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Abstract

Substantial progress has been made toward each of the 3 Specific Aims (SAs) of our research project, "Caffeine, adenosine receptors andestrogen in toxin models of Parkinson's disease (PD)". The overarching hypothesis of the project is that multiple environmental protectants and toxins interact to influence of the health of the dopaminergic neurons lost in PD. To that end we are characterizing the interplay between several environmental agents (pesticides, caffeine and estrogen) that are leading candidate modulators of PD risk.

A major finding and publication of this project (SA #3) in its first year entails our demonstration that estrogen can prevent the neuroprotective effect of caffeine in the mouse MPTP model of PD. We have obtained evidence that endogenous estrogen (in females) andexogenous estrogen (in males and in ovariectomized females) can prevent the protective effect of caffeine on MPTP-induced loss of brain dopamine. Estrogen did not alter caffeine pharmacokinetics arguing for a downstream estrogen-caffeine interaction in the modification of dopaminergic neuron injury. These findings establish an animal model of estrogen-caffeine interactions in the modification of PD risk in humans, along with the opportunity to understand its molecular mechanisms. In addition, our laboratory and human data for this interaction are now sufficiently compelling to influence the design and interpretation of neuroprotection trials of estrogen or caffeine currently underway or under consideration. Ultimately, a better understanding of the interplay between environmental factors like caffeine estrogen may suggest effective preventative as well as therapeutic strategies for this neurodegenerative disorder.

Additional key findings have helped establish novel methodological approaches to the role adenosine receptors in neuroproteciton by caffeine. We have generated a colony of double knockout mice lacking the A1, the A2A or both adenosine receptor subtypes. These mice will be used to clarify the respective receptor subtype roles in neuroprotection by caffeine (SA #1). We have also adapted a powerful virus-based gene delivery system to conditionally knockout adenosine receptors from specific brain regions, enabling us to determine in which brain region(s) adenosine receptors contribute to neurotoxicity in models of PD (SA #2).

Table of Contents

Introduction

Identifying the mechanisms by which caffeine and more specific A_{2A} antagonists protect dopaminergic neurons in multiple toxin models of Parkinson's disease (PD) will advance our knowledge of the pathophysiology, epidemiology and therapeutics of PD.

The *overarching hypothesis* pursued by this proposal is that **multiple environmental protectants and toxins interact to influence of the health of the dopaminergic neurons lost in Parkinson's disease**. Here we endeavor to characterize the interplay between several environmental agents (pesticides, caffeine and estrogen) that are leading candidate modulators of PD risk.
We are pursuing 3 *specific hypotheses:*

1) Caffeine acts through blockade of brain A_{2A}Rs to protect dopaminergic neurons in both acute and chronic toxin models of PD. (***Specific Aim #1***)

2) Caffeine perfusion and focal A_{2A}R inactivation within the striatum are sufficient to attenuate MPTP toxicity, by reducing toxin-induced release of glutamate and/or GABA. (***Specific Aim #2***)

3) Estrogen attenuates the protective effect of caffeine but not the protective of A_{2A}R deletion because it acts by altering caffeine metabolism or A_{2A}R expression. (***Specific Aim #3***)

Statement of Relevance (from our proposal)

A. Parkinson's Disease -

Basic neuroscience significance - The results will improve our understanding of adenosine receptor neurobiology, and will provide insight into the role of endogenous adenosine in basal ganglia biology physiology and PD pathophysiology.

Epidemiological significance - Establishing the ability of caffeine to protect dopaminergic neurons in PD models and identifying a plausible mechanism of action greatly strengthens the hypothesis that a neuroprotective effect of caffeine is the basis for its inverse epidemiological association with risk of PD.

Therapeutic significance - With several specific adenosine A_{2A} antagonists emerging as promising therapeutic candidates based on their motor-enhancing (symptom-relieving) action, the prospects for additional neuroprotective benefit substantiated by this project may considerably enhance their therapeutic potential. In addition, identifying a biological basis for caffeine-estrogen interaction in modifying PD risk could also affect recommendations for estrogen replacement strategies in women with PD taking A_{2A} antagonists or caffeine (and *vice versa*). Furthermore, based on evidence that A_{2A}Rs contribute to the neurotoxicity affecting cortical and striatal neurons (as well as dopaminergic neurons), our findings may support novel A_{2A}R-

based neuroprotective treatments for a wider range of neurological diseases from stroke to amyotrophic lateral sclerosis (ALS) to Alzheimer's disease.

B. Environmental Neurotoxin Exposure in Military Service – By characterizing the neuroprotective effects of caffeine in a chronic pesticide model of PD (as well as the acute MPTP model), the proposed work will define a prototypical interaction between environmental toxins and protectants in determining the extent of a well-characterized neurological lesion (dopaminergic neuron death). Although there has been no compelling evidence to suggest that the incidence of PD will itself increase in association with military service or combat theatre exposures,[1] putative toxin exposure in the military may be linked to the development of another debilitating neurodegenerative disorder, ALS.[2] Moreover, some objective biological measures in veterans diagnosed with a "Persian Gulf War syndrome" have indicated dysfunction of dopaminergic neurotransmission in the basal ganglia,[3] raising the possibility (together with other data[4]) of altered risk for PD in this group. In any event, establishing a biological precedent for neurotoxin-neuroprotectant interplay in the relatively common disorder of PD, may provide a 'roadmap' that can be used should any neurological illness be confirmed to develop in association with prior military exposures.

C. Understanding the Non-stimulant CNS Effects of Caffeine. The psychoactive agent caffeine has been endorsed for military use at relatively high doses to help maintain operational readiness.[5] This recommendation has been based on a large body of evidence demonstrating sustainment of mental task performance by caffeine, and a lack of evidence for substantial harm at these doses. However, adopting the use of any CNS-active drug by protocol warrants careful consideration of newly appreciated neuronal actions of the agent. Accordingly, the proposed investigation of the novel neuroprotective effect of caffeine and its underlying mechanisms (e.g., altered neurotransmitter release) would be of significance for military programs that provide specific doses of caffeine to personnel to enhance cognitive function.

D. Gender Differences in How Environmental Factors Impact Toxin Susceptibility. Our investigation of how caffeine and estrogen exposures interact to modify neurotoxin susceptibility in laboratory models of PD may have substantial significance for the human epidemiology that prompted our pursuit of this line of research. In addition, the proposed studies may provide a prototype for modeling how gender and estrogen status interact with environmental exposures of relevance to the military (i.e., neurotoxins, caffeine). A better appreciation of how gender alters susceptibility to environmental toxins or protectants may ultimately lead to a better understanding (and modification) of the differential risks faced by women and men serving in the same military operations.

Body of the Report

Progress on Specific Aims and experiments as laid out in our Statement of Work (SOW [in blue]) is described here in detail.

STATEMENT OF WORK

Specific Aim #1 – to definitively determine whether brain A_{2A}Rs or A_1Rs contribute to dopaminergic neuron degeneration in acute and chronic toxin models of PD, and whether the brain A_{2A}R is required for caffeine's protective effect in these PD models. **(~576 mice)**

Hypothesis 1: **Caffeine acts through blockade of brain A_{2A} (not A_1) receptors to protect dopaminergic neurons in both acute (MPTP) and chronic (paraquat/maneb) toxin models of PD.**

Exp# 1 – **Effect of the A_1/A_{2A} receptor double KO in MPTP and paraquat/maneb (Pq/Mb) models**

We have generated an A_1-A_{2A} double KO line of mice in collaboration with Drs. Jiang-Fan Chen (Boston) and Bertil Fredholm (Sweden) in which our previously characterized A_{2A} KO mice have been crossed with A_1 KO mice. Importantly, we designed a breeding scheme that has placed the individual KO lines onto a common genetic background (C57Bl/6) using a marker-assisted selection ("speed congenic") strategy that virtually eliminates the common confounder of mixed genetic background in the interpretation of knockout mouse studies. During the past year we have begun to expand the colony through double heterozygote ($[A_1^{+/-}, A_{2A}^{+/-}]$ x $[A_1^{+/-}, A_{2A}^{+/-}]$) matings. This breeding strategy though arduous yields well-matched set homozygous A_1 KO $[A_1^{-/-}, A_{2A}^{+/+}]$, A_{2A} KO $[A_1^{+/+}, A_{2A}^{-/-}]$, double A_1-A_{2A} KO $[A_1^{-/-}, A_{2A}^{-/-}]$ and control WT $[A_1^{+/+}, A_{2A}^{+/+}]$ littermates. (Note that mice of these 4 genotypes comprise only a quarter of all offspring.)

In preparation for the proposed experiments we are expanding this double KO colony through these double heterozygote crosses, with PCR genotyping of the offspring producing the desired KOs and controls in the expected Mendelian distribution as shown in Figure 1 (with lane 2 showing a double KO, for example). We are also completing a stereological analysis of nigral dopaminergic neurons after chronic treatment *in vivo* with the paraquat/maneb pesticide combination (+/- caffeine, a mixed A_1-A_{2A} antagonist), which may further establish the rationale for investigating this chronic pesticide model of PD in the single and double A_1-A_{2A} KO mice.

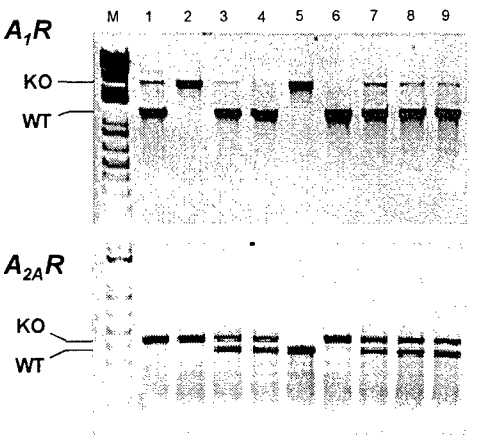

Fig. 1 – ***A_1R* and *$A_{2A}R$* genotyping of offspring from double heterozygote matings.** To detect WT and mutant (KO) alleles of the *A_1R* and *$A_{2A}R$* genes in the same congenic C57BL/6mice, we employed PCR genotyping protocols with one downstream primer targeting the inserted *Neo*-cassette and another targeting the WT *A_1R* or *$A_{2A}R$* gene to distinguish mutant from WT alleles for each gene.

Exp# 2 – **Effect of brain-specific A_{2A} KO in MPTP and Pq/Mb models.**

In preparation for this experiment we have completed the generation and initial characterization of a conditional (Cre/*loxP* system) KO of post-natal forebrain neuronal A_{2A} receptors. As detailed in our proposal the *CamKII*α promoter was used to drive expression of the *cre* recombinase gene in postnatal forebrain neurons, and thus to cause selective depletion of striatal neuron A_{2A} receptors following brain development. In our recent publication Bastia et al, 2005[6] (Appendix D) we confirm successful forebrain-specific recombination by genetic, autoradiographic and behavioral assessments.

Exp# 3 – **Brain A_{2A}R-dependence of caffeine's neuroprotective effect.**

We took advantage of a complementary transgenic Cre mouse line (provided by Dr. David Guttmann, St. Louis) that uses the *GFAP* promoter to drive expression of the *cre* gene in astrocytes. By crossing this mouse with the 'floxed A_{2A}' line of mice, we have generated a set of mice lacking the A_{2A} receptor in astrocytes. This astrocytic A_{2A} KO line has allowed us to ask whether brain astrocytes express the A_{2A} receptors whose blockade by caffeine protects against MPTP toxicity. This possibility has become of particular interest with the recent appreciation that astrocyte A_{2A} receptors can play a role in control extracellular glutamate concentrations.[7-8]

This past year we have conducted two experiments to address the role of astrocyte A_{2A} receptors in neuroprotection by caffeine. The pooled results (see Fig. 2) clearly indicate that the astrocyte A_{2A} receptor is not essential for caffeine's protective effect in the MPTP model because it's neuroprotection by caffeine is undiminished in mice lacking astrocyte A_{2A} receptors. This finding is consistent with our original hypothesis, and we therefore plan to pursue the neuron-specific conditional KO experiment in the coming year.

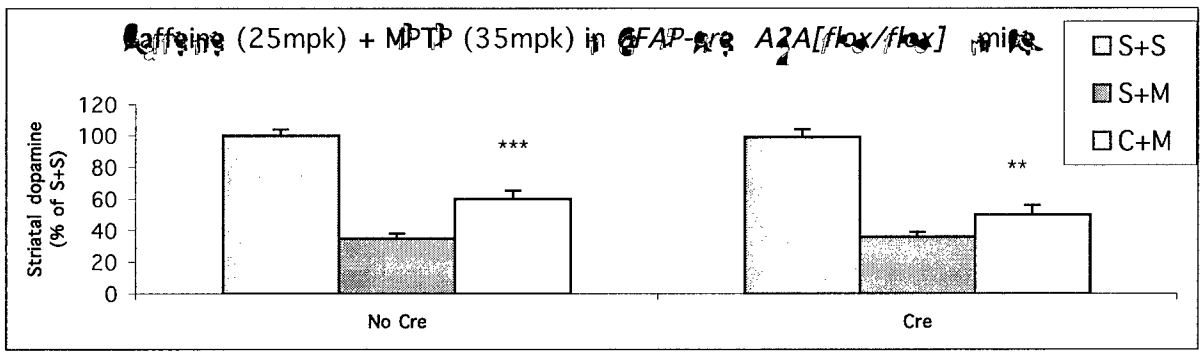

Fig. 2 – **Conditional astrocyte KO of A_{2A} receptors does not eliminate neuroprotection by caffeine.** Caffeine (**C**) attenuates the loss of striatal dopamine induced by MPTP (**M**, 35 mg/kg) not only in control ("**No Cre**") mice (i.e., *A_{2A}[flox/flox]*), but also in astrocyte (conditional) KO littermates ("**Cre**") mice (i.e., *GFAP-cre, A_{2A}[flox/flox]*).

