Adenosine A2A Receptors in Striatal Glutamatergic Terminals and GABAergic Neurons Oppositely Modulate Psychostimulant Action and DARPP-32 Phosphorylation

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Abstract

Adenosine A2A receptors (A2AR) are located postsynaptically in striatopallidal GABAergic neurons, antagonizing dopamine D2 receptor functions, and are also located presynaptically at corticostriatal terminals, facilitating glutamate release. To address the hypothesis that these two A2AR populations differently control the action of psychostimulants, we characterized A2AR modulation of cocaine-induced effects at the level of DARPP-32 phosphorylation at Thr-34 and Thr-75, c-Fos expression, and psychomotor activity using two lines of cell-type selective A2AR knockout (KO) mice with selective A2AR deletion in GABAergic neurons (striatum-A2AR-KO mice), or with A2AR deletion in both striatal GABAergic neurons and projecting cortical glutamatergic neurons (forebrain-A2AR-KO mice). We demonstrated that striatum-A2AR KO mice lacked A2ARs exclusively in striatal GABAergic terminals whereas forebrain-A2AR KO mice lacked A2ARs in both striatal GABAergic and glutamatergic terminals leading to a blunted A2AR-mediated facilitation of synaptosomal glutamate release. The inactivation of A2ARs in GABAergic neurons reduced striatal DARPP-32 phosphorylation at Thr-34 and increased its phosphorylation at Thr-75. Conversely, the additional deletion of corticostriatal glutamatergic A2ARs produced opposite effects on DARPP-32 phosphorylation at Thr-34 and Thr-75. This distinct modulation of DARPP-32 phosphorylation was associated with opposite responses to cocaine-induced striatal c-Fos expression and psychomotor activity in striatum-A2AR KO (enhanced) and forebrain-A2AR KO mice (reduced). Thus, A2ARs in glutamatergic corticostriatal terminals and in GABAergic striatal neurons modulate the action of psychostimulants and DARPP-32 phosphorylation in opposite ways. We conclude that A2ARs in glutamatergic terminals prominently control the action of psychostimulants and define a novel mechanism by which A2ARs fine-tune striatal activity by integrating GABAergic, dopaminergic and glutamatergic signaling.

Introduction

Striatal circuits, composed mainly of GABAergic medium spiny neurons (MSN), are the principal entry point of the basal ganglia and the primary site for processing of motor, motivational and cognitive behaviors [1]. MSN are driven by cortico-thalamic excitatory glutamatergic projections and modulated by nigral dopaminergic inputs. MSN project either directly (striatonigral MSN) or indirectly (striatopallidal MSN) to output nuclei [2]. Adenosine A2A receptors (A2AR) are highly expressed in striatopallidal MSN where they antagonize dopamine D2 receptor (D2-R) function [3]. In addition, A2AR are also located in striatal glutamatergic terminals [4] where they are involved in the modulation of glutamate release and corticostratial synaptic transmission [5,6,7,8]. Notably, blockade of A2AR in extra-striatal forebrain neurons attenuates behavioral responses to psychostimulants such as cocaine [9], amphetamine [10,11] or L-DOPA [12]. This led us to propose that presynaptic A2AR in corticostriatal glutamatergic terminals exert their excitatory effects by facilitating glutamate release to counteract the inhibitory effect of postsynaptic A2AR in GABAergic MNS [3,9]. This working model places A2AR in a unique position, integrating GABAergic, glutamatergic and dopaminergic neurotransmission to fine-tune striatal activity.

Dopamine- and cAMP-regulated phosphoprotein (DARPP-32) is a key signaling molecule coordinating MSN responsiveness, where its activity is regulated by its phosphorylation status on different residues, namely Thr-34 and Thr-75 [13]. The phosphorylation of striatal DARPP-32 at Thr-34 and Thr-75 is
under tight control of dopamine, adenosine and glutamatergic
signalling [13]. DARPP-32 phosphorylation at Thr-34 is
controlled by the Gs/Gi-cAMP-PKA signalling pathway via D1
receptors (D1R) in the direct pathway and A2AR/D2R activation
in the indirect pathway. DARPP-32 phosphorylation at Thr-75 in
MSN is competitively inhibited by and inversely correlated with
the activation of cAMP signalling and is additionally modulated by
glutamate signalling via cdk5 kinase [14]. Studies with global [13]
or striatal pathway-selective genetic deletion of DARPP-32
[15,16,17] confirmed that DARPP-32 activation in the direct
and indirect pathways oppositely determines motor responses to
psychoactive drugs. Specifically, the selective deletion of
DARPP32 in the indirect pathway enhances psychomotor activity
while the selective deletion of DARPP-32 in the direct pathway
attenuates the psychomotor effect [15,16]. Thus, Thr-34 and Thr-
75 phosphorylation of DARPP-32 integrates the glutamatergic
drive with dopaminergic extrinsic modulation as well as with
intrinsinc striatal modulation such as through adenosine [13].
We therefore hypothesize that A2AR in GABAergic and glutamatergic
neurons modulates the action of psychostimulants through a
putative opposite control of striatal DARPP-32 phosphorylation.

