Neuroprotection by caffeine in the MPTP model of Parkinson's disease and its dependence on adenosine A_{2A} receptors

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Abstract

Considerable epidemiological and laboratory data have suggested that caffeine, a nonselective adenosine receptor antagonist, may protect against the underlying neurodegeneration of Parkinson's disease (PD). Although both caffeine and more specific antagonists of the A_{2A} subtype of adenosine receptor (A_{2A}R) have been found to confer protection in animal models of PD, the dependence of caffeine's neuroprotective effects on the A_{2A}R is not known. To definitively determine its A_{2A}R dependence, the effect of caffeine on MPTP neurotoxicity was compared in wild-type (WT) and A_{2A}R gene global knockout (A_{2A} KO) mice, as well as in CNS cell type-specific (conditional) A_{2A}R knockout (cKO) mice that lack the receptor either in postnatal forebrain neurons or in astrocytes. In WT and in heterozygous A_{2A}R KO mice caffeine pretreatment (25 mg/kg ip) significantly attenuated MPTP-induced depletion of striatal dopamine. By contrast in homozygous A_{2A}R global KO mice caffeine had no effect on MPTP toxicity. In forebrain neuron A_{2A}R cKO mice, caffeine lost its locomotor stimulant effect, whereas its neuroprotective effect was mostly preserved. In astrocytic A_{2A}R cKO mice, both caffeine's locomotor stimulant and protective properties were undiminished. Taken together, these results indicate that neuroprotection by caffeine in the MPTP model of PD relies on the A_{2A}R, although the specific cellular localization of these receptors remains to be determined.

Key-words

- Adenosine A_{2A} receptors;
- MPTP;
- Caffeine;
- Neuroprotection;
- Parkinson's disease;

Parkinson's disease (PD) is a neurodegenerative disorder characterized by a progressive loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc). The neuropathological signs of PD occur long before any substantive clinical symptoms appear and it is estimated that at the time of symptom onset there may be 60–80% loss of striatal dopamine (Bernheimer et al., 1973). Like idiopathic PD, parkinsonism induced by acute exposure to the dopaminergic neuron protoxin 1-methyl-4-phenyl-1,2,3,6 tetra-hydropyridine (MPTP) results from degeneration of nigrostriatal dopaminergic neurons and associated loss of striatal dopamine (Langston et al., 1984; Ricaurte et al., 1987). Most of the biochemical, pathological and clinical features that occur following a substantial lesion from MPTP treatment in animal models resemble symptoms observed after losing 80% of total striatal dopamine (Langston et al., 1984; Kopin and Markey, 1988; Jackson-Lewis et al., 2007). Despite some limitations (e.g., the lack of Lewy body-like inclusions or reliable behavioral deficits characteristic of PD), acute MPTP intoxication remains one of the best-characterized animal models of PD and recapitulates neurochemical and anatomical features of the disease (Dawson et al., 2002; Jackson-Lewis et al., 2007).

Caffeine is the most consumed psychoactive drug in the world and, like classical psychostimulants, produces behavioral effects such as increased motor activation, arousal, and reinforcement. Multiple epidemiological studies have also shown that people who consume more caffeinated beverages are substantially less likely to develop PD (Ross et al., 2000, Ascherio et al., 2001, 2004). Although caffeinated coffee, tea and soda comprise many chemical constituents, the finding that regular but not decaffeinated coffee consumption is predictive of reduced PD risk (Ascherio et al., 2001; Palacios et al., 2012) implicates caffeine as the basis of the inverse

association. However, epidemiological studies do not directly address causality and it remains unknown whether caffeine protects against the neurodegeneration underlying PD.