Specific Aim #2 – to localize the region within brain through which caffeine or A_{2A} receptor inactivation produces its neuroprotective effect in the MPTP model of PD. **(~576 mice)**

Hypothesis 2: **Caffeine perfusion and focal A_{2A} receptors inactivation within striatum (but not frontal cortex) are sufficient to attenuate MPTP toxicity, by reducing toxin-induced striatal release of glutamate and/or GABA.**

Exp# 4 – **Effect of intracerebral caffeine perfusion on MPTP-induced neurotransmitter overflow and toxicity**:

In preparation for this experiment with local administration of caffeine, we are continuing to characterize (using microdialysis) MPTP-induced neurotransmitter overflow in the striatum and its modulation by systemic caffeine.

Exp# 5 – **Effect of intracerebral infusion of Cre-expressing adeno-associated virus (AAV-Cre) on MPTP-induced toxicity in floxed A_{2A}R mice**: Homozygous floxed A_{2A}R mice that previously received a stereotactic infusion of AAV-Cre or AAV-green fluorescent protein (AAV-GFP) into the striatum (or frontal cortex or substantia nigra) will be acutely exposed to systemic MPTP. One week later infusion needle track will be localized histochemically, while dopaminergic neuron integrity will be visualized by striatal DAT binding and nigral TH-IR counts will be assessed as in Exp #4.

We have made substantial progress toward this experiment in successfully introducing the a viral (AAV) *cre* recombinase gene into the striatum of 'floxed A_{2A}' mice, leading to the unilateral local recombination and disruption of the A_{2A} gene, and in turn the elimination of striatal A_{2A} receptors. Working with several serotypes of AAV-*cre* and AAV-*GFP* (provided through a collaboration with Dr. Miguel Esteves), Dr. Augusta Pisanu (a post-doctoral fellow who joined the lab earlier this year specifically to pursue this SA) has demonstrated that AAV-*GFP* stereotactically injected into the striatum of wild-type mice leads to the local expression of GFP (green fluorescent protein; Fig. 3). The area of infection/expression was better localized to the targeted striatum when using a serotype 1 AAV-*GFP* (Fig. 3A) compared to a mixed serotype 1/8 AAV-*GFP* (which produced more widespread expression crossing over from the targeted striatum into the overlying cortex; Fig. 3B).

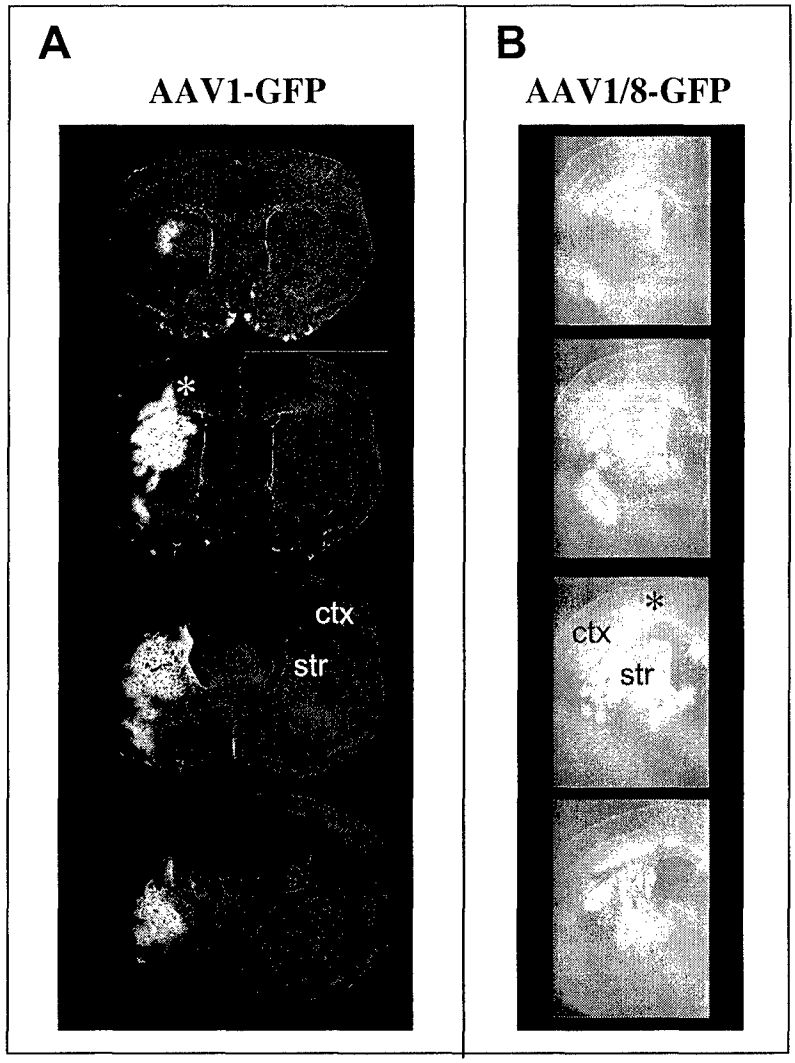

Fig. 3 – **Intrastriatal injection of AAV1-*GFP* leads to local striatal GFP expression.** (**A**) One month after stereotactic unilateral intrastriatal injection of AAV1-*GFP* into wild-type C57BL/6, coronal brain sections were treated with a Hoechst chromatin stain (rich blue background). Green fluorescence was detected in rostral (top) through caudal (bottom) sections only on the side of the injection, and almost entirely within the striatum (**str**). (**B**) By contrast, injection of an AAV1/8-GFP serotype under the same conditions led to a much less discreet distribution of GFP (without Hoechst counter-stain in this mouse) in cortex (**ctx**) as well as striatum. Injection needle tracks are indicated (✻.

Accordingly, we have selected AAV1*cre* (serotype 1 AAV-*cre*) for targeting the selective elimination of striatal A_{2A} receptors in floxed A_{2A} mice. Figure 4A shows successful *cre* expression (with detection of Cre immunoreactivity within striatal nuclei by immunohistochemistry) on the side of AAV1-*cre* injection. Remarkably, adjacent coronal sections demonstrate complete elimination of detectable striatal A_{2A} receptors (Fig. 4B) in the exact region of the Cre recombinase. This close correlation of nuclear Cre expression and A_{2A} receptor disappearance is consistent with functional recombinase producing the expected recombination across the *loxP* sites flanking a critical sequence within the A_{2A} gene in the 'floxed A_{2A}' mice. This sequence deletion was designed to disrupt the gene, leading to discontinued production of functional receptor.

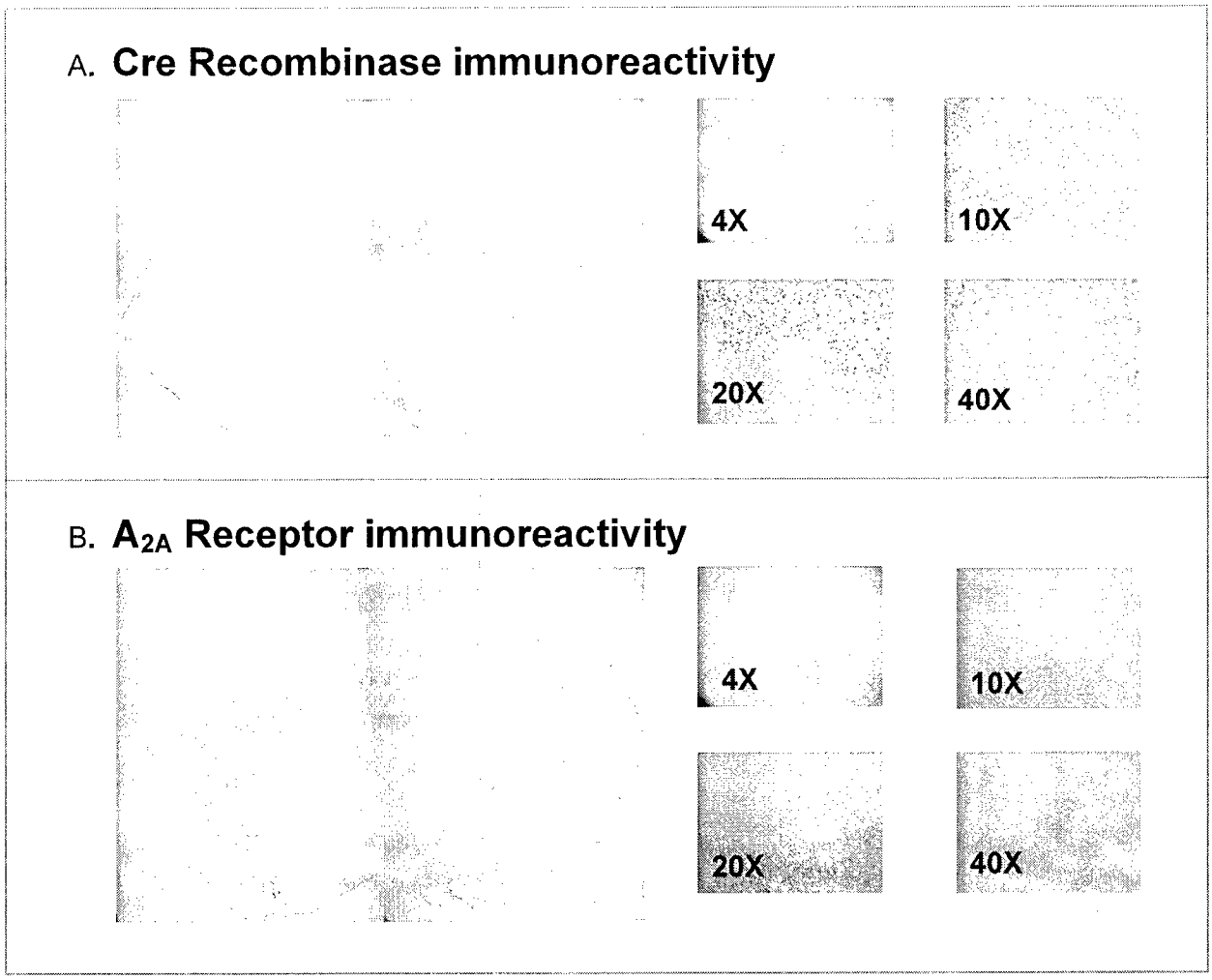

Fig. 4 – **Intrastriatal injection of AAV1-*cre* leads to local striatal Cre expression (A) and the coincident elimination of striatal A_{2A} receptors (B) in 'floxed A_{2A}' mice.** One month after stereotactic unilateral intrastriatal injection of AAV1-*cre* into 'floxed A_{2A}' (*A_{2A}[flox/flox]*) mice, Cre-immunoreactivity was assessed by Cre immunohistochemsitry (**A**) with a HRP-coupled secondary antibody yielding a (brown) reaction product in the nucelei of striatal cells (seen best at 40x magnification on the right). Adjacent coronal brain sections were processed for A_{2A} receptor immunohistochemsitry (**B**) with a HRP-coupled secondary antibody yielding a (brown) DAB reaction product more but specifically staining the striatum, except in the precise location of Cre expression (c.f., contents of red ovals in **A** and **B**).

This methodological advance will allow us to dissect A_{2A} receptor involvement in neurotoxin models of PD with an unprecedented combination of anatomical and molecular precision.

Specific Aim #3 – to investigate caffeine-estrogen interactions in the MPTP model of PD by determining the effect estrogen replacement on the neuroprotective phenotype of A2A KO mice, and exploring potential peripheral and CNS mechanisms contributing to caffeine's reduced neuroprotective efficacy in the presence of estrogen. **(~576 mice)**

With support from this award we have completed our initial study of estrogen-caffeine interaction in the MPTP model of Parkinson's disease, as detailed in the attached manuscript and meeting abstract (Appendices A and B). Our results demonstrate that estrogen reduces caffeine's neuroprotective effect against MPTP toxicity in both male and female mice. In the context of human epidemiology on PD, our findings suggest a biological basis for the interaction between estrogen and caffeine in modifying the risk of PD.

Hypothesis 3: **Estrogen attenuates the protective effect of caffeine but not the protective of A_{2A} receptor deletion because it acts by altering caffeine metabolism or A_{2A} receptor expression.**

Exp# 6 – **Effect of estrogen replacement on MPTP toxicity in OVX A2A KO versus WT mice.**

The clear outcome of *Exp# 7* showing that estrogen did not alter caffeine metabolism (see below) argues against the premise in *Hypothesis 3*. Therefore, it may be that estrogen will in fact attenuate the protective effect of A2A receptor depletion (in the A2A KO) as well as caffeine's protective effect.

Exp# 7 – **Effect of estrogen replacement on caffeine pharmacokinetics and striatal A_{2A} receptor density**: The effects of controlled-release estrogen versus vehicle pellets implanted in OVX female WT mice will be determined on serum and brain concentrations of caffeine at various time points after intraperitoneal caffeine administration (Exp # 7a), and on A2A, D1 and D2 receptor autoradiography in the striatum (Exp # 7b).

