Neuroprotection by Caffeine and A$_{2A}$ Adenosine Receptor Inactivation in a Model of Parkinson's Disease

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Recent epidemiological studies have established an association between the common consumption of coffee or other caffeinated beverages and a reduced risk of developing Parkinson's disease (PD). To explore the possibility that caffeine helps prevent the dopaminergic deficits characteristic of PD, we investigated the effects of caffeine and the adenosine receptor subtypes through which it may act in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxin model of PD. Caffeine, at doses comparable to those of typical human exposure, attenuated MPTP-induced loss of striatal dopamine and dopamine transporter binding sites. The effects of caffeine were mimicked by several A$_{2A}$ antagonists (7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine (SCH 58261), 3,7-dimethyl-1-propargylxanthine, and (E)-1,3-diethyl-8 (KW-6002)-(3,4-dimethoxystyryl)-7-methyl-3,7-dihydro-1H-purine-2,6-dione) (KW-6002) and by genetic inactivation of the A$_{2A}$ receptor, but not by A$_1$ receptor blockade with 8-cyclopentyl-1,3-dipropylxanthine, suggesting that caffeine attenuates MPTP toxicity by A$_{2A}$ receptor blockade. These data establish a potential neural basis for the inverse association of caffeine with the development of PD, and they enhance the potential of A$_{2A}$ antagonists as a novel treatment for this neurodegenerative disease.

Key words: adenosine receptor; methylxanthine; neurotoxin; 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; dopamine transporter; Parkinson's disease; knock-out; mice

Caffeine, arguably the most widely consumed psychoactive dietary component in the world (Fredholm et al., 1999), as well as coffee drinking have been linked to a reduced risk of developing Parkinson's disease (PD) in two large prospective epidemiological studies (Ross et al., 2000, Ascherio et al., 2001) and multiple retrospective reports (Benedetti et al., 2000, and references therein). The incidence of PD declines steadily with increasing levels of coffee or caffeine intake, with the relative risk reduced as much as fivefold over a typical range of caffeine consumption. Despite the strength of these epidemiological correlations, they do not address the key question: does caffeine help prevent PD, or does (presymptomatic) PD or its causes help prevent the habitual use of caffeine? To address the possibility that caffeine may offer neuroprotection against the dopaminergic neurodegeneration that underlies PD, we investigated the effect of caffeine in the well established 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD (Gerlach and Riederer, 1996).

Pharmacological studies indicate that the CNS effects of caffeine are mediated primarily by its antagonistic actions at the A$_1$ and A$_{2A}$ subtypes of adenosine receptors (Fredholm et al., 1999). A$_{2A}$ adenosine receptors (A$_{2A}$Rs) may be particularly relevant because their expression in brain is largely restricted to the striatum (Svenningsson et al., 1999), the major target of the dopaminergic neurons that degenerate in PD. Furthermore, their blockade is known to protect against excitotoxic and ischemic neuronal injury (Ongini et al., 1997; Jones et al., 1998; Monopoli et al., 1998). Accordingly, we also assessed the effects of both A$_1$Rs and A$_{2A}$Rs on MPTP toxicity using complementary pharmacological and genetic approaches to adenosine receptor inactivation.

MATERIALS AND METHODS

MPTP treatment paradigms and adenosine receptor antagonists. Male C57BL/6 mice (25–28 gm; 2–3 months old) received a single intraperitoneal injection of 20–40 mg/kg MPTP (or saline) or four intraperitoneal injections of 20 mg/kg MPTP (or saline) 2 hr apart. All MPTP doses are given for the hydrochloride salt. Pretreated mice also received caffeine, specific adenosine receptor antagonist, or vehicle 10 min before each MPTP dose. Saline served as vehicle for caffeine, whereas a fresh