To test this hypothesis, we developed and characterized two cell
type-selective A2AR knockout (KO) lines with selective deletion of
A2AR either in inhibitory GABAergic striatopallidal neurons
(striatum-A2AR KO, st-A2AR KO) or in excitatory glutamatergic
cortical neurons in addition to GABAergic MSN (forebrain-A2AR
KO, fb-A2AR KO). Their use allowed us to demonstrate that
A2ARs in GABAergic MSN and in corticostriatal glutamatergic
terminals control the action of psychostimulants in opposite
mannersthe levels of (i) DARPP-32 phosphorylation; (ii)
cocaine-induced c-Fos expression; and (iii) cocaine-induced
psychomotor activity. This suggests that A2AR control the action
of psychostimulants through the regulation of DARPP-32
phosphorylation [at Thr-34 and Thr-75] in striatopallidal neurons.
Furthermore, these results define a novel function of A2AR in
Glutamatergic terminals and GABAergic striatopallidal neurons to
fine-tune striatal neuronal activity and the action of psychostim-
ulants through the integration of GABAergic, glutamatergic and
dopaminergic signalling pathways.

Results

1. Selective preservation of A2AR in glutamatergic but not
GABAergic terminals in striatum- (but not forebrain-)
A2AR KO mice

To demonstrate the selectivity of A2AR deletion in st-A2AR KO
and fb-A2AR KO mice, we quantified A2AR immunoreactivity in
Glutamatergic (vesicular glutamate transporters type 1, vGluT1-
positive) and GABAergic (vesicular GABA transporters, vGAT-
positive) terminals from the striatum of st-A2AR KO, fb-A2AR KO
and global A2AR knockout (gb-A2AR KO) mice as well as their
corresponding wild-type (WT) littermates. Quantitative analysis
revealed that A2AR immunoactivity was depleted in GABAergic
terminals from st-A2AR KO and fb-A2AR KO mice to background
levels (n = 4–6 animals per group, p < 0.05, unpaired Student’s t
test) (Figure 1A) similar to those found in gb-A2AR KO mice (not
shown). In contrast, A2AR immunoreactivity in glutamatergic
terminals (about 50% of vGluT-positive terminals contain A2AR, see
[7]) was completely abolished in fb-A2AR KO mice and gb-
A2AR KO mice (n = 4–6 animals per group, p < 0.05, unpaired
Student’s t test), but was selectively preserved in st-A2AR KO mice
(n = 6, p > 0.05, unpaired Student’s t test) due to the presence of
presynaptic A2AR on corticostriatal terminals of extra-striatal
Glutamatergic neurons (Figure 1B). The preservation of presynap-
tic glutamatergic A2AR in st-A2AR KO mice was also consistent
with the normal level of A2AR binding density in total membranes
[9] and synaptosomal membranes (data not shown) of the cerebral
cortex of st-A2AR KO mice. Together, these data demonstrate
that A2AR in glutamatergic terminals of the striatum were
selectively preserved in st-A2AR KO mice but abolished in fb-
A2AR KO mice.

2. Selective preservation of the A2AR R-mediated
facilitation of glutamate release in synaptosomes from
striatum-A2AR KO mice

To examine the functional consequence of A2AR deletion in
Glutamatergic terminals in the striatum, we compared the ability of
the selective A2AR agonist CGS21680 to facilitate glutamate
release from striatal synaptosomes of fb-A2AR KO or st-A2AR KO
mice. In the absence of CGS21680, the elevation of extracellular
K+ concentration induced similar spike releases of glutamate from
striatal synaptosomes from WT mice during two consecutive
stimulation periods (ratio of 0.98 ± 0.02, n = 16). A supra-
maximal but A2AR-selective concentration of CGS21680 (20 nM,
present during the second period of stimulation) enhanced 3H-
glutamate release by 38.2 ± 2.4% and 35.9 ± 1.9% from striatal
synaptosomes of fb-WT and st-WT mice, respectively (n = 6–8,
p < 0.05 compared to 0%, one sample t-test) (Figure 2A and 2B).
In contrast, CGS21680-mediated facilitation of 3H-glutamate release
was completely abolished in striatal synaptosomes from fb-A2AR KO
mice (n = 4, p > 0.05 compared to 0%) (Figure 2C), but was
unaffected in synaptosomes from st-A2AR KO mice (n = 4, p > 0.05
compared to 0%) (Figure 2D). These findings support the
selective preservation of presynaptic A2AR function in glutamatergic
terminals in st-A2AR KO but not fb-A2AR KO mice.