We have completed this experiment with the clear demonstration that chronic exogenous estrogen attenuates caffeine's neuroprotective effect without altering serum or brain levels and kinetics of caffeine or its demethylation metabolites theophylline, paraxanthine and theobromine. These data are provided in the context of a full manuscript under revision for the *J. Neurosci.* (appendix A). We similarly report that A2A receptor expression is unaffected by estrogen treatment in our paradigm.

Key Research Accomplishments (in Year 1)

- We have systematically demonstrated that estrogen can prevent caffeine's neuroprotective effect against dopaminergic neuron injury in the MPTP mosue model of Parkinson's disease (PD).
- In so doing, we have provided a biological basis for the consistent epidemiological finding that caffeine is associated with a reduced risk of PD in men and in women who have not had estrogen replacement therapy, but not in women who have had estrogen replacement therapy.
- We have demonstrated for the first time the use of virally delivered *cre* gene (via an AAV1-*cre* vector) that can discreetly eliminate the A_{2A} receptor in brain. This powerful Cre-*loxP* methodology will allow us to dissect -- with exceptional molecular and anatomical precision -- the role of the adenosine A_{2A} receptor in caffeine's influence on dopaminergic neuron injury in neurotoxin models of PD.
- We have established and expanded a novel double A_1-A_{2A} double receptor knockout (KO) in preparation for experiments that can gauge the relative roles of the two major brain adenosine receptors in toxin models of PD.
- Publications (with acknowledgements citing W81XWH-04-1-0881/ USAMRAA)
    a. Xu K, Xu Y, Brown-Jermyn D, Chen J-F, Ascherio A, Dluzen D, Schwarzschild MA. Estrogen reduces the neuroprotective effect of caffeine in a mouse model of Parkinson's disease. Program No. 665.12. 2005 Washington, DC: Society for Neuroscience. [Abstract]
    b. Hauser R, Schwarzschild MA. 2005. Adenosine A_{2A} receptors for Parkinson's disease. *Drugs and Aging* 22:471-482. [Review]
    c. Xu K, Xu Y, Brown-Jermyn D, Chen J-F, Ascherio A, Dluzen D, Schwarzschild MA. Estrogen prevents neuroprotection by caffeine in the mouse MPTP model of Parkinson's disease. [submitted to *J Neurosci*; status: acceptable pending revision]

Reportable Outcomes

1) Publications (with acknowledgements citing W81XWH-04-1-0881/ USAMRAA)

- Xu K, Xu Y, Brown-Jermyn D, Chen J-F, Ascherio A, Dluzen D, Schwarzschild MA. Estrogen reduces the neuroprotective effect of caffeine in a mouse model of Parkinson's disease. Program No. 665.12. 2005 Washington, DC: Society for Neuroscience. [Abstract]
- Hauser R, Schwarzschild MA. 2005. Adenosine A_{2A} receptors for Parkinson's disease. *Drugs and Aging* 22:471-482. [Review]

- Xu K, Xu Y, Brown-Jermyn D, Chen J-F, Ascherio A, Dluzen D, Schwarzschild MA. Estrogen prevents neuroprotection by caffeine in the mouse MPTP model of Parkinson's disease. [submitted to *J Neurosci*; status: acceptable pending revision]

1) Presentations (with acknowledgements including DoD/USAMRAA/NETRP)

- April 6, 2005 – (Austin) University of Texas, SW – grand rounds, "*Caffeine, adenosine receptors and Parkinson's disease*""
- September 7, 2005 – Polish Neuroscience Society; 9/7/05 symposium speaker, "*Caffeine, adenosine A_{2A} receptors and neuroprotection in PD*"
- September 16, 2005 (New York City) – Michael J. Fox Foundation for Parkinson's Research, "*Dyskinesia Summit*"
- September 26, 2005 (Philadelphia) Univ. of Penn – Dept of Pharmacology seminar, "*Caffeine, adenosine A_{2A} receptors and Parkinson's disease*"

Conclusions

Central hypothesis: *Multiple environmental protectants and toxins interact to influence of the health of the dopaminergic neurons lost in Parkinson's disease.*

Our initial progress under this award supports the central hypothesis, particularly with respect to caffeine-estrogen interactions in models of PD (**SA 3**). Critical to our ability to successfully pursue the Specific Aims of our research program, we have made substantial progress in establishing and charactering the key (global and conditional receptor KO) methodologies of this project (**SA 1 and 2**). We expect to build on our conceptual and technical advances of the first year in pursuing the key experiments of the project as originally proposed in our SOW.

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Abbreviated title: Estrogen, caffeine and MPTP toxicity.

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Abstract

Epidemiological studies have linked caffeine consumption with a reduced risk of Parkinson's disease (PD) in men. Interestingly, in women this inverse association is present only in those who have not used postmenopausal estrogens, suggesting an interaction between the influences of estrogen and caffeine use on the risk of PD. To explore a possible biological basis for this interaction in a mouse model of PD, we investigated the effect of caffeine on MPTP neurotoxicity, first in young or retired breeder (RB) male and female intact mice, then in ovariectomized (OVX) female mice or male mice treated with estrogen or placebo. 1. Caffeine treatment produced a dose-dependent attenuation of MPTP-induced striatal dopamine loss in both young and RB male, but not female mice. Only the highest dose of caffeine attenuated MPTP-induced dopamine loss in female mice. 2. In young female mice, lower doses of caffeine significantly reduced dopamine loss in OVX or OVX+placebo mice, but not in sham or OVX + estrogen mice. Moreover, in RB OVX female mice, none of caffeine treatments attenuated MPTP-induced dopamine loss in estrogen treated mice. 3. Estrogen treatment prevented caffeine's protection against dopamine loss in young male mice. 4. There is no overall difference in brain levels of caffeine and its metabolites between OVX+placebo and OVX+estrogen mice. Taken together, these results demonstrate that estrogen reduces caffeine's neuroprotective effect against MPTP toxicity in both male and female mice, suggesting a biological basis for the interaction between estrogen and caffeine in modifying the risk of PD.

Parkinson's disease (PD) is a progressive neurodegenerative disorder that is pathologically well characterized. However, the etiology of PD remains unclear. Twin studies (Tanner et al., 1999; Viergge et al., 1999; Wirdefeldt et al., 2004) have suggested that non-genetic factors, such as environmental exposures or random cellular events that occur during aging, play a prominent role in promoting the development of typical PD.

A major negative risk factor for PD has been identified recently as the consumption of caffeine. Multiple retrospective as well as several large prospective epidemiological studies have demonstrated that amongst dietary factors, prior coffee or tea drinking are consistently associated with a reduced risk of developing PD even after accounting for smoking and other potential confounding factors (Benedetti et al., 2000; Ross et al., 2000; Ascherio et al., 2001; 2004). The incidence of PD declines steadily with increasing intake of caffeine or of coffee (but not decaffeinated coffee).

The mechanisms that underlie this epidemiological correlation remain unclear. One hypothesis that caffeine might represent a protective environmental factor in PD is supported by our findings that caffeine can protect against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) -induced nigrostriatal neurodegeneration in mice (Chen et al., 2001; Otzas et al., 2002). Moreover, caffeine's metabolites, paraxanthine and theophylline, provide similar attenuations of MPTP-induced dopaminergic toxicity (Xu et al., 2002b). In contrast to its locomotor stimulant effect, caffeine's neuroprotectant effect does not show tolerance after chronic caffeine exposure (Xu et al., 2002a). Recently, caffeine's protection against dopaminergic neuron loss and associated behavior changes was confirmed in the 6-OHDA rat model of PD (Joghataie et al., 2004). Together, the protective effects of caffeine and its metabolites in rodent models of PD support a causal

basis for the inverse relationship between human caffeine consumption and the risk of subsequently developing PD.

Interestingly, the negative link between caffeine intake and risk of developing PD has been observed consistently in men, but not in women (Benedetti et al., 2000; Ascherio et al., 2001, 2004). Overall, there was no clear relationship between PD and caffeine intake in two large prospectively followed populations of women (Ascherio et al., 2001, 2004). When these women were further divided by their estrogen use status after menopause, a negative association between caffeine intake and risk of PD similar to men was observed in those women who had never used estrogen replacement therapy, but not in those who had ever used it (Ascherio et al., 2003, 2004). These results suggest that estrogen replacement therapy may prevent the beneficial effect of caffeine in reducing risk of developing PD.

To address the possibility that estrogen may interfere with neuroprotection by caffeine against dopaminergic neurotoxicity, we investigated their interaction in the well-established MPTP mouse model of PD. We first assessed the difference in neuroprotection by caffeine in male and female mice. Then we systematically investigated the effect of exogenous estrogen on caffeine's neuroprotection. Last we explored the effect of estrogen on caffeine's metabolism as a possible mechanism of interaction between estrogen and caffeine.

Materials and Methods

Animals, ovariectomy and estrogen replacement. Young (~10 week old) or retired breeder (6-9 month old) male and female C57BL/6 mice (Charles River Laboratories,

Wilmington, MA) were used in these experiments. All experiments were performed in accordance with Massachusetts General Hospital and National Institute of Health Guidelines on the ethical use of animals. Mice were housed five per cage with *ad libitum* access to food and water and were maintained at a constant temperature and humidity with 12:12 hour light:dark cycle. To remove the main source of female hormones, bilateral ovariectomies were performed under anesthesia using Avertin (2% 2,2,2-tribromoethanol and 1% amyl alcohol, 20 ml/kg i.p.) at either Charles River Laboratories or Massachusetts General Hospital. Sham operations were also included, in which all the other procedures are the same except for removal of the ovaries. In the experiments where estrogen replacement were used, placebo or estrogen pellets (17β estradiol, 0.1 mg/pellet, 21-day-release, Innovative Research of America, Sarasota, FL) were implanted at the neck of mice under anesthesia seven to ten days after ovariectomy. This estrogen regimen was used as it produces an approximate replacement of physiological levels within the serum in ovariectomized mice (Gao and Dluzen, 2001).

Caffeine and MPTP treatment. Different doses of caffeine (5, 10, 20 or 40 mg/kg) or saline were injected intraperitoneally (i.p.) 10 min before MPTP (40 mg/kg i.p.) or saline injection (N=3-6 for saline treatments and N=6-13 for MPTP treatments). The extent of the caffeine dose range employed across experiments varied depending on the number of mice available. In experiments where ovariectomy and/or estrogen pellets were implanted, caffeine and MPTP treatments were performed ten days after ovariectomy or estrogen implantation whichever comes later to assure that estrogen levels were depleted or maintained at a constant level, respectively.

Dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) measurement. 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 and extracted with 150 mM phosphoric acid and 0.2 mM EDTA. The striatum was homogenized and centrifuged at 12,000 g for 15 min at 4°C. Supernatants were analyzed for dopamine and DOPAC content using standard reverse-phase HPLC with electrochemical detection (ESA, Chelmsford, MA). Biogenic amines were separated on a C-18, 5-μm sphere column (Varian, Palo Alto, CA). The mobile phase consisted of 0.1 M sodium phosphate monobasic, 0.1 mM EDTA, 0.18 mM sodium octyl sulfate, and 8% methanol in filtered distilled water. The final pH of 3.3 was obtained with the addition of concentrated phosphoric acid, and the mobile phase was filtered and degassed before use. The dopamine and DOPAC contents were calculated as pmol/mg of tissue and these values are presented within the figures as percent change from respective Saline-Saline treated controls.

Caffeine and its metabolites measurement. Ten days after estrogen or placebo pellet implantation, OVX retired breeder female mice were treated with saline or caffeine (5 or 40 mg/kg i.p., N=1 for saline, N=5 for caffeine). Mice are killed at 10, 30, 60, 120, 180, 240 or 360 minutes after injection by rapid cervical dislocation. The right cerebral hemisphere was dissected out, frozen on dry ice and stored at –80°C until use. Each brain tissue was homogenized in 0.1 M monobasic sodium phosphate and centrifuged at 12,000 g for 15 min at 4°C. Supernatants were analyzed using LC/MS for determination of caffeine and its three metabolites, paraxanthine, theophylline and theobromine. The

lower limit of quantitation is 30 ng/ml. The analysis of caffeine and its metabolites were gratefully performed by Drs. Foltz and Andrenyak at Center for Human toxicology at University of Utah.

Statistical analyses. The data from striatal dopamine and DOPAC content as well as caffeine and its metabolites measurements were analyzed by two-way ANOVA. Post-hoc comparisons were performed using Fisher's least significant difference test. Data values in the figures present group mean±SEM.