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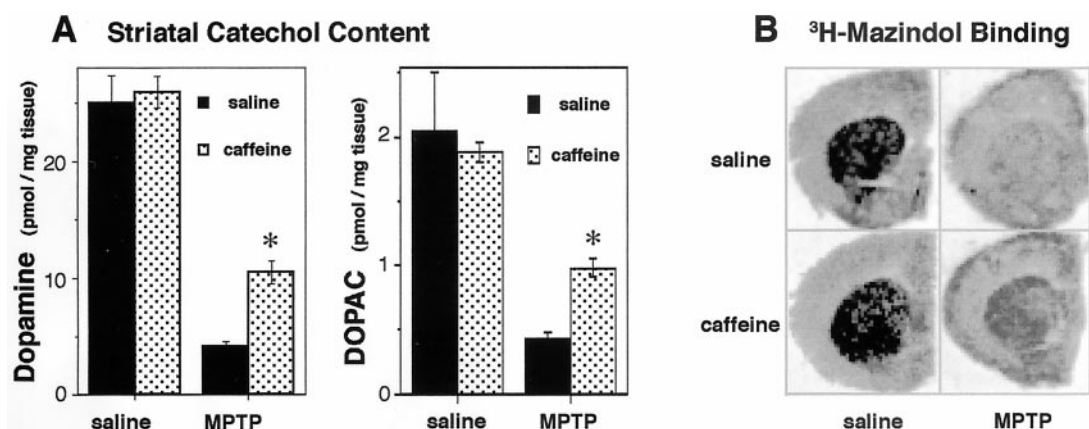
Figure 1. Caffeine attenuates MPTP-induced dopaminergic deficits. Mice were pretreated with caffeine (10 mg/kg, i.p.) or saline 10 min before each of four doses of MPTP (20 mg/kg, i.p.) or saline. One week later, catechol (dopamine and DOPAC) levels in striatum (*A*) and DAT (^{3}H-mazindol) binding density in coronal sections through the striatum (*B*) were determined. *Bars* represent striatal catechol content (mean ± SEM) of mice treated with MPTP (*n* = 13) or saline (*n* = ~5) after pretreatment with caffeine (*stippled*) and saline (*black*). * indicates *p* < 0.01 compared with saline-pretreated control mice; Student's *t* test. ^{3}H-mazindol autoradiography in *B* shows representative sections at the level of striatum. MPTP reduced specific ^{3}H-mazindol binding by 44% (to 182 ± 17 fmol/mg tissue compared with unlesioned control values of 330 ± 26 fmol/mg), whereas MPTP after caffeine reduced specific binding by only 24% (to 250 ± 23 fmol/mg compared with "caffeine-pretreated, unlesioned" values of 330 ± 46 fmol/mg). The administration of caffeine with MPTP significantly increased the residual DAT levels (*p* < 0.05; Student's *t* test).

mixture of dimethylsulfoxide (15%), ethoxylated castor oil (15%; Alkamuls EL-620, Rhodia, Cranberry, NJ), and water was used for specific antagonists. All injection volumes were 7–10 μl/gm. Neuroprotection by A_{2A} antagonists was replicated in two of our laboratories (in Massachusetts and Virginia), with the (E)-1,3-diethyl-8-(3,4-dimethoxystyryl)-7-methyl-3,7-dihydro-1H-purine-2,6-dione (KW-6002) experiment being performed in the latter, in which the treatment protocol differed in several ways. Older C57BL/6 mice (9 months old) received KW-6002 in a vehicle of 0.3% Tween 80 in PBS, pH 7.4, 20 min before a single MPTP dose (35 mg/kg, i.p.). Higher basal striatal catechol levels in this laboratory may have reflected different mouse ages and substrains and dissection techniques. KW-6002 was synthesized as described (Shimada et al., 1997), and its identity was confirmed by melting point (188°C) and ^{1}H-NMR analyses. 7-(2-Phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine (SCH 58261) was a gift of E. Ongini (Schering-Plough).

Catechol measurements and dopamine transporter autoradiography. Seven days after treatment, mice were killed by rapid cervical dislocation, and their striata were dissected and assayed for catechols by standard reverse-phase HPLC with electrochemical detection. For dopamine transporter (DAT) autoradiography (^{3}H-mazindol), a cerebral hemisphere was removed and cut by cryostat into 20 μ coronal sections. These were preincubated for 5 min with ice-cold buffer (0.5 M Tris-HCl, 0.3 M NaCl, and 5 mM KCl, pH 7.9) and then incubated for 60 min in the same buffer containing 6 nM ^{3}H-mazindol and 300 nM desipramine. Washed and air-dried slides were exposed to autoradiographic film along with a tritium-labeled calibration standard for 2–4 weeks. Films were analyzed with an image analysis system, and specific striatal ^{3}H-mazindol binding (fmol/mg tissue) was calculated by subtracting nonspecific binding (^{3}H-mazindol binding in the presence of 100 μM unlabeled nomifensine as well as desipramine) from total binding.

Brain KW-6002, MPTP, and MPTP metabolite determinations. Whole-brain concentrations of KW-6002 were quantified by a modification of a previous method (Nonaka et al., 1993), using reverse-phase HPLC with a 75% acetonitrile mobile phase and 8-(3-chlorostyryl)caffeine as an internal standard. KW-6002 and the standard were monitored at 360 nm. KW-6002 and internal standard calibration curves were linear over the range measured in brain extracts. MPTP and its oxidative metabolite 1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP$^+$) were measured as described previously (Giovanni et al., 1991).