3. A2AR in glutamatergic terminals and GABAergic
eurons oppositely modulate striatal DARPP-32
phosphorylation at Thr-34 and Thr-75

To determine the functional significance of the deletion of A2AR
exclusively in GABAergic striatal neurons (in st-A2AR KO) and of
its additional deletion in glutamatergic terminals (fb-A2AR KO),
we evaluated the phosphorylation status of striatal DARPP-32 at
Thr-34 in fb-A2AR KO mice (two-way ANOVA, drug effect:
F(1,12) = 95.765, p < 0.001; genotype effect: F(1,12) = 7.753,
p = 0.017; drug x genotype: F(1,12) = 9.034, p = 0.011) (Figure
3A, 3B) and in st-A2AR KO mice (two-way ANOVA, drug effect:
F(1,12) = 78.861, p < 0.001; genotype effect: F(1,12) = 25.924,
p < 0.001; drug x genotype: F(1,12) = 12.508, p = 0.002) (Figure
3C, 3D). In addition, we also evaluated the phosphorylation status
of striatal DARPP-32 at Thr-34 in fb-A2AR KO mice (two-way
ANOVA, drug effect: F(1,12) = 78.577, p < 0.001; genotype effect:
F(1,12) = 6.060, p = 0.054; drug x genotype: F(1,12) = 0.717,
p = 0.414) (Figure 3E, 3F) as well as in st-A2AR KO mice
(two-way ANOVA, drug effect: F(1,12) = 624.116, p < 0.001;
genotype effect: F(1,12) = 42.378, p < 0.001; drug x genotype:
F(1,12) = 16.111, p = 0.002) (Figure 3G, 3H). Under basal
condition (i.e. after treatment with vehicle), the level of DARPP-
32 phosphorylation at Thr-75 or at Thr-34 was comparable
between fb-A2AR KO mice and their fb-WT littermates (Figure
3B and 3F) (n = 4–6 per group, p > 0.05, two-way ANOVA post hoc
Bonferroni test). Interestingly, the deletion of A2AR in GABAergic
striatopallidal neurons significantly increased the basal level of
DARPP-32 phosphorylation at Thr-75 (Figure 3H) (n = 4 per
group, p < 0.05) together with a (mild) reduction of the basal level
of DARPP-32 phosphorylation at Thr-34 (Figure 3D) in st-A2AR
KO (but not fb-A2AR KO) mice (n = 6). These observations are
Figure 1. Deletion of A2AR immunoreactivity in glutamatergic terminals of forebrain-A2AR KO and GABAergic terminals of both forebrain A2AR - and striatum-A2AR KO mice. Detection and quantification of the percentage of GABAergic terminals (A, vGAT-positive) and glutamatergic terminals (B, vGluT1-positive) and from forebrain-selective-A2AR KO (fb-KO) or striatum-selective-A2AR KO (st-KO) mice and their wild type (WT) littermates (control) that are endowed with A2AR immunoreactivity. The bar graphs represent the percentage of vGluT1- or vGAT-immunopositive terminals that are also endowed with A2AR immunoreactivity (mean ± SEM, 3 fields per mouse, n = 4-6 animal per group). * p<0.05
consistent with a direct effect of postsynaptic A2AR in GABAergic neurons [18,19]. Also in agreement with previous studies [18,20], acute treatment with cocaine (25 mg/kg, i.p.) produced a marked increase of DARPP-32 phosphorylation at Thr-34 and a concomitant reduction of DARPP-32 phosphorylation at Thr-75 in WT mice (st-WT and fb-WT, Figure 3). As predicted from a direct, postsynaptic facilitatory effect of A2AR in GABAergic neurons, cocaine-induced DARPP-32 phosphorylation at Thr-34 was significantly attenuated in st-A2AR KO mice compared to their WT littermates (n = 6 per group, p < 0.05 comparing cocaine with saline treatment) (Figure 3D). In contrast, the acute treatment with cocaine markedly increased DARPP-32 phosphorylation at Thr-34 in fb-A2AR KO mice compared to WT littermates (n = 4, p < 0.05 comparing cocaine with saline treatment) (Figure 3A and 3B), consistent with a reduced glutamate release and disinhibition of glutamate suppression of DARPP-32 phosphorylation at Thr-34 in fb-A2AR KO mice [14]. Additional fluorescence immunohistochemistry using brain sections showed that DARPP32 phosphorylation at Thr-75 was markedly reduced 45 minutes after cocaine treatment in fb-WT and fb-A2AR KO mice (data not shown), a finding consistent with Western blot analysis. These findings demonstrated that, following cocaine treatment, presynaptic A2AR in glutamatergic terminals exert an opposite and predominant effect over postsynaptic A2AR in GABAergic neurons on striatal DARPP-32 phosphorylation at Thr-34 and Thr-75.