Laboratory studies have supported a true neuroprotective effect of caffeine in PD by demonstrating its biological plausibility in multiple animal models of the disease. When caffeine is co-administered with MPTP to mice at doses comparable with those of typical human exposure, it dose-dependently attenuates the loss of striatal dopamine triggered by MPTP using different exposure paradigms and in several mouse strains (Chen et al., 2001; Xu et al., 2002). It is shown that caffeine can be given up to two hours before or after MPTP and still confer protection against MPTP-induced dopamine loss. Moreover, caffeine's metabolites, paraxanthine and theophylline, also provide protection against MPTP neurotoxicity (Xu et al., 2010). Other preclinical studies have shown that caffeine protects against dopaminergic neuron degeneration, dopamine loss and/or associated behavioral changes induced by 6-hydroxydopamine (6-OHDA) in rats (Joghataie et al., 2004; Aguiar et al., 2006; Kelsey et al., 2009) and by the pesticide combination of paraquat and maneb in mice (Kachroo et al., 2010). Interestingly, the neuroprotective effect of caffeine could be dissociated from its psychomotor stimulant properties (Xu et al., 2002; Yu et al., 2008); whereas the latter showed tolerance to repeated administration, under the same conditions caffeine's neuroprotective action persisted unabated (Xu et al., 2002).

Although the convergence of caffeine's clinical correlations and protective effects in animal models supports a true beneficial effect in reducing PD risk, the mechanisms underlying neuroprotection by caffeine remain a matter of debate. Pharmacological studies indicate that its CNS effects are mediated primarily by its antagonistic actions at the A₁ and A_{2A} subtypes of

adenosine receptors (Fredholm and Persson, 1982; Nehlig et al., 1992; Fredholm and Lindström, 1999; Fisone et al., 2004; Ferré et al., 2008). Interestingly, A_{2A}R blockade, but not A₁R blockade mimics caffeine's protective effects in several experimental models of PD (Schwarzschild et al., 2006). In rodents, selective A_{2A}R antagonists attenuate the loss of dopaminergic neurons in the SNpc or dopamine depletion in the striatum induced by either systemic administration of MPTP or acute infusion of 6-OHDA in the medial forebrain bundle (Chen et al., 2001; Ikeda et al., 2002; Pierri et al., 2005). A critical role of the A_{2A}R in the MPTP model of PD was confirmed by the phenotype of global A_{2A}R KO mice, which show preserved striatal dopamine content after acute MPTP multiple dose administration (Chen et al., 2001).

In order to determine whether protection by caffeine in fact requires the A_{2A}R we investigated the effect of A_{2A}R depletion on the ability of caffeine to protect against MPTP toxicity in A_{2A}R KO and littermate control mice. In addition to a global (constitutive) A_{2A}R KO line we employed conditional (Cre-*loxP* system) KO mice with cell-specific disruption of the A_{2A}R gene either in astrocytes or (cortical and striatal) neurons based on transgenic *cre* expression driven by *GFAP* (Glial fibrillary acidic protein) or *CamKII α* promoters, respectively. Our findings demonstrate an A_{2A}R-dependent mechanism by which caffeine protects against MPTP neurotoxicity. They also show that astrocytic A_{2A}Rs are not required and forebrain neuronal A_{2A}Rs cannot fully account for caffeine's neuroprotective effect in this model of PD.

Experimental procedures

Transgenic animals

Breeding and characterization of global A_{2A}R knockout mice (A_{2A} KO), CaMKII α gene promoter-driven forebrain neuron A_{2A}R cKO mice (CaMKII α -A_{2A}R KO) as well as the GFAP gene promoter-driven astrocyte A_{2A}R cKO (gfap-A_{2A}R KO) mice have been described previously (Chen et al., 1999, 2002; Bastia et al., 2005; Yu et al., 2008; Matos et al., 2015). Briefly, chimeric A_{2A}R KO mice (F0) that were derived from 129-Steel embryonic stem cells were bred to C57BL/6 mice, resulting in mice of mixed C57BL/6 x 129-Steel background. The mixed line was then repeatedly backcrossed to pure C57BL/6 mice over 10 generations, yielding an A_{2A}R KO line congenic for the C57BL/6 background. A_{2A}R KO, heterozygous and wild-type (WT) littermates (male, 4-14 months old) were used in the global KO study. Both fb-A_{2A}R KO and astro-A_{2A}R KO mice were generated using a Cre/*loxP* strategy. Cre recombinase gene expression was controlled by either the forebrain (cortical and striatal) neuron-specific *CaMKII- α* promoter (Bastia et al., 2005) or astrocyte-specific GFAP gene promoter, as previously described elsewhere (Bajenaru et al., 2002). Both transgenic *cre* mice and “floxed” A_{2A}R gene (A_{2A}^{*flox/flox*}) mice were backcrossed for 10–12 generations to C57BL/6 mice (Charles River; Wilmington, MA). Homozygous floxed (A_{2A}^{*flox/flox*}) mice were crossed with *cre*(+) mice, and female *cre*(+) (A_{2A}^{*flox/+*}) offspring were then crossed with A_{2A}^{*flox/+*} males. Their *cre*(+) A_{2A}^{*flox/flox*} and *cre*(-) A_{2A}^{*flox/flox*} offspring (male and females, 2-9 months old) were used in this study. Mice were housed five in each cage in temperature- and humidity-controlled rooms with a 12-h dark:light cycle and had free access to food and water. All experiments were conducted in accordance with Massachusetts General Hospital and NIH Guidelines on the ethical use of animals.