Derivation and breeding of $A_{2A}R$ knock-out mice. $A_{2A}R$ knock-out (A_{2A} KO) mice were generated using a standard displacement target vector as described previously (Chen et al., 1999). Briefly, chimeric A_{2A} 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 × 129-Steel background. To effectively eliminate the potentially confounding influence of the 129-Steel background, the mixed line was then repeatedly back-

crossed to pure C57BL/6 mice over six generations, yielding a near congenic (N6) C57BL/6 line. A_{2A} KO (−/−) and wild-type (WT; +/+) littermates (both male and female) from N6 heterozygote (±) intercrosses were used in this study. Pure 129-Steel mice were derived as described previously (Chen et al., 1999).

Statistical analyses. Single statistical comparisons between two groups were performed using a non-paired two-tailed Student's *t* test. Analysis of dose–response relationships was performed by one-way ANOVA followed by Dunnett's *post hoc* comparisons. Data values in the text present group averages ± SEM.

RESULTS

Caffeine attenuates MPTP-induced dopaminergic toxicity

In the MPTP model, mice exposed to MPTP (multiple or single doses) consistently develop biochemical and anatomical lesions of the dopaminergic nigrostriatal system that parallel characteristic features of PD (Gerlach and Riederer, 1996). Four doses of MPTP administered 2 hr apart depleted striatal dopamine levels measured 1 week later to only 15% of control values (i.e., for mice treated with saline and pretreated with vehicle) (Fig. 1*A*). However, in the presence of a low dose of caffeine (10 mg/kg 10 min before each MPTP dose), dopamine depletion was significantly attenuated, with the residual dopamine increased to 40% of control values. Simultaneous measurement of dihydroxyphenylacetic acid (DOPAC), the major CNS metabolite of dopamine, revealed an 80% depletion after treatment with MPTP alone, whereas residual striatal DOPAC more than doubled in mice pretreated with caffeine (Fig. 1*A*).

In addition to analysis of catechol levels as biochemical markers of dopaminergic nigrostriatal function, the density of dopamine transporter binding sites was measured as an anatomical marker of nigrostriatal innervation. Again, MPTP toxicity was diminished in the presence of caffeine, which significantly attenuated the loss of striatal DAT (^{3}H-mazindol) binding sites induced by MPTP (Fig. 1*B*). The increase in striatal catechol and DAT levels 1 week after caffeine exposure could not be attributed to a direct effect of caffeine on catechol metabolism or innervation density because caffeine alone had no effect on these measures compared with control values (Fig. 1*A*,*B*).
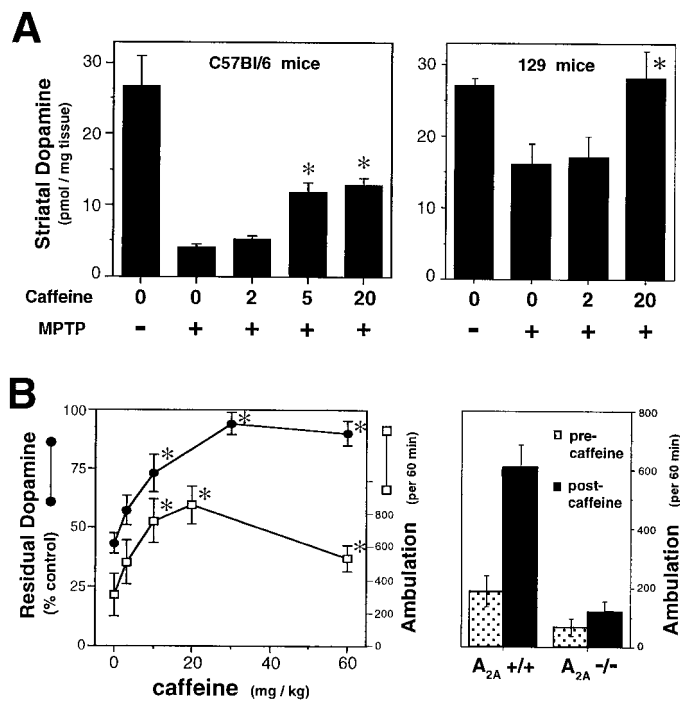
Figure 2. Dose dependence of neuroprotection and motor activation by caffeine. *A,* Caffeine (0–60 mg/kg, i.p.) was administered 10 min before each of four doses of MPTP (20 mg/kg, i.p.) or saline in C57BL/6 (*left*) or 129-Steel (*right*) strains of mice, and striatal dopamine content was determined 1 week later. *B,* Alternatively, a single dose (0–60 mg/kg, i.p.) of caffeine was administered 10 min before one dose of MPTP (40 mg/kg, i.p.) or saline in C57BL/6 mice, and its effects on striatal dopamine content (●) were compared with those of a single dose (0–60 mg/kg, i.p.) of caffeine on locomotion (□) (*B, left*). The effects of caffeine (at a dose producing maximal motor activation; 20 mg/kg, i.p.) on locomotor activity was compared between WT mice and those lacking the $A_{2A}R$ (*B, right*). In this experiment, locomotion, scored as the number of adjacent photobeam breaks (*Ambulation*), was determined for the 60 min before as well as after caffeine administration. For the dose–response curves, * indicates $p < 0.01$ when compared with dopamine content in mice treated with MPTP alone (0 caffeine) or when compared with locomotion after saline (0 caffeine) injection (Dunnett's test after one-way ANOVA). In *A*, $n = \sim10$ and ~7 for MPTP-treated C57BL/6 and 129-Steel mice, respectively. In *B*, $n = \sim9$ for MPTP-treated mice and 6 for caffeine-induced motor activity.