4. Cocaine-induced striatal c-Fos expression and psychomotor activity are enhanced in striatum-A2AR KO but attenuated in forebrain-A2AR KO mice

To evaluate the functional significance of the opposite modulation of striatal DARPP-32 phosphorylation by A2AR in GABAergic striatal neurons and in glutamatergic terminals, we compared cocaine-induced psychomotor activity and c-Fos expression, a measure of MSN activity, in the striatum of st-A2AR KO and fb-A2AR KO mice. Consistent with our previous reports [9], we found that cocaine (25 mg/kg, i.p.)-induced psychomotor activity was enhanced in st-A2AR KO (n = 9) but attenuated in fb-A2AR KO mice (n = 12) compared to their WT littermates (n = 8-12) (two-way ANOVA, drug effect: F(1,24) = 91.892, p < 0.001; genotype effect: F(3,24) = 8.456, p < 0.001; drug x genotype: F(3,24) = 13.297, p < 0.001) (Figure 4A and 4B). The
opposite psychomotor effects of cocaine in st-A2AR KO and fb-A2AR KO mice were also paralleled by similar opposite effects of cocaine on c-Fos gene expression in the striatum of these two transgenic mouse strains. As expected, cocaine treatment (25 mg/kg, i.p.) increased c-Fos expression in the striatum of WT mice (st-WT and fb-WT, Figure 4C) to a similar extent. Interestingly, cocaine-induced striatal c-Fos expression was enhanced in st-A2AR KO mice (p<0.05, Student’s t-test, comparing with st-WT) (Figure 4C) but reduced in fb-A2AR KO mice compared to their corresponding WT littermates (p<0.05, Student’s t-test, comparing with fb-WT mice) (Figure 4C). Furthermore, double immunohistochemical analysis showed that the cocaine-induced increase of striatal c-Fos immunoreactivity in st-A2AR KO mice was restricted to dynorphin-positive cells (Figure 4D). As shown in
Figure 4D, the majority of c-Fos-positive cells (black arrows) in the
striatum were also stained with dynorphin, whereas some neurons
were stained with dynorphin (white arrow heads) or c-Fos (black
arrow heads) only.

Lastly, we performed double fluorescence immunohistochemical
study to investigate if the cocaine-induced c-Fos expression mostly
occurred in enkephalin (Enk)-positive or Enk-negative cells in fb-
A2AR KO mice. The basal level of c-Fos expression in fb-A2AR KO mice was comparable with their WT littermates after saline
injection (Figure 5, A and B), while enkephalin-negative cells constituted about 50% of the total cell population. Cocaine
treatment markedly increased striatal c-Fos expression in fb-WT
and fb-A2AR KO mice (two-way ANOVA, drug effect: F(1,34) = 234.289, p<0.001; genotype effect: F(1,34) = 70.643, p<0.001;
drug x genotype: F(1,34) = 56.521, p<0.001) (Figure 5, A and B). This induction largely occurs in Enk-negative cells (drug effect:
F(1,34) = 202.149, p<0.001; genotype effect: F(1,34) = 33.480, p<0.001; drug x genotype: F(1,34) = 21.888, p<0.001) (i.e. in the
direct pathway, Figure 5D); this finding is consistent with our
results using two color, sequential immunohistochemistry of c-Fos
dynorphin (Figure 4) and also agrees with previous reports
that cocaine induces c-Fos expression predominantly in the D1R-
containing striatonigral neurons [e.g. [17]]. In fb-WT animals, we
also observed a cocaine-induced c-Fos expression in the D2R-
containing indirect pathway, likely attributed to a postsynaptic
(striatopallidal) A2AR effect since cocaine-induced c-Fos expression
was reduced in fb-A2AR KO mice (Figure 5C). Thus fb-A2AR KO
mice displayed a reduced cocaine-induced c-Fos expression in the
direct pathway as well as the indirect pathway, although the
majority of cocaine-induced modifications of c-Fos expression in
fb-A2AR KO mice were attributed to the direct pathway (see
Figure 5). This finding suggests that the elimination of presynaptic
glutamatergic A2AR mainly affects the direct pathway to control
psychomotor activity and c-Fos expression.
A2AR in glutamatergic corticostriatal terminals modulate psychomotor activity