Drug treatment

In all experiments involving MPTP, a single injection of caffeine (25 mg/kg dissolved in saline) or saline was administered 10 min before a single intraperitoneal injection of MPTP (35mg/kg, HCl salt of MPTP dissolved in saline; corresponding to a MPTP•HCl dose of 29 mg/kg).

Locomotor activity

Horizontal locomotor activity was assessed in standard polypropylene cages (15 × 25 cm) placed into frames equipped to generate and detect five evenly spaced, parallel infrared light beams across the width of each cage just above its floor (San Diego Instruments, San Diego). Mice were habituated for more than 12 hours overnight in the testing cages that were placed in a behavioral suite. Basal spontaneous locomotion was recorded for at least 180 min. Then locomotor behavior was monitored during the light phase for another 240 min after caffeine (25 mg/kg or saline) ip injection. Locomotion, scored as the number of adjacent photobeam breaks (ambulation), was determined as described before (Xu et al., 2002).

Measurement of dopamine

One week after MPTP treatment, mice were killed by rapid cervical dislocation. The striatum was dissected out from the right cerebral hemisphere, frozen on dry ice and stored at –80°C until use. Each striatum was weighed, homogenized with 150 mM phosphoric acid and 0.2 mM EDTA and centrifuged at 12,000 *g* for 15 min at 4°C. Supernatants were analyzed for dopamine content using standard reverse-phase HPLC with electrochemical detection, according

to our previously published protocol (Chen et al., 2001; Xu, et al., 2006). The dopamine content was calculated as picomoles per milligram of tissue, and these values are presented within the figures as percentage of change from respective saline–saline-treated controls.

Statistics

All values are expressed as mean $\pm$ SEM. Differences among means in dopamine content after MPTP treatment as well as peak locomotion were analyzed using two-way ANOVA with treatment (saline or caffeine) and genotype as independent factors. When ANOVA showed significant differences, pair-wise comparisons between means were tested by Fisher's Least Significant Difference (LSD) post hoc testing. In all analyses, the null hypothesis was rejected at the 0.05 level. The analysis was generated using GraphPad.

Results

1. Caffeine's protection against MPTP-induced dopamine depletion is lost in A_{2A}R global KO mice.

We first evaluated the effect of global genetic deletion of A_{2A}R on caffeine's neuroprotection against MPTP-induced dopamine depletion. MPTP (35 mg/kg ip single injection) significantly depleted striatal dopamine content measured one week later in WT, heterozygous and global A_{2A}R KO mice. As reported previously (Xu et al. 2006), caffeine (25 mg/kg single ip injection 10 minutes before MPTP) significantly attenuated MPTP-induced dopamine depletion in WT mice. Similarly, caffeine also significantly attenuated MPTP-induced dopamine loss in heterozygous mice. However, caffeine pretreatment did not attenuated MPTP-induced dopamine loss in global A_{2A}R KO mice (Figure 1). Although the global A_{2A}R KO can

have protective phenotype in multi-dose MPTP toxicity paradigms (Chen et al, 2001; Yu et al, 2008) no appreciable effect of the KO itself was observed here using a single MPTP injection paradigm (69% vs 75% loss of striatal dopamine induced by MPTP in A_{2A}R KO vs WT mice, respectively; $p>0.05$).

2. Caffeine's protection against MPTP-induced dopamine loss persists, although its motor stimulant effect is lost in forebrain neuronal A_{2A} KO mice.