As a first step to understanding the pharmacological basis of the neuroprotective actions of caffeine in the MPTP model, we determined the dose dependency of caffeine's attenuation of dopaminergic toxicity and compared it with that of its stimulation of motor activity. At a low dose of 5 mg/kg caffeine (which in rodents is roughly equivalent to the caffeine exposure in humans provided by a single cup of coffee or ~100 mg caffeine) (Fredholm et al., 1999), residual dopamine levels nearly tripled compared with those in C57BL/6 mice that received saline pretreatment (Fig. 2*A*). Although caffeine produced similar attenuation at higher concentrations (10 and 20 mg/kg), at doses of 30 mg/kg and above, this combination of caffeine and MPTP (4 ×) produced excessive systemic toxicity, and few mice survived. An attempt to clarify the dose–response relationship at high caffeine doses was undertaken in mice of a 129-Steel genetic background (which can be more resistant than C57BL/6 to systemic toxicity of neurotoxins) (Schauwecker and Steward, 1997). In 129-Steel mice, caffeine (at 20 mg/kg) nearly reversed the more modest dopamine depletion produced by MPTP (20 mg/kg, 4 ×) (Fig.

2*A*). However, this dose–response experiment was also limited by excessive systemic toxicity with caffeine doses above 20 mg/kg. Accordingly, we next adopted a milder treatment paradigm entailing a single dose of MPTP (40 mg/kg, i.p.) 10 min after a single dose of caffeine (or saline) in C57BL/6 mice (Fig. 2*B*). As expected, MPTP (without caffeine) produced a smaller lesion using this paradigm in C57BL/6 mice (with just over 50% depletion of striatal dopamine), and mice survived without significant systemic toxicity even when MPTP was combined with the highest dose (60 mg/kg) of caffeine tested (Fig. 2*B*). Under these conditions, intraperitoneal caffeine at a dose of 10 mg/kg (but not 3 mg/kg) significantly attenuated MPTP toxicity, and at higher doses caffeine produced similar, nearly complete reversals of MPTP toxicity (Fig. 2*B*).

Interestingly, the potency of the neuroprotective effect of caffeine closely matched that of its motor-activating effect (Fig. 2*B*), which is likely mediated by the $A_{2A}R$ (Ledent et al., 1997; Fredholm et al., 1999). In fact, the peak locomotor stimulatory effect of caffeine at ~20 mg/kg in C57BL/6 mice was completely abolished in the absence of the $A_{2A}R$ [in $A_{2A}R$ KO mice (Chen et al., 1999)] (Fig. 2*B*), confirming its critical role in the motor stimulating effect of caffeine. Moreover, the comparable caffeine dose–response curves for neuroprotection and motor activation are consistent with a contribution from the $A_{2A}R$ in caffeine's attenuation of MPTP toxicity. However (and despite the affinity of caffeine for the $A_{2A}R$ exceeding that known for any other receptor or enzyme), caffeine still may activate other receptors (such as the A_1 adenosine receptor) or unknown targets (Fredholm et al., 1999).

A_{2A} but not A_1 adenosine antagonists mimic the neuroprotective effect of caffeine

Because the A_1 and A_{2A} subtypes of adenosine receptors are the most likely targets of the action of caffeine in brain (Fredholm et al., 1999), we compared the ability of A_1 and A_{2A} antagonists to mimic caffeine's protection against MPTP toxicity (Fig. 3). The relatively A_1-specific antagonist 8-cyclopentyl-1,3-dipropylxanthine (CPX) over a range of doses did not attenuate the dopamine depletion induced by a single MPTP dose (20, 30, or 40 mg/kg, i.p.) administered 10 min later (Fig. 3*A*). On the contrary, CPX tended to slightly exacerbate MPTP toxicity, particularly with the smaller lesions produced by lower MPTP doses. CPX alone had no effect on control striatal dopamine content.