Postsynaptic A2AR in GABAergic striatopallidal neurons are involved in the modulation of motor activity due to the concentrated expression of A2AR in striatopallidal neurons and their antagonistic interactions with D2R [21]. In addition, A2AR are also present and functional in presynaptic glutamatergic terminals that play a primordial role in driving striatal circuits [4,5,6,8,22]; however their role in the control of the action of psychostimulants remains largely unexplored, due to the low expression of the A2AR in the cerebral cortex and the inability to selectively manipulate A2AR in distinct cellular elements. Furthermore, it is currently unknown if the presynaptic A2AR might differentially affect the direct versus indirect pathways. The comparative analysis of the phenotypes of st-A2AR KO and fb-A2AR KO mice allowed us to dissect the effects of A2AR in glutamatergic terminals from those of A2AR in GABAergic striatopallidal neurons. Our main findings demonstrate that presynaptic A2AR in corticostriatal glutamatergic terminals facilitate glutamate release (by its presynaptic action alone or in combination with the action of postsynaptic A2AR) and play a predominant role in the control of DARPP-32 phosphorylation, striatal c-Fos expression, and consequent enhanced psychomotor activity upon cocaine exposure. Specifically, we showed that, in contrast to st-A2AR KO, fb-KO mice display a markedly reduced cocaine-induced c-Fos expression mainly in the MSN of the direct but also of the indirect pathway. Together with the finding that the deletion of A2AR in glutamatergic terminals in fb-A2AR KO abolished the A2AR-mediated enhancement of glutamate release, these results suggest that presynaptic A2AR control glutamate release, affecting the activity of both the direct and indirect pathways (with c-Fos expression as a marker for neuronal activity). This indicates that forebrain A2AR exert their control of cocaine action predominantly through the regulation of glutamate release, which challenges previous views attributing those actions to the control of the responsiveness of striatal GABAergic neurons. The most intriguing aspect of A2AR function in glutamatergic terminals is their ability to over-ride the effect of A2AR in striatopallidal neurons, which have a nearly 20-fold higher A2AR density [3]. This preferential engagement of A2AR in glutamatergic terminals is heralded by the observations that psychostimulants [23,24,25,26] as well as NMDA receptor activation [27,28] can enhance the local striatal extracellular levels of adenosine, preferentially near glutamatergic but not GABAergic terminals [25]. Thus, the pattern of generation of adenosine by psychostim-

Figure 5. Immunofluorescence double staining of c-Fos and enkephalin in forebrain-WT and forebrain-A2AR KO mice after saline or cocaine treatment. (A) Representative merged images of immunofluorescence double staining of c-Fos (red) with enkephalin (Enk, green) in cocaine- vs. saline-treated fb-A2AR KO and fb-WT mice. (B) Quantitative analysis demonstrating the percentage of total c-Fos positive [c-Fos(+) ] cells out of the total cells. (C) Quantitative analysis showing the percentage of c-Fos and Enk double positive [c-Fos(+)Enk(+)] stained cells out of the total cells. (D) Quantitative analysis demonstrating the percentage of c-Fos positive but Enk negative [c-Fos(+)Enk(−)] cells out of the total cells. Data in the bar graphs are mean ± SEM, n = 6-10 per group. ∗ p < 0.05, vs. groups of same genotype with saline treatment; # p < 0.05 vs. cocaine-treated WT groups. ∗ p < 0.05, vs. saline-treated fb-A2AR group. Scale bar = 50 μm. Yellow arrows indicate neurons with c-Fos positive but Enk negative [c-Fos(+)Enk(−)] staining; Red arrows indicate neurons with c-Fos and Enk double positive [c-Fos(+)Enk(+) ] staining.
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Discussion

A2AR in glutamatergic corticostriatal terminals modulate psychomotor activity

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A2AR in glutamatergic terminals and GABAergic neurons of the indirect pathway differentially modulate the action of psychostimulants through opposite control of DARPP-32 phosphorylation

Our current findings mechanistically dissociate the role of A2AR in glutamatergic terminals and in GABAergic neurons controlling DARPP-32 phosphorylation in the indirect pathway. Strikingly, the two different subsets of A2AR modulate the actions of psychostimulants via DARPP-32 phosphorylation in an opposite manner. In parallel with enhanced cocaine-induced c-Fos expression and psychomotor activity, the selective inactivation of A2AR in striatal GABAergic neurons (in st-A2AR KO mice) reduced DARPP-32 phosphorylation at Thr-34 and enhanced DARPP-32 phosphorylation at Thr-75. This modulation is consistent with a direct effect of A2AR on GABAergic striatopallidal neurons, since the inactivation of A2AR in GABAergic neurons reduces protein kinase A activity and in turn reduces DARPP-32 phosphorylation at Thr-34, with a parallel increase of DARPP-32 phosphorylation at Thr-75 [19,29]. Furthermore, the increase of DARPP-32 phosphorylation at Thr-75 in fb-A2AR KO mice is best explained by the selective changes of DARPP-32 in the indirect pathway since the attenuation of cocaine-psychomotor activity is strongly correlated with enhanced DARPP-32 in the indirect pathway (not the direct pathway) as clearly demonstrated by the elegant work using cell-type specific DARPP-32 KO [15,16]. Our findings are also in line with the concept that the striatopallidal pathway exerts a general inhibitory effect on behavior such as instrumental learning [30], psychostimulant activity [9], and aversive behavior [31], as revealed by selective destruction of the indirect pathway using targeted toxin expression [32] and by optogenetic silencing [33,34].