Results

1. Gender differences in caffeine's attenuation of MPTP toxicity.

The dose-dependence of caffeine's neuroprotecitive effect in the MPTP model of PD was first compared between intact male and female mice. *a. Young mice (Fig. 1A).* MPTP treatment depleted striatal dopamine level measured one week later down to 38% of control (saline-treated) in ~10 week old male mice. Caffeine pre-treatment attenuated MPTP-induced dopamine loss in a dose-dependent manner in these young males, with a maximal effect (of doubling residual dopamine levels) achieved at 10 mg/kg. In female mice of the same age, MPTP depleted striatal dopamine levels to 62% of control levels (in saline-treated females). This reduction is significantly less than that observed in their male counterparts ($p<0.05$), agreeing with previous reports (Brooks et al., 1989; Freyaldenhoven et al., 1996; Miller et al., 1998) that MPTP induced less dopaminergic toxicity in female than male mice. However, in contrast to male mice, female mice showed no attenuation of MPTP toxicity after lower doses (5, 10 or 20

mg/kg) of caffeine pre-treatment. Only the highest dose (40 mg/kg) of caffeine protected against MPTP. Simultaneous measurement of DOPAC (*Supplemental Fig.1A*), the major metabolite of dopamine in mouse, revealed that lower doses of caffeine pretreatment (10 or 20 mg/kg) significantly attenuated MPTP-induced depletion in male mice, while only the highest dose of caffeine pretreatment (40 mg/kg) provided similar protection in female mice. *b. Retired breeders (Fig. 1B).* We also examined caffeine's neuroprotection against MPTP toxicity in older (6-9 month old), retired breeder mice. Because these mice have passed their peak reproductive age, their comparison may be more relevant to gender differences in epidemiological studies of PD, which is usually diagnosed in people older than 50 years old. Not surprisingly, MPTP treatment induced a greater biochemical lesion in older mice for either gender (c.f., *Figs 1A and B*), as reported previously (Irwin et al., 1992). In male retired breeders, MPTP treatment depleted striatal dopamine levels to 8% of unlesioned controls (*Fig. 1B*). As in the younger male mice, caffeine pre-treatment at and above 10 mg/kg again significantly attenuated MPTP-induced dopamine depletion. In female retired breeders, MPTP treatment depleted striatal dopamine levels to 23% of control levels. Again, female retired breeder mice also showed less MPTP toxicity compared to that of male mice. However, just as in young mice, pre-treatment with only the highest dose of caffeine (40 mg/kg) significantly attenuated the dopamine loss. Caffeine pre-treatment, at all doses tested (10, 20 or 40 mg/kg), also attenuated loss of DOPAC in male mice, whereas only higher doses provided similar protection in female mice (*Supplemental Fig.1B*).

2. Ovariectomy increases the potency of caffeine's protective effect.

To investigate whether the gender difference in caffeine's neuroprotective effects may relate to differences in female hormone status, we examined the effect of caffeine on MPTP toxicity in young female mice whose ovaries, the main site of female hormone production, were removed (ovariectomized, OVX) or in sham-operated female littermates (*Fig. 2*). MPTP treatment depleted striatal dopamine levels in both OVX and sham-operated mice. However, the lower dose of caffeine tested significantly increased residual striatal dopamine levels only in OVX females (as seen in males, *Fig. 1*). By contrast, sham-operated females (like intact females) required pre-treatment with a higher dose of caffeine for attenuation of MPTP-induced dopamine loss.

3. Estrogen attenuates caffeine's neuroprotection in ovariectomized female mice.

a. Young mice (*Fig. 3A*). To determine whether estrogen, the main female sex hormone, can account for the above-identified ovarian influence on neuroprotection by caffeine, we examined the effect of prolonged estrogen replacement on neuroprotection by caffeine in female mice depleted of endogenous estrogen. These OVX mice were implanted with subcutaneous pellets containing placebo or estrogen (17β –estradiol), which is continuously released to maintain steady-state concentrations for 21 days. MPTP treatment reduced striatal dopamine content to 27% of control in placebo-treated mice (OVX+P). The same MPTP exposure in OVX mice treated with estrogen pellets (OVX+E) depleted striatal dopamine levels, but to a significantly lesser extent (down to 39% of control), consistent with previous reports (Dluzen et al., 1996; Miller et al., 1998). In these OVX+P mice, pre-treatment with caffeine, both at 10 and 20 mg/kg, significantly attenuated MPTP-induced striatal dopamine loss. In mice that were

recciving estrogen replacement (OVX + E), however, only the higher dose of caffeine (20 mg/kg) provided significant protection. The striatal levels of DOPAC (*Supplemental Fig.2A*) demonstrated a similar phenomenon. (*Fig. 3B*)To better model estrogen replacement in the menopausal state, a parallel experiment was conducted in retired breeder (rather than young) mice. Again estrogen by itself significantly attenuated MPTP-induced striatal dopamine loss. MPTP depleted dopamine levels to 23% of control in OVX+P mice while it reduced them to 36% of control in OVX+E mice. In keeping with observations in young mice, caffeine pre-treatments (5, 20 or 40 mg/kg) significantly attenuated MPTP-induced dopamine loss in the OVX+P retired breeder mice, with complete reversal achieved at the highest dose of caffeine. However, in the setting of estrogen replacement in these OVX retried breeders, caffeine pre-treatment did not confer protection at any dose tested. The measurement of DOPAC (*Supplemental Fig.2B*) yielded a similar set of findings, with caffeine pretreatment (20 or 40 mg/kg) significantly reducing DOPAC loss in OVX+P, but not OVX+E, mice. Together these results demonstrate that estrogen replacement can reduce or abolish caffeine's neuroprotective effect on MPTP toxicity in young and older OVX females.

4. Estrogen can prevent neuroprotection by caffeine in male mice.

The above effects of manipulating endogenous and exogenous estrogen in female mice suggest that estrogen status may be the key factor accounting for the gender difference in caffeine's neuroprotective action in the MPTP model of PD. To determine whether the neuroprotective effect of caffeine in males indeed relies on the absence or relatively low levels of estrogen, we assessed caffeine's effects on MPTP toxicity in male

mice implanted with placebo or estrogen pellets (*Fig. 4*). MPTP treatment depleted striatal dopamine levels to 44% and 55% of their respective control level in male mice pretreated for 21 days with placebo or estrogen, suggestive of a mild protective estrogen effect as repeatedly observed above. In the placebo-implanted males, caffeine pretreatments significantly attenuated striatal dopamine loss. However, no dose of caffeine pretreatment protected the male mice treated with estrogen pellets. These results demonstrate that estrogen can abolish caffeine's neuroprotection not only in female mice but also in male mice.

5. The effect of estrogen on caffeine metabolism in ovariectomized female mice.

As a first step to understanding the mechanism of estrogen's influence on neuroprotection by caffeine, we measured the brain levels of caffeine (trimethylxanthine) and its major demethylation metabolites (dimethylxanthines, i.e., paraxanthine, theophylline and theobromine) at multiple time points after treatment with caffeine in OVX retired breeder female mice treated with placebo or estrogen pellets. Because estrogen status most consistently modulated the protective effect of lower doses of caffeine, we assessed estrogen's effect on the CNS pharmacokinetics of caffeine administered at 5 mg/kg i.p. (*Fig. 5A*). Brain concentrations of caffeine, which were maximal within 10 min of injection and then declined with a half-life of approximately 50 min, were indistinguishable between estrogen and placebo groups at all time points measured. When caffeine was administered at this low dose, the concentrations of its metabolites were below the limits of reliable detection.

As demonstrated above (*Fig. 3B*), in these OVX retired breeder mice caffeine pretreatments attenuated dopamine depletion in placebo but not estrogen treated mice, with the biggest difference found in caffeine 40mg/kg group. Therefore, we also determined brain concentrations of caffeine and its metabolites after injection of caffeine at 40 mg/kg i.p. (*Fig. 5B*). There was no difference in brain caffeine concentrations between OVX+E and OVX+P mice. There was a small effect of estrogen on brain paraxanthine concentrations, which were slightly but significantly higher in OVX+P than in OVX+E mice at 120, 180 and 360 min. There was also a minor effect on brain theophylline concentrations, which were slightly higher in OVX+P than OVX+E mice 60 min after caffeine injection. There was no difference in brain theobromine concentrations after caffeine injection. Thus exposure to estrogen, under conditions that attenuated the neuroprotective effect of caffeine. had no effect on brain concentrations of caffeine. Moreover, the overall concentrations of caffeine's three dimethyl metabolites even when combined are much (an order of magnitude) lower than that of caffeine such that the slight differences in metabolite concentrations found at some time points are not sufficient to explain the difference in caffeine neuroprotection between OVX+E and OVX+P mice.

Discussion

The present data reveal a novel interaction between estrogen and caffeine in a mouse model of PD – an interaction that recapitulates the relationship between estrogen and caffeine exposures in the human epidemiology of PD. In male but not female mice low doses of caffeine attenuated MPTP-induced depletion of striatal dopamine and

DOPAC content, functional markers of the nigrostriatal neurons that degenerate in PD. Remarkably, neuroprotection by low doses of caffeine was abolished in the presence of estrogen (either endogenous after sham operation, or exogenous when introduced by estrogen pellet after OVX) in young female mice. This attenuation was even more apparent in OVX retired breeders. Moreover, estrogen replacement in male mice effectively replicated the female phenotype of attenuated neuroprotection by caffeine.

Estrogen treatment by itself protects against MPTP toxicity

Our data in agreement with previous reports (Brooks et al., 1989; Freyaldenhoven et al., 1996; Miller et al., 1998) demonstrated that MPTP induced greater striatal dopamine loss in male than female mice. This phenomenon was observed in both young and retired breeder mice, suggesting that the gender difference in the susceptibility to MPTP toxicity is present regardless of age. These experimental findings are consistent with epidemiological studies, which have generally demonstrated a higher prevalence of PD in men than women (Baldereschi et al., 2000; Ascherio et al., 2001, 2004; also see Dluzen et al., 1998 for a review). The gender differences observed in PD patients and animal models, together with epidemiological data suggesting a link reduced estrogen states in women to an increased risk of PD (Benedetti et al, 2001), support the possibility that estrogen may play a protective role in dopaminergic neurodegeneration. Indeed, our current data, which demonstrated that estrogen replacement significantly attenuated MPTP-induced dopaminergic toxicity, confirms the neuroprotective effect of estrogen replacement therapy in this mouse model of PD (Dluzen et al., 1996, Callier et al., 2001; Ramirez et al., 2003).

Caffeine's attenuation of MPTP toxicity is diminished in the presence of estrogen

The current data confirm our previous findings (Chen et al., 2001; Xu et al., 2002a, b) that caffeine dose dependently attenuates MPTP-induced striatal dopamine loss in male mice. Moreover, our data demonstrate that this phenomenon -- observed in retired breeder as well as young mice – persists as animals age. These findings support a biological basis for a causal epidemiological correlation between caffeine consumption and reduced risk of developing PD.

Strikingly, the protection by lower doses of caffeine against MPTP-induced dopamine depletion was lost in female mice. The gender difference in caffeine's dose-response relationship with neuroprotection can be described pharmacologically as a reduction in caffeine's potency in female compared to male mice. In both young and retired breeder mice (*Fig. 1*), an ED_{50} of $\leq$5 mg/kg is apparent in males compared to $\geq$20 mg/kg in females. As we discussed above, female mice are less vulnerable than males to MPTP toxicity, raising the possibility that attenuated protection by caffeine in females may also reflect a 'ceiling effect' rather than reduced potency. However, the loss of protection in both moderate and severe lesions together with repeatedly significant protection achieved by the highest dose of caffeine in female mice of different ages argue against an impenetrable 'ceiling' and support a lower potency in females. The current data correlate well with the epidemiological studies that showed no inverse association between caffeine or coffee intake and risk of PD in women (Benedetti et al., 2000; Ascherio et al., 2001). The neuroprotection observed in female mice was restricted to the highest caffeine dose of 40 mg/kg, and may not be relevant to PD epidemiology. This dose in rodents may be comparable to a human exposure to the caffeine in some 10 cups

of coffee (Fredholm et al, 1999), which exceeds the daily consumption of almost any human subject in PD epidemiology studies.

The most notable finding of the current study is that the presence of the major female steroid hormone estrogen (either endogenous or exogenous) prevents neuroprotection by caffeine (or reduces its potency) in female and male mice. The estrogen effects observed in young mice were more robust in mice past their reproductive prime, highlighting the relevance of these findings to epidemiological studies linking PD risk to caffeine and estrogen exposures of middle-aged adults. These data systematically demonstrate that estrogen treatment attenuates caffeine's protection against MPTP toxicity in this mouse model of PD, providing a possible biological basis for the epidemiological findings that an interaction between estrogen and caffeine exposure modulates the risk of PD (Ascherio et al., 2003, 2004).

Mechanism of interaction between caffeine and estrogen

In pursuing the mechanism, a pharmacokinetic or metabolic interaction is an important consideration. Human data (Patwardhan et al., 1980; Abernethy and Todd, 1985; Pollack et al., 1999) suggest that estrogens can in fact alter caffeine pharmacokinetics. In those studies, caffeine's metabolism was inhibited in women taking estrogen-containing oral contraceptives or estrogen replacement therapy after menopause, suggesting that caffeine's actions would be enhanced rather than attenuated by estrogen. However, our data show no difference in brain caffeine concentrations over the time course (extending beyond 3 half-lives) of measurement in OVX retired breeder female mice treated with either estrogen or placebo.

Interestingly, two metabolites of caffeine (paraxanthine and theophylline) have been shown to provide protection against MPTP-induced dopaminergic toxicity with potencies comparable to caffeine (Xu et al., 2002b). So paradoxically, delaying or inhibiting the metabolism of caffeine could result in decreased level of these metabolites, which might cause reduced protection against neurotoxicity after estrogen therapy in human or animals. In the present study however, although there were slight differences in the brain concentrations of paraxanthine and theophylline between estrogen and placebo treated mice, the levels of these metabolites were much lower (>25 times) than that of caffeine, therefore arguing against their involvement.