By contrast, the relatively specific A_{2A} antagonists SCH 58261, 3,7-dimethyl-1-propargylxanthine (DMPX), and KW-6002 all significantly attenuated striatal dopamine depletion under various conditions (Fig. 3*B*). In the same single-dose MPTP paradigm used for CPX, SCH 58261 dose dependently increased residual levels of striatal dopamine, with significant attenuation of the lesion produced by a SCH 58261 dose as low as 0.5 mg/kg (Fig. 3*B*). Similarly, DMPX (2 and 5 mg/kg) partially reversed the depletion of striatal dopamine (Fig. 3*B*) induced by the four-dose MPTP regimen described above.

Another A_{2A} antagonist, KW-6002, which has shown potential as a symptomatic anti-parkinsonian agent in non-human primates (Richardson et al., 1997; Kanda et al., 1998; Grondin et al., 1999), was also tested for its effect on dopaminergic toxicity. Pretreatment with KW-6002 (3.3 mg/kg, i.p.) before a single dose of MPTP attenuated the partial dopamine and DOPAC depletions measured in striata 1 week later (Fig. 3*B*). Of note, relatively aged mice (9 months old) were used in this experiment (compared with
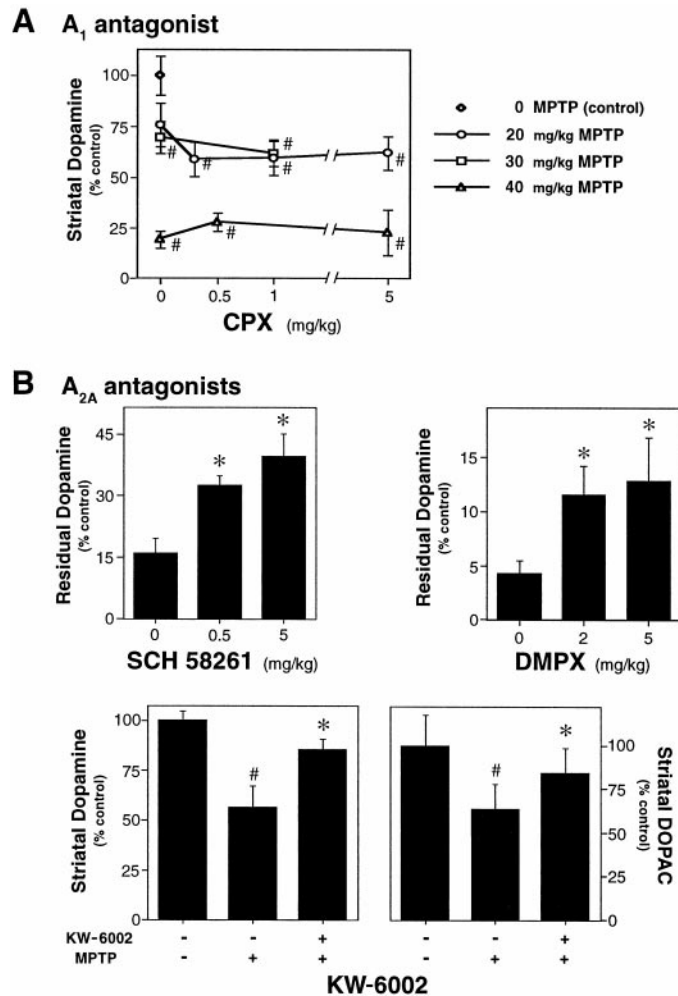
Figure 3. Effects of A_1 and A_{2A} adenosine receptor antagonists on MPTP-induced dopamine depletion. Mice were pretreated with the A_1 antagonist (*A, CPX*, 0–5 mg/kg, i.p.) or with one of three A_{2A} antagonists (*B, SCH 58261* and *DMPX* as indicated, and *KW-6002* at 3.3 mg/kg, i.p.) before receiving a single intraperitoneal dose of 20–40 mg/kg MPTP. In *A*, control striatal dopamine content was 28 ± 3 pmol/mg tissue. For SCH 58261 and DMPX experiments the MPTP dose was 40 mg/kg $\times$ 1 ($n = 7$) or 20 mg/kg $\times$ 4 ($n = 6$–8), respectively, and control striatal dopamine content was 32 ± 2 pmol/mg tissue. For the KW-6002 experiment a single dose of 35 mg/kg MPTP ($n = 10$–11) was used in older mice, and control striatal dopamine and DOPAC content were 121 ± 6 and 11 ± 2 pmol/mg tissue, respectively. Control doses of CPX, SCH 58261, and DMPX (each at 5 mg/kg, i.p.) were also administered before saline treatment (i.e., in unlesioned mice) and produced no effect on striatal dopamine content (data not shown). # indicates $p < 0.05$ when compared with unlesioned controls (Dunnett's test after one-way ANOVA). * indicates $p < 0.05$ when compared with MPTP-treated mice without antagonist pretreatment (Dunnett's test after one-way ANOVA).

