_1$R-mediated effect in $NSEA_{2A}$. These results further demonstrate the hypofunctionality of $CB_1$Rs and provide additional evidence that in the presence of an up-regulation of $A_{2A}$Rs, ZM 241385 is no longer able to influence WIN-mediated effects.

In order to verify whether the alterations in $CB_1$R-mediated effects observed in corticostriatal slices and in striatal synaptosomes from $NSEA_{2A}$ rats were evident also *in vivo*, we compared the effects of WIN on locomotor activity in WT and $NSEA_{2A}$ rats. As known, high doses of WIN are associated with a motor depressive action in rodents (Drews *et al.* 2005; Järbe *et al.* 2006; Polissidis *et al.* 2013) and, in agreement, significant reduction in motor behaviour

in WT rats was found after the treatment with WIN. This effect, however, was not present in $NSEA_{2A}$ rats (Fig. 4), strengthening previous results of a reduced response to $CB_1$R stimulation in the presence of a neuronal over-expression of $A_{2A}$Rs. It is interesting to note that—while in naïve, not treated, rats, a significant reduction was observed in spontaneous motor activity in $NSEA_{2A}$ with respect to WT rats (Figure S1b)—this difference is much less evident in the experiments with WIN. A possible explanation for this apparent discrepancy can be found in the different effect of the i.p. injection in the two genotypes. As seen by comparing the number of beams recorded in the automated activity motor cage, in WT animals the injection with the vehicle caused a remarkable reduction in the motor activity compared with unhandled rats, while in $NSEA_{2A}$ animals, this effect was not evident. This different response to the i.p. injection in the two genotypes could reflect a reduced stress response in the $A_{2A}$ over-expressing rats compared with WT animals.

The finding of a blunted motor depressant effect of WIN in $NSEA_{2A}$ rats is apparently at odds with previous studies, showing that genetic inactivation or pharmacological blockade of $A_{2A}$Rs reduced the motor depressant effects of $CB_1$R agonists (Andersson *et al.* 2005; Carriba *et al.* 2007). These studies rather indicated that $A_{2A}$R activation is required for the $CB_1$R-mediated motor depression to occur, although others have suggested that $A_{2A}$Rs are more involved in physical dependence and place conditioning than in motor depression induced by delta-9-tetrahydrocannabinol (Soria *et al.* 2004). Although there is no definite explanation for the current results, it is important to consider the recent achievements in the field of GPCR, which identify negative crosstalk and cross-antagonism as common properties of GPCR heteromers (Ferré *et al.* 2014). Recently, the existence of these properties has been demonstrated in the $A_{2A}$-$D_2$ heteromer, providing a mechanistic explanation, which would depend on a heterotetrameric structure of the heteromer (Bonaventura *et al.* 2015). Under this view, the ability of the $A_{2A}$R antagonist to block $CB_1$ signalling could be related to cross-antagonism within the $A_{2A}$-$CB_1$ heteromer while the ability of an $A_{2A}$R agonist to reduce $CB_1$-mediated inhibition of glutamatergic transmission could depend on the ability of other $A_{2A}$Rs, not forming heteromers with $CB_1$, to functionally counteract the effect of a $CB_1$ agonist. Thus, it is likely that in $NSEA_{2A}$ rats (which expressed increased levels of $A_{2A}$Rs but normal levels of $CB_1$Rs), the proportion of $A_{2A}$Rs forming or not forming heteromers with $CB_1$Rs may be changed. This is relevant, since in the $A_{2A}$/$CB_1$ heteroreceptor complexes, the activation of $A_{2A}$Rs protomers has been reported to facilitate the effects mediated by $CB_1$Rs protomers (Carriba *et al.* 2007) and, on the other hand, the signalling of adenosine $A_{2A}$Rs existing as monomers and as protomers in $A_{2A}$ homo- and heteroreceptor complexes of different types, and their balance, can also be altered in the $NSEA_{2A}$ rats and participate in the observed blunting of $CB_1$R signalling, in addition to the $A_{2A}$/$CB_1$ heteroreceptor complexes (Ferré *et al.*, 2010; Fuxe *et al.* 2015). Nevertheless, it can be hypothesized that the facilitatory role of $A_{2A}$Rs on $CB_1$R-mediated effects may occur mainly at the level of $A_{2A}$/$CB_1$ heteroreceptor complexes. The lack of effects of the $A_{2A}$R antagonists observed in the current study might indicate a decrease in the expression of $A_{2A}$/$CB_1$ heteroreceptor complexes, leading to potential changes in their pharmacology in the striatum, as a consequence of $A_{2A}$R over-expression, and to an overall reduction in $CB_1$-mediated effects. A recent article demonstrated that chronic treatment with L-DOPA in primates disrupts the expression of $A_{2A}$/$CB_1$ heteromers in basal ganglia (Bonaventura *et al.* 2014), highlighting the possibility that the expression of heteroreceptor complexes can be modified by pharmacological interventions.

As a whole, the present study demonstrates a functional impairment of $CB_1$R-mediated effects in the striatum of rats with a genetic up-regulation of $A_{2A}$Rs. Additional studies are needed to identify the exact mechanism, especially by studying the $A_{2A}$/$CB_1$ heteroreceptor complexes by means of techniques like the proximity ligation assay. In spite of the current mechanistic uncertainties, however, these findings confirm and strengthen the fundamental role of the $A_{2A}$/$CB_1$ interaction in the regulation of striatal functions.

## Acknowledgments and conflict of interest disclosure

We thank Adriano Urcioli and Alessio Gugliotta for assistance with animal work. The authors have no conflict of interest to declare.

All experiments were conducted in compliance with the ARRIVE guidelines.

## Supporting information

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

**Figure S1**. (a) $NSEA_{2A}$ rats show a reduction in body weight as compared with WT animals. Results are expressed as mean ± SEM. M, males (n = 16 and n = 14, WT and $NSEA_{2A}$, respectively); F, females (n = 10 and n = 12, WT and $NSEA_{2A}$, respectively). *$p < 0.05$ vs. WT (Student's *t*-test). (b) $NSEA_{2A}$ rats displayed a reduced spontaneous motor activity with respect to WT rats. Bar graph shows the total number of beams recorded during 60 min in the activity metre cage in naïve animals. Data represent mean ± SEM from 15 to 18 animals. *$p < 0.05$, Student's *t*-test. (c) Western blotting showing neuronal over-expression of $A_{2A}$ receptor ($A_{2A}$) in the hippocampus (Hippo), cortex and striatum of $NSEA_{2A}$ rats, as compared with WT animals.

**Figure S2**. Influence of ZM 241385 (ZM) on 4-aminopyride (4-AP)-induced paired-pulse inhibition of the synaptic response in corticostriatal slices from WT and $NSEA_{2A}$ rats.

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