We next examined the contribution of the A_{2A}R in forebrain neurons to the neuroprotection by caffeine in MPTP-induced neurotoxicity. MPTP induced significant and similar dopamine loss in fb-A_{2A}R KO mice and their non-transgenic littermate controls. In contrast to our findings with the global A_{2A}R KO, caffeine pretreatment significantly attenuated MPTP-induced dopamine depletion in both fb-A_{2A}R KO and control mice (Figure 2. The amount of residual dopamine with caffeine pretreatment was ~180% greater than without caffeine in control mice, whereas it was only ~64% greater in fb-A_{2A}R KO mice ($p=0.052$, *t*-test; comparing the 'Caffeine + MPTP' mice without and with the *cre* transgene as shown in Figure 2). These data would suggest that caffeine's attenuation of MPTP-induced striatal dopamine loss is at least partially independent of forebrain neuronal A_{2A}R, though it may contribute partially to caffeine's protective effect. In contrast to what was found for caffeine's neuroprotective effect, caffeine stimulated locomotor activities in fb-A_{2A}R WT but not fb-A_{2A}R KO mice (Figures 3).

3. Caffeine's neuroprotective and motor stimulant effects are unaltered in astrocyte-specific A_{2A}R KO mice.

Finally, we investigated the roles of A_{2A}R in astrocytes in caffeine's neuroprotection and motor stimulant effect. MPTP induced significant striatal dopamine loss in gfap-A_{2A}R KO and their WT littermates. Caffeine pre-treatment significantly and similarly attenuated MPTP-induced dopamine loss in both gfap-A_{2A}R WT and gfap-A_{2A}R KO mice (Figure 4). We also studied caffeine's motor stimulant effect in these gfap-A_{2A}R KO and their WT littermates. In contrast to what was found in fb-A_{2A}R KO mice, caffeine treatment stimulated locomotion in both gfap-A_{2A}R KO mice to the same extent as in their matched controls (Figure 5).

Discussion

The main finding of our study is the demonstration that caffeine's neuroprotection depends on adenosine A_{2A}R. Although the use of a global A_{2A}R KO helps establish an essential role of this receptor in caffeine's neuroprotective effect, constitutive KO methodology has limitations such as its inability to distinguish between a role for the receptor during development or adulthood (Bockamp et al., 2002). Our results also suggest that caffeine's protective effect is mostly dependent on A_{2A}R other than those located on forebrain (striatal and cortical) neurons and on astrocytes.

Adenosine receptor antagonism has been shown to be the main mechanism of action responsible for the CNS effects of caffeine. Several studies have indicated that caffeine exerts its psychostimulant effects acting as a nonselective adenosine A₁R and A_{2A}R receptor antagonist

(Fredholm and Persson, 1982; Nehlig et al., 1992; Fredholm and Lindström, 1999; Fisone et al., 2004; Ferré, 2008). Interestingly, the expression of these two adenosine receptors in the brain display very different patterns: whereas A₁R are present throughout the brain, the expression of the A_{2A}R is largely restricted to the striatum. Although brain A_{2A}R were initially thought to be exclusively located in this region, several studies provided evidence also for the presence of A_{2A}R in the hippocampus and neo-cortex (Rebola et al., 2005; reviewed in Cunha et al., 2001) at a lower density (Lopes et al., 2004).

Adenosine A_{2A}R in the striatum act on the striatopallidal pathway to control locomotor activity, whereas the sparser extra-striatal A_{2A}R may serve other functions such as facilitation of neurotransmitter release and modulation of neurodegeneration. In addition, compared to the predominant postsynaptic role, there may be a modulation of the pre-synaptic terminals by facilitating the evoked release of neurotransmitters (reviewed in Cunha et al., 2001). Moreover, several studies have focused on the importance of forebrain A_{2A}R for PD neuroprotection (Carta et al., 2009) and possible extra-neuronal protective effects of A_{2A}R antagonist (Yu et al., 2008).

Previous reports have suggested that adenosine may contribute to the pathological changes of Parkinson's disease by triggering the activation of surrounding glial cells (Hirsch et al. 1999). A_{2A}R activation is in fact partially responsible for cerebral inflammation and excitotoxicity (Popoli et al. 1995; Okusa et al. 1999; Sullivan et al. 1999) and A_{2A}Rs located on glial cells might play a role in neuroprotection mediated by A_{2A}R antagonists against acute (Yu et al., 2008) and chronic MPTP-induced striatal dopamine depletion (Sonsalla et al., 2012) MPTP-induced striatal dopamine depletion.