It is interesting that although both estrogen and caffeine individually protect nigrostriatal dopaminergic neurons, when combined, estrogen diminished the potency (rather than enhancing the efficacy) of caffeine's neuroprotective action. This finding suggests that these two agents may work through a common mechanism to prevent MPTP toxicity. Estrogen may compete with caffeine for its activation of a protective pathway, effectively shifting its dose-response curve for neuroprotection to the right. It is worth noting that estrogen and caffeine as well as MPTP share a cytochrome P450, CYP1A2, for their metabolism or detoxification (Gu et al., 1992; Tassaneeyakul et al., 1994; Yamazaki et al, 1998; Forsyth et al., 2000). Therefore, metabolism of caffeine and/or estrogen could alter MPTP metabolism. However, because CYP1A2 contributes to hepatic detoxification of MPTP (Forsyth et al., 2000), competition for this enzyme by caffeine or estrogen would be expected to exacerbate rather than ameliorate MPTP toxicity. Moreover, since the brain MPTP levels were unaffected by acute caffeine treatment (Chen et al., 2001), it is unlikely that estrogen modulates caffeine's protection

through its action on MPTP metabolism.

Caffeine at the doses of 5-40 mg/kg used in the present study produced peak brain concentrations of 400-4000 ng/ml (equaling about 2-20 μM), most likely functions as an antagonist of adenosine A_1 and A_{2A} receptors (Fredholm et al., 1999). Our previous study (Chen et al, 2001) indicates that the attenuated neurotoxicity of caffeine likely involves its antagonism at the A_{2A} receptor ($A_{2A}R$). Therefore, estrogen might directly modulate caffeine's neuroprotection through an action on the $A_{2A}R$, although there is no information currently on such an interaction. It is reported (Rose'Meyer et al., 2003) that $A_{2A}R$ expression measured by quantitative PCR is significantly decreased after OVX. However, we found no difference in $A_{2A}R$ binding densities between OVX mice treated with estrogen or placebo (data not shown). Amongst the potential mechanisms for neuroprotection by caffeine (and more specific antagonists of the $A_{2A}R$; see review in Xu et al, 2005), the attenuation of glutamate release and of resultant excitotoxicity may be a protective mechanism shared with estrogen (O'Neil et al., 2004; Ritz et al., 2004; Saleh et al., 2004). Whether this or another shared mechanism of neuroprotection by caffeine and estrogen can account for their interaction in the MPTP model and in the epidemiology of PD remains to be clarified.

Relevance of an estrogen-caffeine interaction for PD

The attenuation of caffeine's neuroprotective effect by estrogen in the MPTP model of PD establishes a potential neural basis for the epidemiological association between estrogen replacement therapy, caffeine consumption and risk of developing PD in women. This convergence of laboratory and human epidemiological findings

strengthens the contention that widely used exogenous estrogens may interact with the nearly ubiquitously consumed $A_{2A}R$ antagonist caffeine to modify the risk of developing PD. The present study also establishes an animal model of this interaction in humans, along with the opportunity to pursue its molecular mechanism(s). In addition, the cumulative evidence for this interaction is sufficiently compelling to influence the design and interpretation of neuroprotection trials of estrogen or caffeine currently underway or under consideration (Ravina et al, 2003). Ultimately, a better understanding of the interplay between environmental factors like caffeine and estrogen may suggest effective preventative as well as therapeutic strategies for this neurodegenerative disorder.

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Figure legends

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Figure 2. **Low dose of caffeine attenuates MPTP-induced dopamine depletion in ovariectomized (*OVX*) but not sham-operated (*SHAM*) young female mice.** Ten days after ovariectomy or sham operation, mice received caffeine (5 or 20 mg/kg, i.p.) or saline administeration 10 min before a single dose of MPTP (40 mg/kg, i.p. N=8) or saline (N=5-7). One week later, striatal dopamine content was determined. Bars represent striatal dopamine levels (mean ± SEM) calculated as percentage of their respective controls (i.e., *S*+*S* group). *S*, saline; *M*, MPTP; *C5 or C20*, Caffeine 5 or 20 mg/kg. Data were analyzed by two-way ANOVA followed by Fisher's LSD test. **p<0.01, ***p<0.001, compared with respective *S*+*M* group.

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Supplemental Figures

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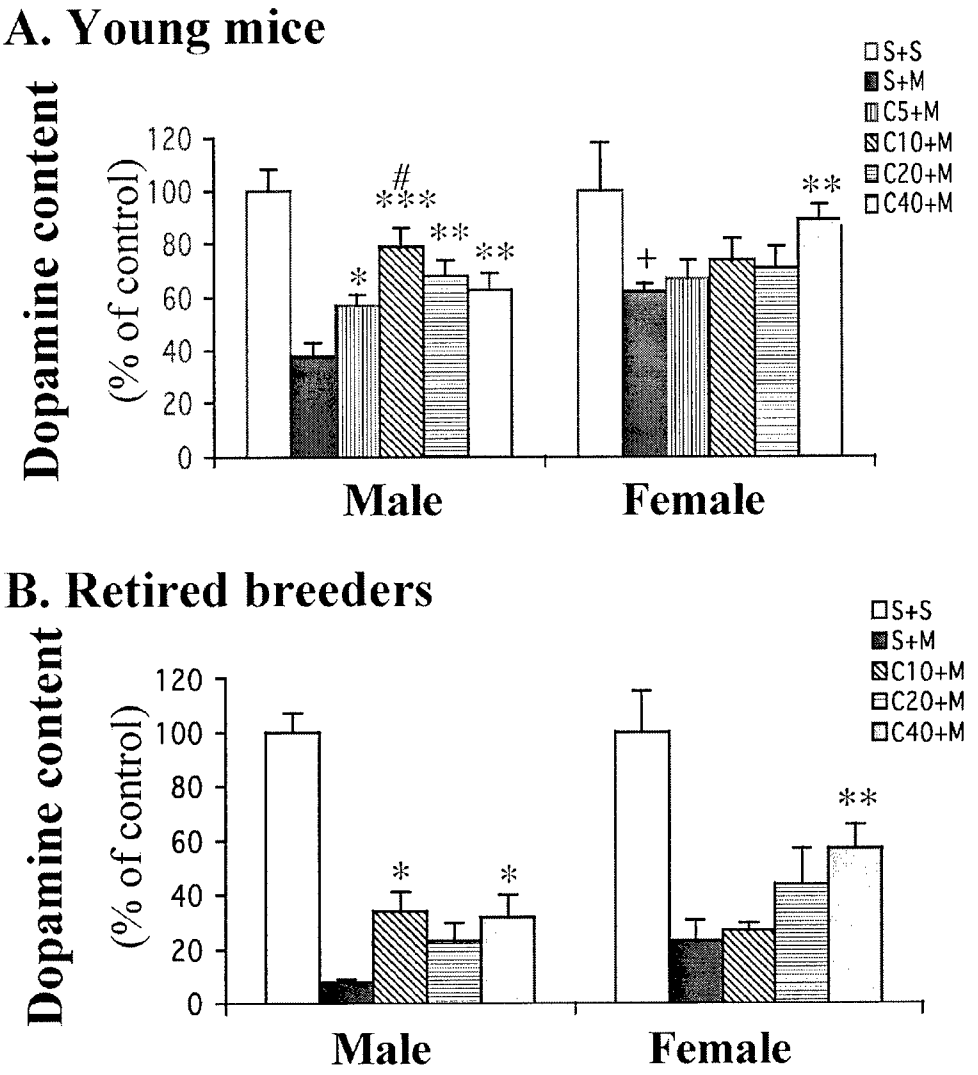

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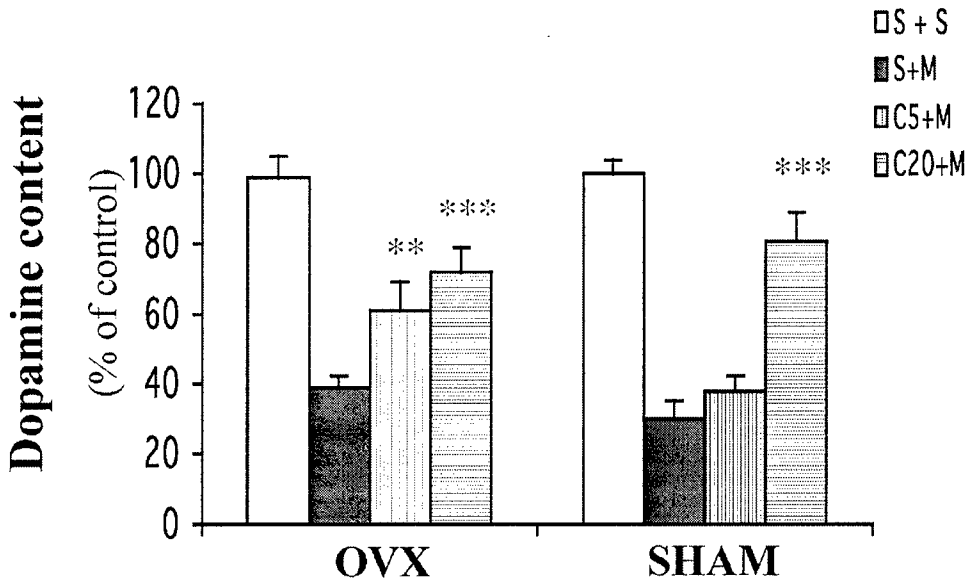

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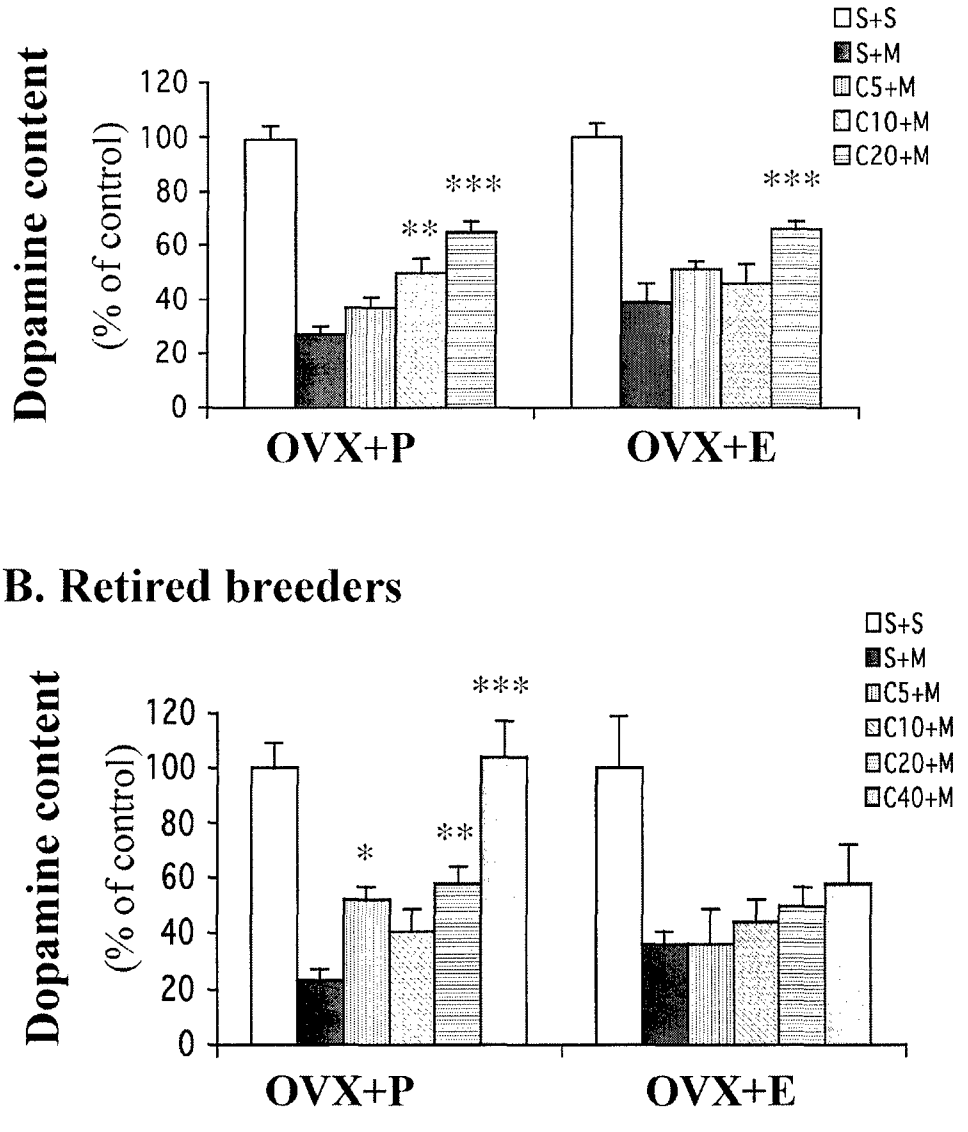