However, the A2AR control of c-Fos expression in the striatum seems to result mainly from the c-Fos response in the direct pathway since we now demonstrated that cocaine-induced c-Fos expression was detected mainly in dynorphin-positive neurons. This effect could either result from recurrent collateral connections between striatopallidal and striatonigral MSN [35] or from an enhanced D2R-mediated release of endocannabinoids, which would decrease glutamate release from corticostriatal terminals projecting to both the indirect as well as the direct pathway [36]. This also explains the ability of A2AR to control D1R-mediated responses such as rotational behavior [37,38], c-Fos expression in striatopallidal neurons [39] and DARPP-32 phosphorylation [29,40]. In addition, the c-Fos expression may also be a secondary functional consequence of the enhanced psychomotor activity by selective deletion of A2AR in the indirect pathway. Although only derived from the use of a single dose of cocaine at single time point, the present findings nonetheless provide an important snapshot of the A2AR modulation of cocaine-induced molecular responses at the level of DARPP-32 phosphorylation and c-Fos expression in the striatum.

The additional elimination of A2AR from glutamatergic terminals in fb-A2AR KO mice reduced the basal Thr-75 phosphorylation of DARPP-32 and caused an enhancement of cocaine-induced Thr-34 phosphorylation of DARPP-32, in contrast to our findings in st-A2AR KO mice. This suggests that tonic activation of A2AR in glutamatergic corticostriatal terminals exerts opposite effects (compared to A2AR in GABAergic striatopallidal neurons) on striatal DARPP-32 phosphorylation. Since the major biochemical and neurochemical differences between fb-A2AR KO and st-A2AR KO mice is the deletion of A2AR in glutamatergic terminals (Figure 1) and the consequent abolition of A2AR-facilitated glutamate release from striatal nerve terminals (Figure 2), the different regulation of DARPP-32 phosphorylation by A2AR in fb-A2AR KO mice likely results either from the impact of presynaptic A2AR on glutamate release alone or from the combined effect of presynaptic A2AR and postsynaptic A2AR actions, an issue that will require the use of selective deletions of A2AR in presynaptic glutamatergic corticostriatal terminals to be resolved. In fact, we are concluding that the differences between the phenotypes of fb-A2AR KO and st-A2AR KO mice are mostly due to the effects of presynaptic A2AR in glutamatergic corticostriatal terminals since the most evident differentiating factor in fb-A2AR KO mice is the deletion of presynaptic A2AR and the abolishment of A2AR-mediated facilitation of glutamate release. Since increased DARPP-32 phosphorylation at Thr-34 in the direct pathway is expected to produce enhanced cocaine psychomotor activity [15,17], the increased DARPP-32 phosphorylation at Thr-34, together with the attenuation of cocaine-induced psychomotor activity in fb-A2AR KO mice strongly suggests that glutamate release by A2AR in corticostriatal terminals preferentially affects DARPP-32 phosphorylation in the indirect pathway. Conversely, fb-A2AR KO mice display an altered c-Fos expression in the direct and indirect pathways with the direct pathway being prominent one. Overall, the molecular and behavioral responses found in fb-A2AR KO mice suggest a selective modification of DARPP-32 phosphorylation in the indirect pathway and a prominent modification of cocaine-induced c-Fos expression in the direct pathway in tight correlation with cocaine-induced psychomotor activity. This is in line with the findings from cell-type specific deletion of DARPP-32, which showed that cocaine-induced psychomotor activity was attenuated by selective inactivation of DARPP-32 in the indirect pathway [15]. While these results suggest that A2AR activity in glutamatergic terminals and GABAergic neurons may influence the action of psychostimulants by controlling DARPP-32 phosphorylation selectively in the indirect pathway, with the c-Fos response being secondary to the psychomotor effect, additional experiments are clearly warranted to clarify the cellular substrate linking the presynaptic A2AR control of glutamate release and its impact on psychomotor activity.

Neurobiological and therapeutic implications

Based on the opposite phenotypes of cocaine-induced molecular and behavioral changes in st-A2AR KO and fb-A2AR KO mice, and their association with glutamatergic, GABAergic and dopaminergic systems at presynaptic and postsynaptic sites, we propose a new model for A2AR function in the control of striatal circuits: A2AR in glutamatergic terminals and GABAergic neurons provide a “fine-tuning” mechanism, whereby they integrate and regulate dopaminergic and glutamatergic signaling in the striatum. The integrated function of A2AR is accomplished through the opposing actions of A2AR in GABAergic striatal neurons (through A2AR-D2R antagonistic interactions) and in glutamatergic corticostriatal terminals (by modulating glutamate release). The novelty of this model is that the “fine-tuning” provided by A2AR may serve to prevent over- or under-stimulation of striatal neurons, and illustrates an essential aspect of the integrated function of the adenosine neuromodulation system [41]. Since decreased glutamatergic neurotransmission and increased dopaminergic activity contribute to the pathophysiology of schizophrenia and related psychiatric disorders, the ability of A2AR to integrate dopaminergic and glutamatergic systems indicates that adenosine acting at
A2AR may modulate both positive (by preventing hyper-dopaminergic activity) and negative (by preventing hypo-glutamatergic activity) symptoms of schizophrenia [42]. Thus, the selective manipulation of presynaptic A2AR in glutamatergic terminals [43] may have a therapeutic value to manage a variety of neuropsychiatric behaviors such as anxiety, depression, psychosis and schizophrenia [44].