Whereas several studies have reported a clear role of A₁R in the behavioral effects of acutely administered caffeine with involvement of A_{2A}R preferentially under conditions of chronic caffeine treatment (Karcz-Kubicha et al., 2003; Antoniou et al., 2005; Orrúet al., 2013), we have confirmed (Figure 3) that caffeine's acute locomotor stimulant effect also requires the A_{2A}R, specifically those expressed in forebrain (e.g., striatal) neurons (Yu et al, 2008).

Although the basis of caffeine's neuroprotective properties appear to be in some ways distinct from those of its behavioral actions (Xu et al, 2002; Yu et al, 2008), we have now provided definitive evidence that caffeine's ability to protect dopaminergic neurons can be entirely dependent on the A_{2A}R, as previous suggested by other studies. For example, selective A_{2A}R antagonists mimicked the protective effects of caffeine by reducing both MPTP (Chen et al., 2001) and 6-OHDA (Ikeda et al., 2002) induced neurotoxicity, mimicking the protective effects of caffeine. The findings suggest that caffeine's protective effects, which can be mimicked by A_{2A}R depletion (Chen et al. 1999; Li et al, 2009), are in fact mediated by the A_{2A}R, as described in other neurotoxicity models like those for stroke (Fredholm et al. 1996) and traumatic brain injury (Li et al, 2008).

While our main results are generally in agreement with the findings of Chen et al. in 2001 and Yu et al. in 2008, the depletion of A_{2A}R in the global A_{2A}R KO mice in the current study was not sufficient to attenuate MPTP neurotoxicity in contrast to the earlier studies. This apparent discrepancy could be related to the different MPTP•HCl doses and dosing paradigms used across these experiments (a single injection of 35 mg/kg ip here compared to multiple injections of 20

mg/kg ip hours apart in the earlier studies). Differences in MPTP dose amounts and timing are known to induce different patterns of neuronal death. (e.g., apoptotic vs necrotic; Meredith et al., 2011), and thus potentially different dependencies on the A_{2A}R. Interestingly, recent evidence that caffeine may produce some CNS effects through inverse agonism (rather than competitive antagonism of endogenous adenosine) at the A_{2A}R (Fernández-Dueñas et al., 2014) suggests a possible explanation for how caffeine's protective effect could be A_{2A}R-dependent, without A_{2A}R depletion having an effect of its own, in the MPTP paradigm employed in our study.

Selective deletion of A_{2A}R in forebrain neurons showed that caffeine's attenuation of MPTP-induced striatal dopamine loss is at least partially independent of forebrain neuronal A_{2A}Rs, although locomotion appeared fully dependent upon them. These results are in agreement with previous studies that suggested that selective A_{2A}R antagonists (KW-6002) did not require forebrain neurons to protect against MPTP neurotoxicity (Yu et al., 2008). Extra-striatal A_{2A}R, characterized in Bastia *et al.* 2005, have been shown to have a critical role in providing a prominent effect on psychomotor activity induced by amphetamine, cocaine and phencyclidine (Bastia et al., 2005; Shen et al., 2008) and a recent study has also considered this extra-striatal A_{2A}R to have a fundamental role of in PD neuroprotection (Carta et al., 2009).

In our study, selective depletion of fb-A_{2A}R did not abolish the effect of caffeine on MPTP-induced dopamine loss, suggesting that caffeine neuroprotection on acute MPTP toxicity does not fully depend on A_{2A}R in forebrain neurons. On the other hand, we found out that caffeine's locomotor activating properties are still entirely dependent upon the presence of fb-A_{2A}R,

suggesting that caffeine stimulates motor activity through A_{2A}R in forebrain neurons, likely those in the striatum. In complete agreement with these results, the selective deletion of A_{2A}R in forebrain neurons abolished motor effects of A_{2A}R antagonist KW-6002 (Yu et al., 2008). They are, however, in contrast with a previous report on the selective deletion of A_{2A}R from forebrain neurons preventing dopaminergic neuron loss and gliosis in the SNpc following multiple MPTP injections (Carta et al., 2009). These discrepancies might involve other paradigm variables, such as different MPTP treatment paradigms as above, or different readouts for dopaminergic toxicity (TH-positive SNpc cells vs striatal dopamine content).