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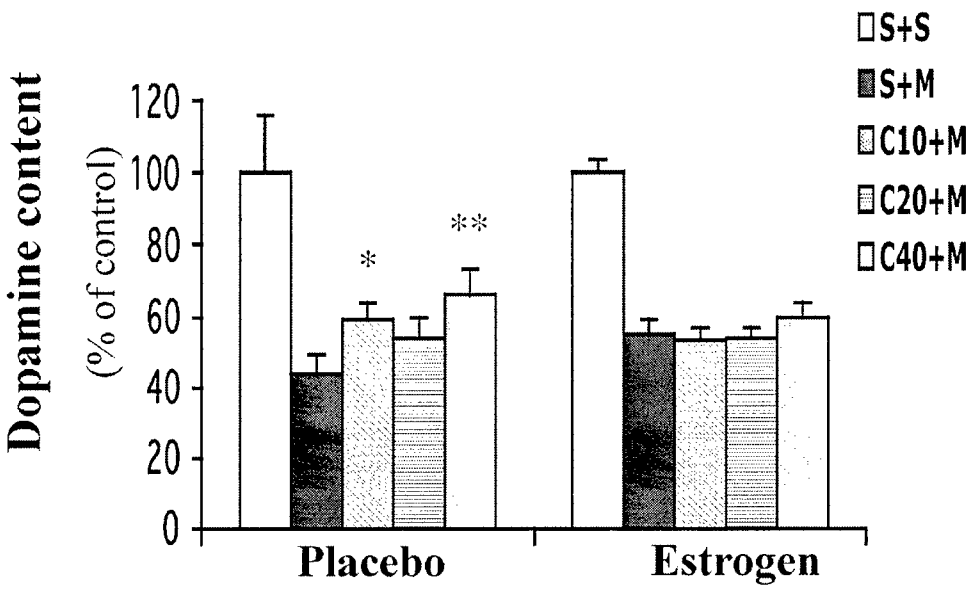

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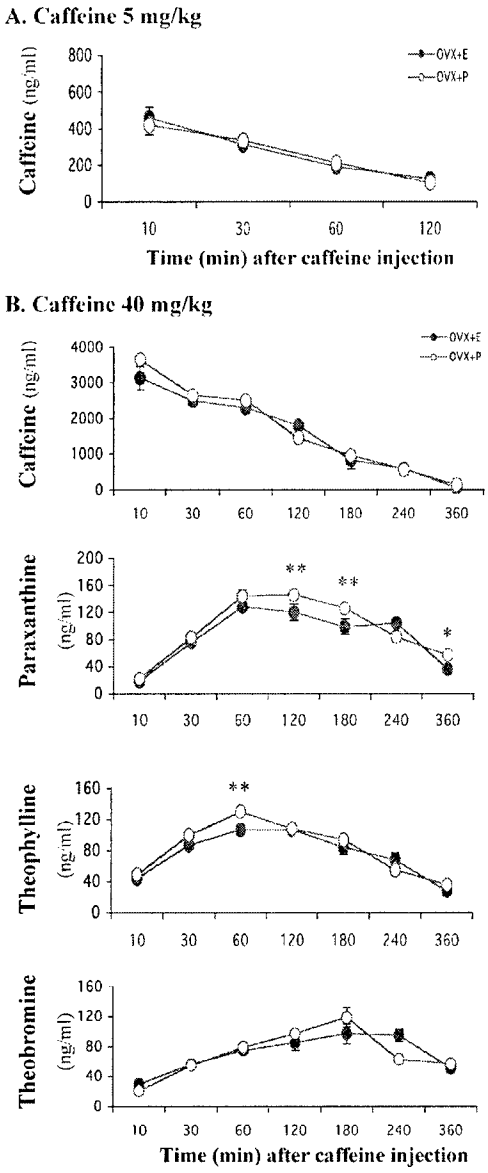

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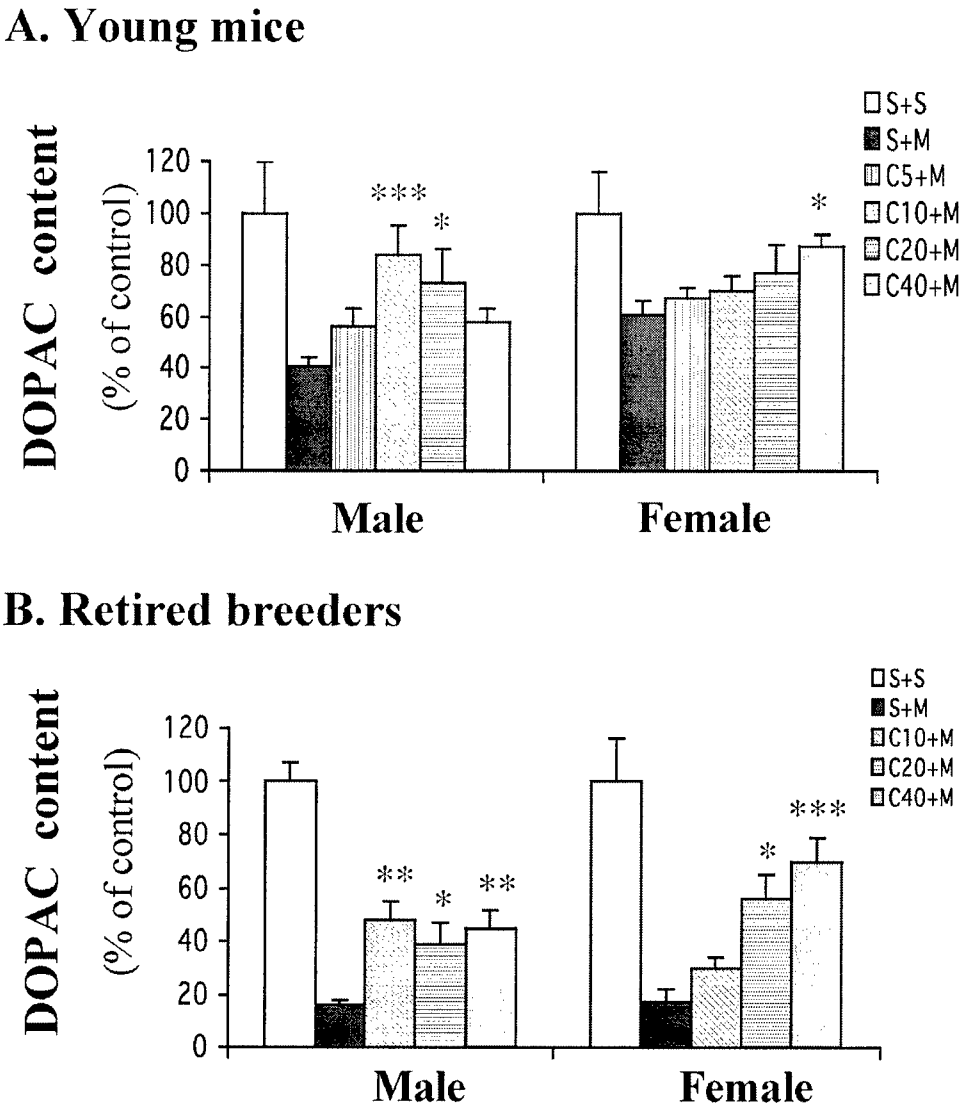

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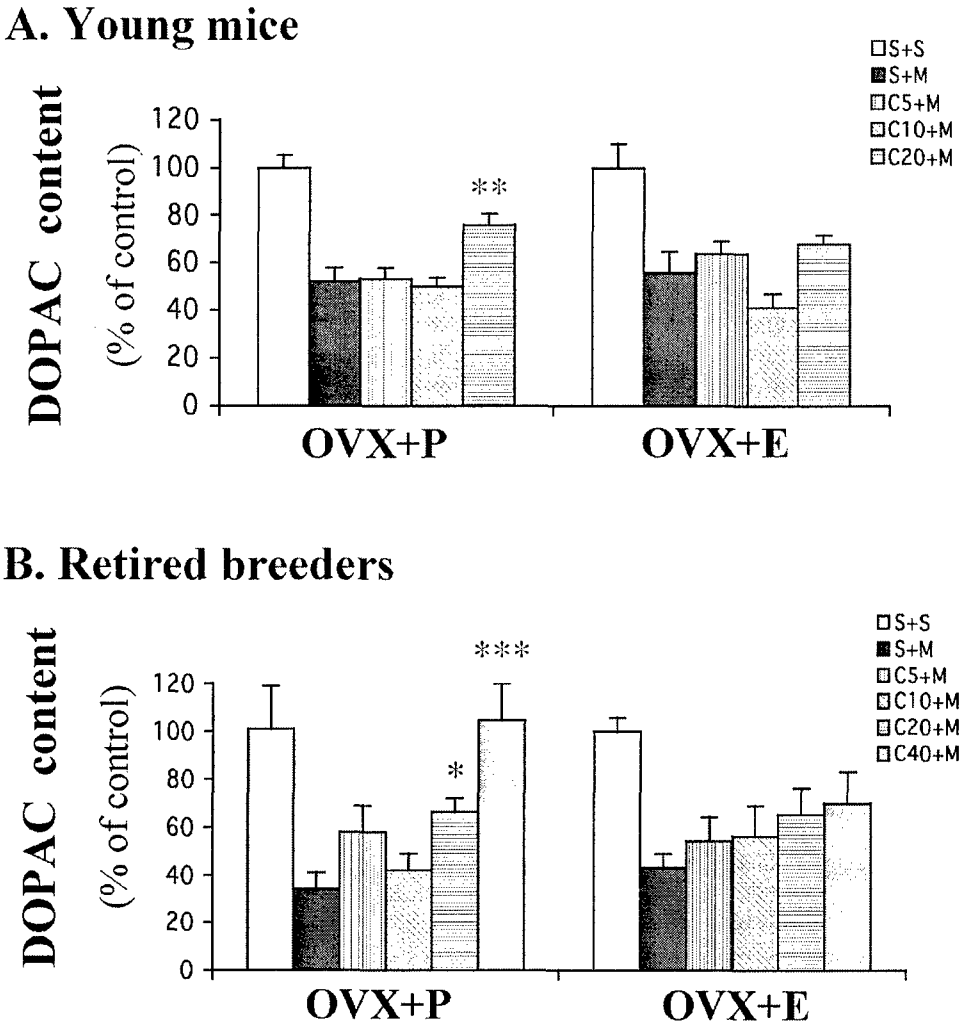

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Program Number: 665.12 **Day / Time:** Tuesday, Nov. 15, 11:00 AM - 12:00 PM

Estrogen reduces the neuroprotective effect of caffeine in a mouse model of Parkinson's disease

K.Xu[1*]; Y.Xu[1]; D.Brown-Jermyn[1]; J.F.Chen[2]; A.Ascherio[3]; D.E.Dluzen[4]; M.A.Schwarzschild[1]

1. MGH-MIND, Charlestown, MA, USA; 2. BU, Boston, MA, USA; 3. HSPH, Boston, MA, USA; 4. NEOUCOM, Rootstown, OH, USA

Epidemiological studies have linked caffeine consumption with a reduced risk of Parkinson's disease (PD) in men. Interestingly, in women this inverse association is present only in those who have not used postmenopausal estrogens, suggesting an interaction between the influences of estrogen and caffeine use on the risk of PD. To explore a possible biological basis for an estrogen-caffeine interaction in a mouse model of PD, we investigated the effect of caffeine on MPTP neurotoxicity, first in young or retired breeder (RB) male and female mice, then in ovariectomized (OVX) female mice or intact male mice implanted with estrogen or placebo pellets. Caffeine (0-40 mg/kg ip) was administered 10 min prior to a single MPTP injection (40 mg/kg ip). One week later striatal dopamine content was determined. 1. Caffeine treatment produced a dose-dependent attenuation of MPTP-induced striatal dopamine loss in both young and RB male mice. However, in female mice, only the highest dose of caffeine provided similar protection. 2. Lower doses of caffeine significantly reduced dopamine loss in OVX or OVX+placebo mice, but not in sham-operated or OVX + estrogen young female mice. Moreover, in OVX RB female mice, caffeine treatment, both lower and higher doses, significantly attenuated MPTP-induced dopamine loss only in placebo, but not in estrogen treated mice. 3. Caffeine's protection against dopamine depletion in male mice was lost after estrogen treatment. 4. There is no overall difference in brain levels of caffeine and its metabolites between OVX+placebo and OVX+estrogen mice. Taken together, these results demonstrate that estrogen reduces caffeine's neuroprotective effect in both male and female mice in the MPTP model of PD. They suggest a biological basis for the interaction between estrogen and caffeine in modifying the risk of PD, and provide a model for exploring further the mechanism of this interaction.

Support Contributed By: NIEHS,Beeson Program/AFAR & USAMRAA.

Citation: K.Xu, Y.Xu, D.Brown-Jermyn, J.F.Chen, A.Ascherio, D.E.Dluzen, M.A.Schwarzschild. Estrogen reduces the neuroprotective effect of caffeine in a mouse model of Parkinson's disease. Program No. 665.12. *2005 Abstract Viewer/Itinerary Planner.* Washington, DC: Society for Neuroscience

LEADING ARTICLE

Drugs Aging 2008; 25 (4)
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Adenosine A_{2A} Receptor Antagonists for Parkinson's Disease

Rationale, Therapeutic Potential and Clinical Experience

Robert A. Hauser[1] and *Michael A. Schwarzschild*[2]

1 Departments of Neurology, Pharmacology and Experimental Therapeutics, University of South Florida and Tampa General Healthcare, Tampa, Florida, USA
2 Department of Neurology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts, USA

Abstract

Long-term disability in Parkinson's disease (PD) is related to progression of the underlying disease and the emergence of complications of chronic levodopa therapy. There is a need for new medications that can slow the underlying progression of degeneration, improve PD symptoms in early disease without inducing dyskinesia, and improve motor fluctuations and 'off' time in advanced disease without worsening dyskinesia. Much interest has focused on the development of nondopaminergic therapies, with antagonists of the adenosine A_{2A} receptor emerging as leading candidates. A_{2A} receptors are selectively expressed in the basal ganglia and specific A_{2A} antagonists reverse motor deficits without causing dyskinesia in animal models of PD. The antiparkinsonian potential of A_{2A} receptor blockade has been expanded further by convergent epidemiological and laboratory findings suggesting a possible neuroprotective effect of A_{2A} receptor antagonists in PD. Istradefylline (KW-6002) is the first of several adenosine A_{2A} receptor antagonists in development for PD to advance to phase III clinical trials. Initial studies indicate that in patients with motor fluctuations on levodopa, addition of istradefylline reduces 'off' time. Additional studies are necessary to evaluate the benefit of istradefylline as monotherapy in early disease, its effect on the development of dyskinesia, and its effect on disease progression.