Materials and Methods

1. Generation and genotyping of striatum-A2AR KO mice and forebrain-A2AR KO mice

Animals were handled according to the NIH Guide for the Care and Use of Laboratory Animals and in accordance with the protocol approved by the IACUC at the Boston University School of Medicine and by the Faculty of Medicine of the University of Coimbra. The Cre-loxP strategy was used to generate fb-A2AR KO and st-A2AR KO mice. The generation and genotyping of fb-A2AR KO mice has been described recently [10]. Briefly, transgenic mice expressing the Cre recombinase under control of the CaMKIIα gene promoter were crossbred with homozygous floxed A2ARflox/+ mice (F10 generation in congenic C57BL/6 background). Their Cre (+) A2ARflox/+ offspring display an A2AR deletion in postnatal forebrain neurons (including cortex, hippocampus and striatum). Similarly, homozygous floxed A2ARflox/+ mice (F5 generation in mixed 129-Steel and C57BL/6 background) were crossed with Dlx5/6-Cre transgenic mice expressing Cre recombinase under control of the Dlx5/6 gene promoter, which is active exclusively in striatal neurons during development [45,46,47], to generate st-A2AR KO mice [Dlx5/6-Cre(+);A2ARflox/+;] mice [9]. Genotyping was conducted by 3 primer PCR analysis of tail DNA [10]. Fb-A2AR KO and st-A2AR KO mice were characterized for their selective Adora2a deletion in the forebrain (i.e., cortex, hippocampus and striatum) [10,48] or exclusively in striatal [9] neurons, as shown in our previous studies. The selectivity in the two lines was further validated by Cre-expression by X-gal staining of LacZ in a Rosa26 reporter transgenic line, PCR analysis of Cre-mediated Adora2a deletion, A2AR immunohistochemistry and 3H-ZM241385 radioligand binding of A2AR density [9,10,48,49]. Our early studies showed that the behaviors of two WT genotypes [Cre(-);A2ARflox/+; or Cre(+);A2ARflox/+;] were not distinguishable (data not shown) and so we used either WT or in some cases two WT types were pooled in to one group referred to as simply st-WT or fb-WT, accordingly.

2. Drug treatments and psychomotor activity assessments

Before drug treatment, all mice were habituated in the testing environment and mice were injected with a single dose of cocaine (25 mg/kg, i.p.; Sigma, St. Louis, MO, USA). Horizontal locomotor activity was monitored for 180 min after drug administration and analyzed as described previously [9].

3. Glutamate release from striatal synaptosomes

3H-glutamate release experiments were performed as previously described after purification of striatal nerve terminals using a sucrose/Percoll fractionation method [22]. Briefly, nerve terminals were equilibrated at 37°C for 10 min, loaded with 3H-glutamate (0.2 μM, specific activity of 45 Ci/mmol, Amersham, Piscataway, NJ, USA) for 3 min at 37°C, washed, layered over Whatman GF/C filters and superfused with oxygenated Krebs solution for 20 min before starting collection of the superfusate. Synaptosomes were stimulated with 20 mM K+ at 3 min (S1) and 9 min (S2) after starting sample collection, triggering a release of tritium that was mostly 3H-glutamate, released in a Ca2+-dependent manner [22]. The A2AR agonist CGS21680 (Tociris, Bristol, UK), tested at a concentration that is supra-maximal but selective to activate A2AR [22], was added 2 min before S2 onwards and its effect was quantified by modification of the S2/S1 ratio compared to control chambers. Normalized facilitation by CGS21680 of the K+ -evoked 3H-glutamate release was tested by the one-sample t-test against the hypothetical value of 0% compared to paired control experiments carried out in the same batch of nerve terminals in the absence of added drugs. P ≤ 0.05 was considered to represent a significant difference.