2–3 months old in the other experiments reported here). Caffeine similarly produced significant protection against MPTP-induced striatal dopamine depletion in aged (11 month old) C57BL/6 mice (J. P. Petzer, K. Castagnoli, N. Castagnoli Jr, unpublished observations). The consistent demonstration of attenuated MPTP toxicity by caffeine and A_{2A} antagonists both in the young and aged nervous systems and in independently run laboratories (see Materials and Methods) serves to substantiate this finding.

To correlate the neuroprotective effect of KW-6002 with its CNS concentration, we determined brain concentrations of KW-6002 20 min after intraperitoneal administration of (3.3 or 10 mg/kg) KW-6002, and observed averages of 2.6 μM ($n = 2$, 2.6, and 2.7) and 3.6 μM ($n = 2$, 3.6, and 3.7), respectively. The reported KW-6002 K_i of 2.2 nM for the $A_{2A}R$ (Shimada et al., 1997) suggests that the brain concentration of KW-6002 produced by a typical intraperitoneal dose of 3.3 mg/kg is >1000-fold greater than its K_i at the time of MPTP administration. Thus, adequate concentrations of KW-6002 were likely achieved to ensure $A_{2A}R$ inactivation. However, because the affinity of KW-6002 for the $A_{2A}R$ is only 70-fold greater than that for the A_1 receptor (Shimada et al., 1997), these pharmacokinetic data highlight concerns regarding the selectivity of A_{2A} antagonists (Ongini and Fredholm, 1996) at the typical doses used here.

A neuroprotective phenotype of A_{2A} KO mice parallels the effect of caffeine

To clarify the role played by $A_{2A}Rs$ in MPTP toxicity and in its attenuation by caffeine, we took advantage of the complete specificity for $A_{2A}R$ inactivation offered by the A_{2A} KO model (Ledent et al., 1997; Chen et al., 1999). The severe dopamine depletion produced by four doses of MPTP in WT C57BL/6 mice was significantly attenuated in littermates lacking the $A_{2A}R$ (Fig. 4*A*). One week after MPTP treatment, striata from A_{2A} KO mice contained nearly fourfold higher levels of residual dopamine than striata from WT mice. Again, receptor autoradiographic analysis of DAT binding sites showed that this MPTP exposure markedly reduced ³H-mazindol binding in striatum of C57BL/6 WT mice (Fig. 4*B*). However, A_{2A} KO mice exhibited significantly higher levels of residual ³H-mazindol binding, compared with those of WT mice, 1 week after MPTP treatment. Thus genetic deficiency as well as pharmacological antagonism of the $A_{2A}R$ reproduces caffeine's attenuation of MPTP toxicity, confirming the role of this receptor in facilitating MPTP toxicity and supporting its contribution to the neuroprotective effect of caffeine. However, these data do not rule out the participation of other receptors or signaling molecules targeted by caffeine.

In an initial assessment of how caffeine and the $A_{2A}R$ alter the sequence of events underlying MPTP toxicity, we considered whether caffeine or $A_{2A}R$ deficiency affects the accumulation of MPTP and its oxidative metabolite MPDP$^+$ in the striatum shortly after its systemic administration. This is an important consideration because $A_{2A}Rs$ located on the endothelial cells of systemic and cerebral blood vessels can alter vascular function (Phillis, 1989; Ledent et al., 1997) and thus could alter MPTP entry into the CNS. Also, because conversion of MPTP to MPDP$^+$ by monoamine oxidase (MAO) B is required for MPTP biotransformation to the active toxin MPP$^+$ (for review, see Gerlach and Riederer, 1996), a change in striatal MPDP$^+$ levels may suggest a mechanism involving altered MAO B activity. Striatal MPTP and MPDP$^+$ levels were measured 15 min after systemic MPTP administration (20 mg/kg, i.p), at a time when striatal concentrations of both are known to peak in C57BL/6 mice (Giovanni et al., 1991). Striatal MPTP content was indistinguishable between mice pretreated with saline and those pretreated with caffeine (10 mg/kg) 10 min before the MPTP, with values of 28.4 ± 2.3 and 26.5 ± 3.0 pmol MPTP/mg striatal tissue, respectively ($n = 9$). Similarly, WT and A_{2A} KO mice displayed essentially identical MPTP levels in striatum 15 min after the same MPTP injection paradigm, with values of 33.8 ± 6.6 and 32.3 ± 6.5 pmol MPTP/mg tissue, respectively ($n = 8$). Striatal MPDP$^+$ content was also indistinguishable between the mice pretreated with saline and caffeine (29.2 ± 3.9 and 28.5 ± 4.3 pmol MPDP$^+$/mg striatal tissue, respectively; $n = 8$–9) and