Although current medication treatment of Parkinson's disease (PD) provides good benefit for a number of years, long-term treatment remains inadequate. The underlying neuronal degeneration continues to progress and many patients develop long-term complications of dopamine replacement therapy.[1] Continued neuronal degeneration can lead to the emergence of dementia or imbalance, problems that can cause substantial disability and that are poorly responsive to symptomatic treatment. In addition, many patients develop fluctuations in response to treatment with levodopa and experience dyskinesia (twisting, turning movements) during peaks of levodopa-derived dopamine in the brain. Dyskinesia itself can be disabling,[2] and also commonly prevents the administration of additional dopaminergic medication to improve symptoms when levodopa has worn off ('off' time).

Because of these limitations of current therapy, an intense search for new medications to treat PD is ongoing. There is a need for medications that can slow the underlying progression of degeneration,

improve PD symptoms in early disease without inducing dyskinesia, and improve motor fluctuations and 'off' time in advanced disease without worsening dyskinesia. Much interest has focused on the development of nondopaminergic therapies, especially adenosine A_{2A} receptor antagonists. Istradefylline (KW-6002) is an adenosine A_{2A} receptor antagonist that is now in phase III clinical trials for PD.

1. Preclinical

1.1 Neurobiology of A_{2A} Receptors

The case for developing adenosine A_{2A} receptor antagonists as antiparkinsonian therapy has been built on a solid foundation of preclinical evidence. Here we review the distinctive neurobiology of this receptor, and then highlight findings in animal models of PD that have justified clinical trials of A_{2A} receptor antagonists. For more comprehensive assessments, the reader should see Kase et al.[3] and Xu et al.[4]

Adenosine is a ubiquitous purine that plays multiple fundamental roles within all cells, conveying genetic information (as an elemental component of DNA and RNA), storing metabolic energy (in adenosine triphosphate [ATP]) and transducing cytoplasmic signals (through cyclic adenosine monophosphate [cAMP]). In addition, adenosine functions outside of neurons and other cells to carry information between them.

The sources of extracellular adenosine acting on CNS neurons are poorly understood, but vary depending on neuronal locale (synaptic vs ambient) and conditions (normal vs pathological). Under basal conditions, ambient adenosine in the fluid bathing neurons may mirror intracellular levels because of passive bidirectional adenosine transporters, and in turn may exert a homeostatic influence on neuronal function.[5] By contrast, transient synaptic elevations in adenosine occur locally during vesicular neurotransmission with the co-release of ATP and classical neurotransmitters. ATP released into the synaptic cleft is rapidly converted to adenosine by the extracellular ectonucleotidase pathway.

Adenosine modulates neuronal function in mammals through four G protein-coupled adenosine receptor subtypes: A_1, A_{2A}, A_{2B} and A_3. The A_1 and A_{2A} subtypes bind adenosine with high affinity, are coupled to adenylate cyclase (negatively and positively, respectively) and are expressed in high levels in the brain. However, A_1 and A_{2A} receptors differ dramatically in their CNS distributions. Whereas A_1 receptors are widely expressed throughout the brain, the A_{2A} receptor is highly enriched in the basal ganglia[6] (figure 1a). The relatively selective expression of the A_{2A} receptor also contrasts with the broad distribution of neurotransmitter receptors (e.g. acetylcholine and glutamate receptors), antagonists of which are established nondopaminergic antiparkinsonian drugs. Although striatal receptors likely mediate the symptomatic benefits of anticholinergic agents (e.g. trihexyphenidyl) and putative antiglutamatergic agents (e.g. amantadine), their often prohibitive cognitive adverse effects presumably result from blockade of the high levels of extrastriatal (particularly cortical) receptors for these drugs (figure 1a, outer columns). The relatively restricted expression of the A_{2A} receptor may thus account for the low propensity for CNS adverse effects noted thus far for A_{2A} receptor antagonists in PD patients.[7-9]

The CNS pattern of A_{2A} receptor expression is notable not only for its relative restriction to the striatum (figure 1a), but also because of its distinctive cellular localisation within the striatum (figure 1b). Adenosine A_{2A} receptor messenger RNA (mRNA) is richly expressed in the subpopulation of medium-sized spiny GABAergic neurons forming the 'indirect' striatal output pathway, part of the basal ganglia circuitry that is disrupted in PD (as schematised in figure 2, and reviewed in more detail by Albin et al.,[10] Kase et al.,[11] and Wichmann and DeLong[12]). In the striatum, A_{2A} receptor mRNA co-localises with dopamine D_2 receptor mRNA[13] as well as preproenkephalin mRNA,[14] in the striato-pallidal neurons of the indirect pathway.

The molecular and cellular signals generated by A_{2A} receptor stimulation are just beginning to be understood. Biochemically, it is clear that the A_{2A}

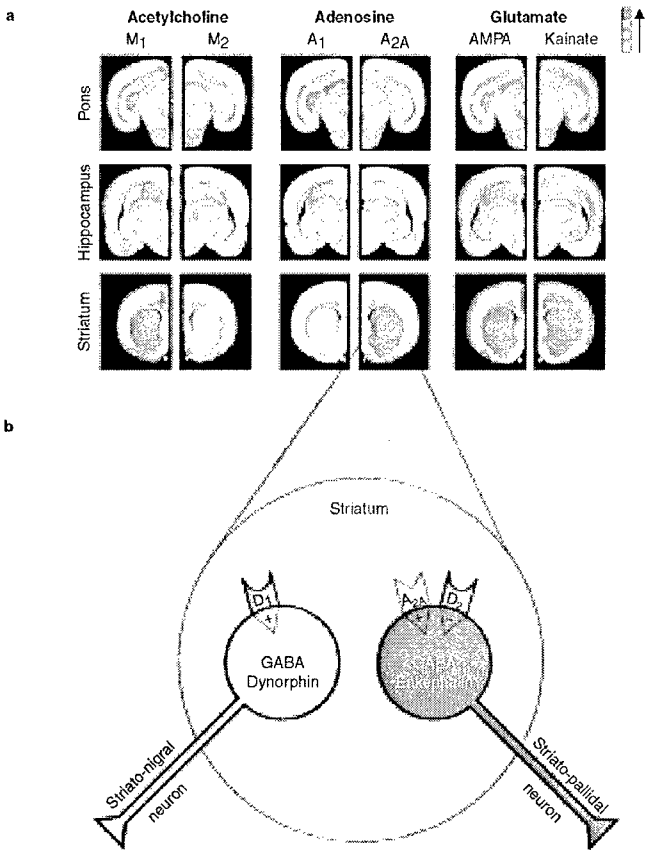

Fig. 1. Restricted regional and neuronal localisation of the adenosine A_{2A} receptor in brain. **(a)** Brain expression of subtypes of three neurotransmitter receptors targeted for nondopaminergic therapy in Parkinson's disease. Composite distributions of specific radioligand binding to subtypes of muscarinic cholinergic (M_1 and M_2), adenosinergic (A_1 and A_{2A}) and ionotropic glutamatergic (1-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid [AMPA] and kainate) receptors are shown in coronal sections from the caudal, mid- and rostral rat brain (containing pons, hippocampus and striatum, respectively). Increasing density of radioligand-binding is colour-coded over a spectrum from white to blue to yellow to red. Most striatal receptors that modulate dopaminergic neurotransmission are also widely distributed throughout the brain, whereas the A_{2A} subtype of adenosine receptor is relatively restricted in its expression to the striatum and the underlying olfactory tubercle (reproduced from Tohyama and Takatsuji[6] with permission from Oxford University Press and Igaku-Shoin Ltd). **[b]** Schematic of A_{2A} receptor distribution at the cellular level between the two major types of output neurons of the striatum. Striatal A_{2A} receptors are largely restricted to the GABAergic medium spiny neurons co-expressing enkephalin and dopamine D_2 receptors, and projecting to the globus pallidus. A_{2A} receptors through G protein-coupled signalling pathways generally activate (+) neuronal activity, an influence that is counterbalanced by the inhibitory (−) effects of D_2 receptors on these neurons.

receptor can couple through stimulatory G proteins to activate adenylate cyclase, thereby enhancing production of the second messenger cAMP. By contrast, the D_2 receptor is negatively coupled to adenylate cyclase through an inhibitory G protein. These opposing actions of A_{2A} and D_2 receptors co-localised to the striato-pallidal neuron (figure 1b) likely contribute to their counterbalancing behavioural as well as cellular effects.

The excitatory cellular effects of A_{2A} receptor activation on striato-pallidal neurons are manifest as enhanced GABA release from their nerve terminals in the globus pallidus pars externa (figure 2).[15] Increased pallidal GABA then suppresses the inhibitory (GABAergic) pallidal projections to the subthalamic nucleus (STN), thereby activating (disinhibiting) the excitatory effects of glutamatergic STN projections to the globus pallidus pars interna

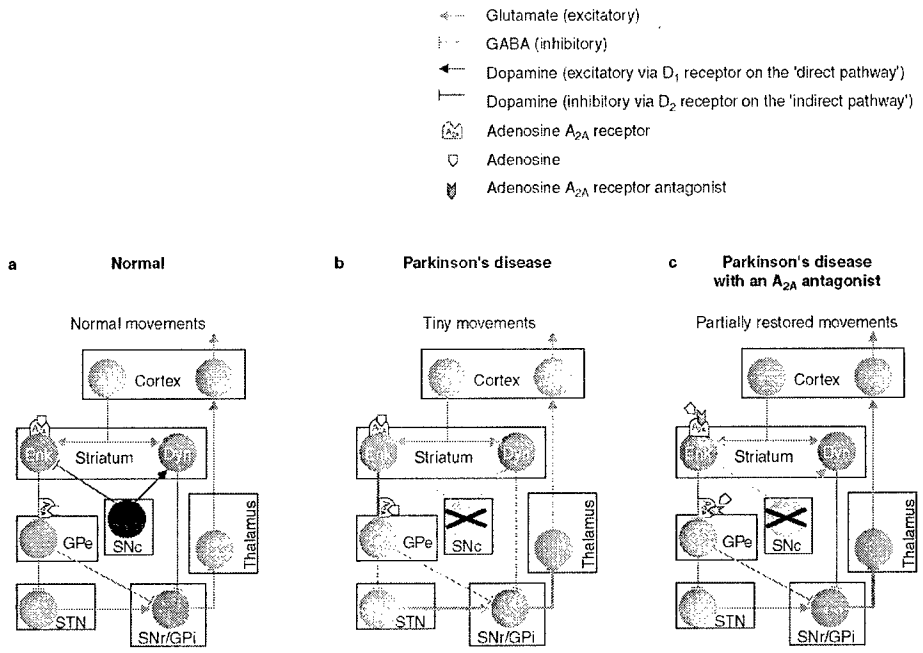

Fig. 2. Schematic of the probable mechanism by which A_{2A} receptor antagonists relieve motor symptoms in Parkinson's disease (PD). As depicted in a simplified diagram of basal ganglia activity and its normal modulation of movement (**a**), dopamine acts at the striatal level to facilitate movement through stimulatory D_1 receptors on striato-nigral neurons of the 'direct' pathway and through inhibitory D_2 receptors on striato-pallidal neurons of the 'indirect' pathway. Adenosine acts on stimulatory A_{2A} receptors on striato-pallidal neurons at postsynaptic sites in the striatum, and also at presynaptic sites on GABAergic nerve terminals in the globus pallidus pars externa (GPe). Loss of striatal dopamine in PD (**b**) disinhibits these striato-pallidal projection neurons, leading to GPe suppression and therefore disinhibition of the subthalamic nucleus (STN). Depletion of dopamine also leads to a parallel inhibition of striato-nigral neurons in the direct pathway. The resulting imbalance between the activities of the direct and indirect pathways in turn enhances inhibitory output from the internal GP (GPi) and substantia nigra pars reticulata (SNr) with excess inhibition of thalamo-cortical neurons, resulting in the characteristic reduced movement of PD. A_{2A} receptor blockade in PD (**c**) should result in recovery of GPe activity. This in turn would reduce the excessive excitatory drive from the STN to the GPi/SNr complex, thereby partially restoring balance between the direct and indirect pathways. Accordingly, overactivity of GPi/SNr output neurons (and the resultant motor deficits) in PD may be partially reversed by A_{2A} antagonists. Thickened or broken lines represent increased or decreased neural activity, respectively, along a pathway (reproduced from Albin et al.,[10] with permission; see also Xu et al.[4] and Kase et al.[11]). **Enk** = enkephalin; **Dyn** = dynorphin; **SNc** = substantia nigra pars compacta. **((Author: in our current house style we do not use variant font sizes. Is this suggested solution OK and, if not, do you have any other suggestions?))**

and substantia nigra pars reticulata. Activation of these GABAergic output nuclei of the basal ganglia leads to inhibition of the thalamic motor nuclei, which in turn leads to a reduction in the facilitative influence of thalamo-cortical activity onto the motor cortex. Through this mechanism, A_{2A} receptors may contribute to motor inhibition in PD. Conversely, A_{2A} antagonists partially reverse motor deficits through their inactivation of A_{2A} receptors on striato-pallidal neurons. In support of this model, it has recently been demonstrated that the motor stimulant action of the A_{2A} antagonist KW-6002 is eliminated after conditional knockout of the A_{2A} receptor gene in neurons of the striatum.[16]