4. Immunocytochemical detection of A2AR in glutamatergic and GABAergic nerve terminals

Striatal nerve terminals were purified through a discontinuous Percoll gradient and plated over poly-L-lysine-coated cover-slips for immunocytochemical analysis, using antibodies that were previously validated [22,50]. Permeabilized nerve terminals were incubated for 1 h with rabbit anti-A2AR (1:500, Upstate Biotechnology, Lake Placid, NY, USA), and guinea pig anti-vesicular GABA transporters (vGAT, 1:1,000, Calbiochem, San Diego, CA, USA) or guinea pig anti-vesicular glutamate type 1 transporters (vGlut1, 1:1,000, Chemicon, Temecula, CA, USA) antibodies followed by a 1 h incubation with different AlexaFluor-labeled secondary antibodies (1:2,000, Molecular Probes, Leiden, The Netherlands), which did not yield any signal in the absence of the corresponding primary antibodies. After washing and mounting onto slides with Prolong Gold Antifading (Invitrogen, Eugene, OR, USA), preparations were visualized in a Zeiss fluorescence microscope and analyzed with MetaFluor 5.0. Each coverslip was analyzed by counting three different fields and in each field a total amount of 150 individualized elements excluding elements based on their insufficient or excessive pixel intensity and excessive size, as previously described [22,50]. Note that this approach can only globally distinguish glutamatergic from GABAergic terminals, but the anti-vGlut1 and anti-vGAT antibodies used cannot distinguish between the different types of glutamatergic terminals (projecting to the direct or indirect pathways) or GABAergic terminals (direct projections or collaterals).

5. Western blot analysis of DARPP-32 phosphorylation at Thr-32 and Thr-75

DARPP-32 immunoreactivity was analyzed as previously described [51] with modifications. Mice were sacrificed by decapitation (45 min after i.p. injection of vehicle or drug) and then heads were immediately immersed in liquid nitrogen for 6 sec. The striata were rapidly (within 20 sec) dissected out on an ice-cold surface, sonicated in 750 μL of 2% sodium dodecylsulfate, and boiled for 10 min. After protein determination, 30 μg protein from each sample was loaded and separated by Western blot to quantify phosphorylated DARPP-32 (Thr32) (1:1,000, antibody kindly provided by Dr. Greengard) and phosphorylated DARPP-32 (Thr75) (1:1,000, Cell Signaling, Danvers, MA, USA), normalized to total DARPP-32 immunoreactivity (1:1,000, Cell Signaling).

6. Immunohistochemistry of c-Fos expression and double labeling of c-Fos with dynorphin or enkephalin

Sequential antibody detection of c-Fos and dynorphin. Free-floating brain coronal sections (30 μm) were double stained immunohistochemically with anti-c-Fos and anti-dynorphin polyclonal antibodies using standard avidin–biotin procedures follow-
ing a sequential antibody detection protocol as described previously [52,53]. For this procedure, the first antibody, i.e., a goat anti-dynorphin polyclonal antibody (1:200, sc-46313, Santa Cruz, CA, USA) was detected first, using immunoperoxidase staining enhanced with 0.00% nickel ammonium sulfate, which yields a dark grayish color. After completion of the first staining, the same sections were incubated with an avidin/biotin blocking solution in order to block free avidin/biotin sites from the first biotinylated goat anti-rabbit IgG antibody. Then, sections were processed for immunolabeling with the second primary antibody, i.e., a rabbit anti-c-Fos polyclonal antibody (1:5,000, PC-38, Calbiochem) following standard protocols using DAB, yielding a bright brown color. This method has been repeatedly shown to lack cross-labeling [52,53]. Moreover, the nuclear localization of c-Fos staining, as opposed to the cytoplasm/neuropil staining of dynorphin, makes it easy to differentiate the two types of staining.

Fluorescence double immunohistochemistry of c-Fos and enkephalin. Coronal brain sections (30 μm) were double stained overnight with primary antibodies, namely rabbit anti-c-Fos polyclonal antibody (1:5,000, PC-38, Calbiochem) and mouse anti-enkephalin monoclonal antibody (1:50, sc-47705, Santa Cruz). After washing, slices were incubated for 1 h at room temperature in a solution containing a goat anti-rabbit secondary antibody, conjugated to Cy3 (1:750, 111-165-144, Jackson Immuno Research, West Grove, PA, USA) and a goat anti-mouse secondary antibody, conjugated to FITC (1:200, 115-095-166, Jackson Immuno Research). The sections were then washed 3 times and mounted on gelatin-coated slides and cover slipped with Vectashield fluorescent mounting medium with DAPI (H-1200, Vector Lab, Burlingame, CA, USA).

7. Statistical analysis

Statistical comparisons between st-A2AR KO vs st-WT or ib- A2AR KO vs ib-WT were analyzed (independently for their different genetic backgrounds) using a paired or unpaired Student’s t test, according to the experimental design. To determine the effect of genotype, drug treatment and their interaction, we applied a two-way ANOVA for repeated measurements followed by Bonferroni post hoc comparison.

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Author Contributions

Conceived and designed the experiments: JFC RAC RM. Performed the experiments: HYS PC PG JQL. Analyzed the data: HYS PC PG. Contributed reagents/materials/analysis tools: JFC RAC RM DB. Wrote the paper: HYS JFC RAC DB.

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