A Striatal Dopamine

B ³H-Mazindol Binding
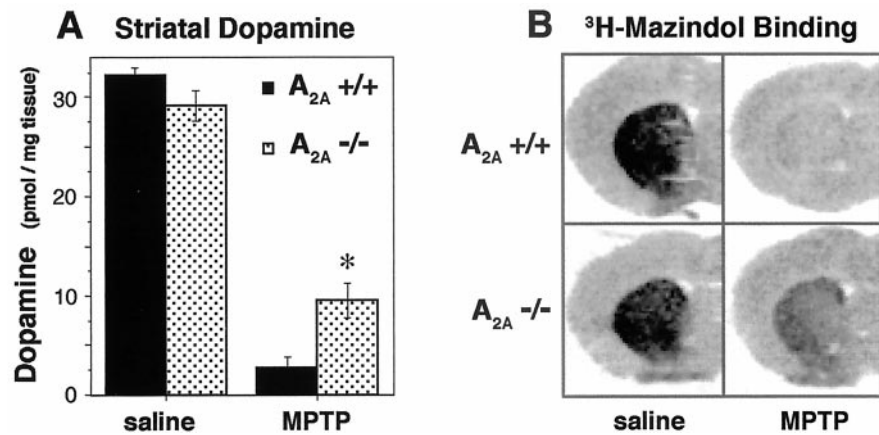
Figure 4. Attenuation of MPTP-induced depletion of dopamine content and dopamine transporter in A_{2A} KO mice. One week after MPTP treatment, striatal dopamine (*A*) or DAT (*B*) levels were determined in WT mice and their A_{2A} KO littermates. *Bars* represent striatal dopamine content (mean ± SEM) of WT (+/+, *black*; n = 8) and KO mice (−/−, *stippled*; n = 8). * indicates $p < 0.01$ when comparing A_{2A} KO with WT mice (Student's *t* test). ³H-mazindol autoradiography in *B* shows representative sections at the level of the striatum in WT and KO mice after saline or MPTP treatment. Specific striatal ³H-mazindol binding densities were 123 ± 19 and 70 ± 11 fmol/mg tissue in A_{2A} KO and WT mice, respectively, after MPTP treatment (n = 9–10; $p < 0.05$; Student's *t* test) and were reduced from 346 ± 27 and 342 ± 15 fmol/mg tissue, respectively, in unlesioned striata (i.e., after saline treatment).

between the WT and A_{2A} KO mice (22.1 ± 4.4 and 33.0 ± 7.1 pmol $MPDP^+$/mg striatal tissue, respectively; n = 7–8; $p = 0.2$). Thus, caffeine and the $A_{2A}R$ likely alter MPTP toxicity by impinging on the cascade of events triggered by MPTP at a point downstream of its entry into the CNS and its initial metabolism.

DISCUSSION

The present data reveal a novel protective effect of caffeine on the pathophysiological responses of dopaminergic nigrostriatal neurons in a mouse model of PD. Caffeine dose dependently attenuated the MPTP-induced depletion of functional and anatomical markers of the nigrostriatal neurons targeted in PD. The neuroprotective actions of this nonspecific adenosine receptor antagonist were mimicked by multiple A_{2A}-specific antagonists but not by an A_1-specific antagonist. MPTP toxicity was also blunted in mice lacking the $A_{2A}R$, confirming the role of this receptor in facilitating MPTP toxicity and supporting its contribution to the neuroprotective effect of caffeine. Although protection against dopaminergic neurotoxicity (as measured here by striatal dopamine and DAT levels) generally correlates well with protection against nigral neurodegeneration, exceptions have been noted (Kupsch et al., 1995). Thus, the effect of caffeine on MPTP-induced neurodegeneration remains to be tested.