Astrocytic A_{2A}Rs also warranted consideration as candidate mediators of neuroprotection by caffeine. Brain glial cells may express A_{2A}Rs (Fiebich et al. 1996; Saura et al. 2005), and A_{2A}Rs on astrocytes may contribute to excitotoxic neurodegeneration (Nishizaki, 2004; Matos et al, 2012). However, using astrocyte-directed conditional A_{2A}R KO mice generated by a *Cre-loxP* system based on the specificity of GFAP gene promoter, we showed that the selective depletion of A_{2A}R from astrocytes affected neither caffeine's motor effect nor its protection against MPTP-induced neurotoxicity. Thus at least in the paradigms we employed, caffeine does not require astrocyte A_{2A}Rs to protect dopaminergic neurons or stimulate motor activity.

Other possible cellular sources of A_{2A}Rs should be considered in addition to striatal and cortical neurons and astrocytes. These include A_{2A}R-expressing microglial cells (Fiebich et al., 1996; Hasko et al., 2005) and oligodendrocytes (Stevens et al., 2002). Caffeine or selective A_{2A}R antagonists were shown to attenuate microglia recruitment to sites of injury and to reduce the production of pro-inflammatory cytokines (Brambilla et al., 2003; Brothers et al., 2010; Rebola

et al., 2011). Microglial activation during inflammatory processes observed in the brains of older rats is partially reversed by chronic caffeine (Brothers et al., 2010). Furthermore, activation of A_{2A}Rs has been associated with release of brain-derived neurotrophic factor (BDNF) and proliferation of microglial cells, which are intrinsically related events of neuroinflammation. There is also evidence that non-CNS cells expressing the A_{2A}R, such as bone marrow cells, may contribute to ischemic brain cell injury, as suggested by Yu et al, in 2004. In addition, it seems that these receptors in peripheral cells may be modulators of inflammatory cytokine production (Ran et al., 2015).

Conclusion

The finding of the complete loss of neuroprotection by caffeine in global A_{2A}R KO mice establishes the adenosine A_{2A}R as a critical mediator of caffeine's neuroprotective effects in the MPTP model of Parkinson's disease. Our conditional KO data suggest that caffeine's neuroprotection is at least partially independent of A_{2A}Rs in the forebrain neurons or astrocytes. The exact location of A_{2A}R that is responsible for caffeine's neuroprotection is still unknown. Understanding the neurobiological basis of caffeine's putative benefit is of increasing translational significance not only in explaining the epidemiological between caffeine use and reduced risk of PD (Liu et al, 2012, Ascherio et al., 2001, 2003). The findings also support therapeutic development of caffeine and more specific adenosine A_{2A}R antagonists as candidate disease-modifying agents, for example, with a long-term clinical trial of caffeine having been initiated in PD (<http://clinicaltrials.gov/show/NCT01738178>). Continued research is warranted to identify CNS A_{2A}Rs other than those residing on striatal and cortical neurons that may contribute

to neurodegeneration.

Acknowledgements:

Source of support: This work is supported by NIH grants 5R01ES010804 and 5K24NS60991 and DOD grant W81XWH-11-1-0150. The Parkinson's Disease Foundation has provided funding supporting to Daniel Garbin Di Luca as a summer research fellow.

Disclosure of potential conflicts of interest:

None reported.

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Figure Legends

Figure 1. Caffeine attenuated MPTP-induced striatal dopamine loss in WT and HZ, but not global A_{2A}R KO male mice. Saline or Caffeine (25 mg/kg ip) were administered 10min before saline or MPTP (35 mg/kg ip single injection). Dopamine content was determined one week after drug treatments. S, saline; M, MPTP; C, Caffeine. N=4 for saline treatments and N=5-14 for MPTP treatments. Bars represent striatal dopamine levels (mean $\pm$ SEM) calculated as percentage of their respective control (i.e., *saline + saline treatment* group). Statistically significant differences among the means of dopamine content in MPTP treated mice were determined by a two-way analysis of variance followed by Fisher's Least Significant Difference test. There is a significant difference in caffeine versus saline treated groups ($F[1,53]=7.11$, $p<0.05$). * $p<0.05$ and ** $p<0.01$ compared with respective S+M group.

Figure 2. Caffeine's attenuation of MPTP-induced striatal dopamine loss is at least partially independent of forebrain neuronal A_{2A}Rs. Saline or Caffeine (25 mg/kg ip) was administered 10 min before saline or MPTP (35 mg/kg ip single injection). Striatal dopamine was determined one week after drug treatments. N=3-7 for saline treatments and N=11-22 for MPTP treatments. Bars represent striatal dopamine levels (mean $\pm$ SEM) calculated as percentage of their respective control (i.e., *saline + saline treatment* group). Statistically significant differences among the means of dopamine content in MPTP treated mice were determined by a two-way analysis of variance followed by Fisher's Least Significant Difference test. There is a significant difference in caffeine versus saline treated groups ($F[1,60]=42.28$, $p<0.0001$). ** $p<0.01$, **** $p<0.0001$ compared with respective S+M group.

Figure 3. Dependence of caffeine-induced locomotion on neuronal A_{2A}Rs. Ambulation was scored as the number of adjacent photobeam breaks (mean $\pm$ SEM, N=8 for each group). Mice were habituated overnight and basal spontaneous locomotion was recorded for at least 180 min. Ambulation was recorded for another 240 min after caffeine (25 mg/kg ip) or saline injection. **A.** Locomotion after saline injection is similar in WT and forebrain neuron A_{2A}R cKO (*CaMKII α -cre*, *A_{2A}^{flx/flx}*) mice. **B.** Caffeine-stimulated locomotion is significantly

reduced in forebrain neuron A_{2A}R cKO mice compared to that of WT mice. **C.** Bars represent peak locomotion (mean $\pm$ SEM) 180 minutes after caffeine or saline injection. Statistically significant differences among the peak locomotions were determined by a two-way analysis of variance followed by Fisher's Least Significant Difference test. There is a significant difference in Cre versus No Cre mice ($F[1,28]=6.77$, $p<0.05$). There is also a significant difference in caffeine versus saline treated groups ($F[1,28]=18.46$, $p<0.001$). *** $p<0.001$ compared with caffeine treated group No Cre mice.

Figure 4. Caffeine attenuated MPTP-induced striatal dopamine loss in both WT and astrocyte-directed A_{2A}R cKO mice. Saline or Caffeine (25 mg/kg ip) was administered 10 min before saline or MPTP (35 mg/kg ip single injection). Striatal dopamine was determined one week after drug treatments. N=3-6 for saline treatments and N=9-20 for MPTP treatments. Bars represent striatal dopamine levels (mean $\pm$ SEM) calculated as percentage of their respective control (i.e., *saline + saline treatment* group). Statistically significant differences among the means of dopamine content in MPTP treated mice were determined by a two-way analysis of variance followed by Fisher's Least Significant Difference test. There is a significant difference in caffeine versus saline treated groups ($F[1,51]=17.69$, $p=0.0001$). * $p<0.05$, *** $p<0.001$ compared with respective S+M group.

Figure 5. Lack of dependence of caffeine-induced locomotion on astrocytic A_{2A}Rs. Ambulation was scored as the number of adjacent photobeam breaks (mean $\pm$ SEM, N=8 for each group). Mice were habituated overnight and basal spontaneous locomotion was recorded for at least 180 min. Ambulation was recorded for another 240 min after caffeine (25 mg/kg ip) or saline injection. **A.** Locomotion is similar after saline injection in WT and astrocyte-directed A_{2A}R cKO (*gfap-cre*, A_{2A}^{flox/flox}) mice. **B.** Caffeine also stimulated similar locomotion in WT and astrocyte-directed A_{2A}R cKO mice. Bars represent peak locomotion (mean $\pm$ SEM) 180 minutes after caffeine or saline injection. There is no statistical difference in locomotion after either saline or caffeine injection between Cre and No Cre mice.

Figure 1.

Figure 2.

Figure 3A.

Figure 3B.

Figure 3C

Figure 4.

Figure 5A.

Figure 5B.

Time (10 min)

Figure 5C