Mechanism of neuroprotection by $A_{2A}R$ inactivation

Although the attenuated neurotoxicity of caffeine likely involves its antagonism at the $A_{2A}R$, the mechanism by which the $A_{2A}R$ alters MPTP toxicity is unknown. Nevertheless, the evidence that MPTP accumulation in the striatum is unchanged in the presence of caffeine or in the absence of the $A_{2A}R$ suggests that a CNS (rather than systemic) mechanism is involved. On entry into the striatum, MPTP is metabolized to $MPDP^+$ by MAO B before spontaneous conversion to the active toxin MPP^+ (Gerlach and Riederer, 1996). MAO also appears to be an unlikely target of caffeine because it has been shown to be a poor inhibitor of MAO activity *in vitro*, and because MAO B (K_m and V_{max}) activities in brain homogenates from A_{2A} KO and WT mice are identical (Fernstrom and Fernstrom, 1984) (S. Steyn, J.-F. Chen, M. A. Schwarzschild, K. Castagnoli, N. Castagnoli Jr, unpublished observations). Furthermore, our findings of unaltered striatal levels of $MPDP^+$ (as well as of MPTP) after systemic MPTP administration in caffeine-treated or A_{2A} KO mice also argue against the modulation of MAO B *in vivo*.

The starkly restricted expression of brain A_{2A}Rs almost exclusively to basal ganglia neurons receiving dopaminergic input and the lack of evidence for their expression on dopaminergic neu-

rons themselves (Svenningsson et al., 1999) suggest that $A_{2A}R$ modulation of dopaminergic neurotoxicity is indirect. The $A_{2A}R$-expressing striatal output neurons could influence dopaminergic neurotoxicity either by an alteration in their retrograde neurotrophic influence on nigrostriatal neurons (Siegel and Chauhan, 2000) or more likely through a feedback circuit impinging on dopaminergic nigral neurons (Rodriguez et al., 1998). In the latter scenario, stimulation of A_{2A}Rs on striatopallidal neurons enhances GABA release in the globus pallidus (Mayfield et al., 1996) and thus may facilitate the so-called "indirect" pathway's disinhibition of subthalamic nucleus activity, which in turn may contribute to excitotoxic injury of dopaminergic neurons in the substantia nigra (Piallat et al., 1996). Inactivation of A_{2A}Rs, on the other hand, would blunt the proposed dopaminergic toxicity produced through this circuit. Alternatively, A_{2A}Rs on excitatory neurons (Sebastiao and Ribeiro, 1996) or on glial cells (Brodie et al, 1998), despite their relatively low levels of expression, may promote dopaminergic toxicity in the MPTP model. For example, $A_{2A}R$ stimulation is known to enhance glutamate release (Popoli et al., 1995; Sebastiao and Ribeiro, 1996), such that blockade of this receptor may attenuate excessive glutamate release and in so doing lessen an excitotoxic component of MPTP toxicity. A similar mechanism has been proposed for the neuroprotective effects of $A_{2A}R$ blockade in models of ischemic brain injury (Ongini et al., 1997; Chen et al., 1999).

Relevance of protection by caffeine and $A_{2A}R$ antagonists for PD

The demonstration of a neuroprotective effect of caffeine in the MPTP model of PD establishes a potential neural basis for the epidemiological association between caffeine consumption and a reduced risk of developing PD. Furthermore, by identifying the $A_{2A}R$ as a plausible receptor target for the neuroprotective influence of caffeine on MPTP toxicity, the present study raises the possibility of an important role for the $A_{2A}R$ in the development of PD. It also points to the $A_{2A}R$ as a novel therapeutic target in the pursuit of neuroprotective strategies for PD. Specific A_{2A} antagonists may provide greater efficacy than nonspecific antagonists such as caffeine, because A_1R blockade may exacerbate dopaminergic toxicity (Lau and Mouradian, 1993; Delle Donne and Sonsalla, 1994) (Fig. 3*A*) and thus could undermine the benefits of $A_{2A}R$ blockade.

A_{2A} antagonists, which have previously been shown to have neuroprotective potential in models of ischemic and excitotoxic brain injury (Jones et al., 1998; Monopoli et al., 1998), may offer particular advantages in the treatment of PD. At present, the

mainstay of PD therapy relies on dopamine replacement strategies. Despite the considerable symptomatic relief that they offer, the disease continues to progress, often complicated by disabling motor side effects of the dopaminergic therapies. $A_{2A}R$ antagonists have been proposed as a potential anti-parkinsonian treatment and are now entering human trials for PD, on the basis of their ability to enhance motor function without producing motor complications in rodent or non-human primate models of PD [primates (Richardson et al., 1997; Kanda et al., 1998; Grondin et al., 1999)]. When coupled with these well established short-term motor benefits, the neuroprotective properties of $A_{2A}R$ antagonists may substantially enhance the therapeutic potential of these agents for the treatment of PD and other neurodegenerative disorders.

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