2. Influence of A_{2A} Receptor Antagonists on Parkinson's Disease (PD) Pathophysiology

2.1 Symptomatic Actions

Although the anatomy of the A_{2A} receptor distinguishes it from other nondopaminergic targets in the quest for improved antiparkinsonian therapy, it is the behavioural pharmacology of A_{2A} receptor antagonists that has provided the central rationale for their development as antiparkinsonian agents. Relatively specific A_{2A} receptor antagonists consistently reverse motor deficits or enhance dopaminergic treatments in animal models of PD. For example, in rats with unilateral 6-hydroxydopamine (6-OHDA) lesions of the dopaminergic pathway, A_{2A} receptor antagonists including SCH 58261, KW-6002 and MSX-3 all potentiated the contralateral turning behaviour induced by levodopa or a dopamine agonist.[17-20] In addition, motor stimulation by an A_{2A} receptor antagonist in this model showed no tolerance after repeated treatment.[21]

Blockade of dopamine receptors with haloperidol and depletion of dopamine by reserpine are also used to model the hypodopaminergic motor dysfunction of PD. Under these conditions, rodents enter an akinetic cataleptic state, which can be reversed by the administration of A_{2A} receptor antagonists.[22-24] Moreover, KW-6002 in combination with levodopa synergistically improves motor function in both haloperidol- and reserpine-treated rodents.[24]

Rigidity and rest tremor are also cardinal features of PD, and can be as disabling as bradykinesia. A_{2A} blockade has been found to improve abnormalities of muscle tone and tremors in rodents, extending the potential benefit of A_{2A} blockade for PD symptoms.[25-27] Again, combining an A_{2A} receptor antagonist with levodopa produced a synergistic antiparkinsonian effect, in this case by alleviating the muscle rigidity induced in rodents by drugs that disrupt dopaminergic neurotransmission.[24]

With rodent studies suggesting the considerable potential of A_{2A} receptor antagonists as symptomatic therapy for PD, evaluation of their efficacy was warranted in nonhuman primate models of PD. Initial investigations[28-30] confirmed the potential for facilitating motor activity while also demonstrating a low potential for inducing or exacerbating dyskinesias. In monkeys treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), KW-6002 produced a significant improvement in motor disability which persisted unabated over at least 3 weeks of treatment. Moreover, the observed reduction in motor disability scores was not accompanied by abnormal or excessive movements such as stereotypies or dyskinesia.[28,29] In the same animal model, KW-6002 was found to synergistically enhance the antiparkinsonian action of a low dose of levodopa or D_2 receptor agonist without exacerbating dyskinesia.[29,30] These latter studies helped establish the rationale for the initial clinical trials of an A_{2A} receptor antagonist in patients with relatively advanced PD.

3. Disease-Modifying Potential

3.1 Neuroprotection

3.1.1 Caffeine in the Epidemiology of PD

Recent epidemiological findings have converged with laboratory studies to suggest that A_{2A} receptor blockade by caffeine, a nonselective adenosine receptor antagonist, protects against the underlying neurodegeneration of PD. Coffee or tea drinking has

emerged as the dietary factor most consistently associated with a lowered risk of developing PD.[31] An inverse relationship between coffee drinking and PD was first demonstrated in case-control studies in the 1990s. Subsequent prospective studies strengthened the link between caffeine consumption and a reduced risk of later developing PD.[32,33] Thirty years after some 8000 Japanese-American men reported their dietary caffeine consumption, over 100 of them developed PD. Higher coffee intake at enrollment was dose-dependently associated with a reduced incidence of PD, including a >5-fold lower risk in those who drank over four medium (6oz) cups per day.[32] Confirmation of these findings was obtained in a prospective study of a larger, multi-ethnic population of men.[33] Coffee drinking, tea drinking, non-coffee caffeine consumption and total caffeine consumption were inversely correlated with the incidence of subsequent PD. By contrast, consumption of decaffeinated coffee was not associated with an altered risk of PD, clearly implicating caffeine as the component responsible for the inverse relationships.

Interestingly, comparison of the female and male cohorts by Ascherio et al.[33] exposed a stark gender difference, with no clear association between PD and caffeine intake apparent in women (as had been previously observed).[34] However, subsequent stratification of the women by estrogen exposure history in two independent prospectively followed cohorts[35,36] implicated a hormonal basis for this gender difference. In each study, in the subset of women who did not use postmenopausal estrogens, caffeine was significantly associated with a reduced risk of subsequent PD, just as in men.

3.1.2 Neuroprotection by Caffeine and More Specific A_{2A} Receptor Antagonists

Despite their strengths, these epidemiological investigations cannot directly address the critical question: does caffeine prevent PD, or does early PD (or its causes) prevent the use of caffeine? Though the question of causality is difficult to answer in humans, animal models can offer useful clues. The hypothesis that caffeine protects humans from dopaminergic neurodegeneration has been strengthened by the finding that caffeine and more specific antagonists of the A_{2A} receptor protect against dopaminergic neuron toxicity in rodent models of PD.

The effect of caffeine on the demise of nigrostriatal dopaminergic neurons has been investigated in the MPTP[37,38] and 6-OHDA[39] models of PD. Caffeine, when administered to mice at doses corresponding to those of typical human use, dose-dependently reversed the loss of striatal dopamine induced by MPTP.[37] Caffeine also prevented MPTP- or 6-OHDA-induced loss of dopaminergic nerve terminals in the striatum, and of dopaminergic cell bodies in the substantia nigra.[37,39,40]

The neuroprotective effects of caffeine appear to be mediated primarily by its antagonistic action at the A_{2A} subtype of the adenosine receptor. MPTP- or 6-OHDA-induced nigro-striatal lesions were attenuated by all A_{2A} receptor antagonists tested to date, including KW-6002[37,41,42] and SCH 58261.[37] Furthermore, A_{2A} knockout mice (lacking functional A_{2A} receptors as a result of targeted gene disruption)[43,44] were assessed for their susceptibility to dopaminergic neuron toxins. MPTP- (but not 6-OHDA-) induced losses of striatal dopamine and dopamine transporter binding sites were significantly attenuated in A_{2A} knockout mice compared with their wild-type littermates.[37,45] Thus, complimentary genetic and pharmacological approaches have generally demonstrated that A_{2A} receptor inactivation, like caffeine, can protect dopaminergic neurons. Overall, these data suggest that caffeine can protect against dopaminergic neuron injury and death through its antagonistic action at the adenosine A_{2A} receptor. How the A_{2A} receptor contributes to dysfunction and death of dopaminergic neurons remains unclear, but several plausible hypotheses[4] are being pursued.

3.2 Preventing Dyskinesias

A major impetus for developing novel nondopaminergic pharmacological approaches to PD has been the need to treat parkinsonian deficits without inducing or exacerbating dyskinesias. A_{2A} receptor antagonists have indeed demonstrated a low potential for triggering dyskinesias in nonhu-

man primates. In addition, laboratory studies have raised the possibility that A_{2A} receptor blockade may provide an additional benefit of preventing the development of dyskinesias in the first place.

Using a variety of rodent models of levodopa-induced dyskinesia in PD, several laboratories have evaluated the antidyskinetic potential of A_{2A} receptor antagonists.[4] Some of these investigations suggest that ongoing A_{2A} blockade may prevent the development of the maladaptive basal ganglia neuroplasticity that underlies dyskinesias.[21,45,46] Other studies that did not find evidence that A_{2A} receptor antagonists can prevent the development of sensitised motor responses to L-dopa did, however, suggest that A_{2A} blockade may reduce their expression – either directly[47] or indirectly (by allowing the use of lower levodopa doses to achieve the same antiparkinsonian benefit).[48]

To better assess the prophylactic antidyskinetic potential of A_{2A} receptor antagonists, Bibbiani et al.[46] evaluated the primate model of dyskinesias. Monkeys previously lesioned (and made parkinsonian) by MPTP were treated daily with apomorphine (a short acting dopamine agonist) together with oral KW-6002 or vehicle for approximately 3 weeks, after which the daily treatments of apomorphine alone were continued for 3 more weeks. These investigators found that, in control animals, dyskinesias consistently developed 1–2 weeks after initiating dopaminergic treatment and persisted for the duration of the experiment. In contrast, monkeys that were initially treated for 3 weeks with dopamine agonist plus KW-6002 did not develop any abnormal movements during this co-treatment period, and only began to manifest dyskinesias 1–2 weeks after discontinuing the A_{2A} receptor antagonist. This study supports a role for the A_{2A} receptor in the development of dyskinesia (rather than in its expression) in primates, and provides additional rationale for targeting early PD patients in future human trials of A_{2A} receptor antagonists.

3.3 Clinical Trials

Based on preclinical studies, adenosine A_{2A} receptor antagonists have the potential to improve symptoms when taken as monotherapy in early PD and to reduce 'off' time without causing or worsening dyskinesia when taken as an adjunct to levodopa in advanced disease. Istradefylline (KW-6002) is now in phase III clinical trials for advanced PD, and results of several trials of this agent are available.

Bara-Jimenez et al.[7] at the US National Institutes of Health (NIH) conducted a 6-week, randomised, blinded, 'proof-of-principle' study using intravenous levodopa infusions to evaluate istradefylline in PD patients with moderate to advanced disease. Fifteen patients participated in the study. All were experiencing motor fluctuations on levodopa and six also exhibited peak-dose dyskinesias. Twelve patients were randomised to istradefylline treatment and three to placebo. One istradefylline subject withdrew from the study because of the need to take a prohibited medication. Subjects randomised to istradefylline received placebo for 2 weeks, istradefylline 40 mg/day for the next 2 weeks, and istradefylline 80 mg/day for the final 2 weeks. Efficacy evaluations were performed at the end of weeks 2 (baseline), 4, and 6. Oral antiparkinsonian medications were withheld on the night prior to the evaluation day and until assessments were completed. At each evaluation visit, function was assessed using the motor subsection of the Unified Parkinson's Disease Rating Scale (UPDRS) and the Abnormal Involuntary Movement Scale (AIMS). The effect of istradefylline administration was evaluated: (i) alone; (ii) with a suboptimal levodopa infusion; (iii) with an optimal levodopa infusion; and (iv) periodically following discontinuation of levodopa infusion. Results were analysed within active treatment subjects, comparing observations at weeks 4 and 6 to those at week 2.

When administered alone, istradefylline had no effect on parkinsonian signs and did not cause dyskinesias. Similarly, during optimal levodopa infusions, istradefylline had no effect on parkinsonian signs or dyskinesias. In contrast, during suboptimal levodopa infusions, istradefylline significantly improved parkinsonian signs. Motor UPDRS scores improved by 24% with istradefylline 40mg (from 30 ± 2.8 to 23 ± 3.6, $p < 0.05$) and by 36% with

istradefylline 80mg (from 30 ± 2.8 to 19 ± 4.3, $p < 0.02$). Of note, the antiparkinsonian response to istradefylline 80mg plus suboptimal levodopa infusion was similar to that produced by an optimal levodopa infusion, but with significantly less dyskinesia (45%, $p < 0.05$). Benefit was also identified for each of the individual cardinal signs, namely tremor ($p < 0.002$), rigidity ($p < 0.01$) and bradykinesia ($p < 0.05$). In addition, the time for the antiparkinsonian effect to decline by 50% following discontinuation of an optimal-dose levodopa infusion was increased by 76% ($p < 0.05$), from 62 ± 14 minutes to 109 ± 24 minutes. Istradefylline was relatively well tolerated. The most frequent adverse events were nausea (3), increased stiffness (3), headache (2) and increased dyskinesias (2).[7]

It is not clear why no antiparkinsonian benefit was identified when istradefylline was administered alone in this study and no other experience is currently available with istradefylline monotherapy in PD patients. It is possible that a higher istradefylline dose administered alone might provide antiparkinsonian benefit in this population. Perhaps more importantly, istradefylline monotherapy might provide benefit in early disease, when endogenous dopamine production and release are still present, although this has not yet been studied. The main findings of the NIH study, namely improved response when istradefylline is administered with a suboptimal levodopa infusion, and extension of the half-time of benefit following discontinuation of a levodopa infusion, suggest that istradefylline might be of benefit to reduce 'off' time in advanced patients with motor fluctuations, and possibly with reduced dyskinesia.

Hauser et al.[8] performed a 12-week, double-blind, placebo controlled, exploratory study of istradefylline as an adjunct to levodopa in PD patients with both motor fluctuations and dyskinesias (US-001 study). Patients were required to have at least 90 minutes of 'off' time between the first dose of levodopa after waking up for the day and 8 hours later to be eligible for the trial. Eighty-three subjects entered the study and were randomised to placebo, istradefylline 5 mg/day during weeks 1 to 4, 10 mg/day during weeks 5 to 8 and 20 mg/day during weeks 9 to 12 (5/10/20 group), or istradefylline 10 mg/day during weeks 1 to 4, 20 mg/day during weeks 5 to 8 and 40 mg/day during weeks 9 to 12 (10/20/40 group). Evaluations were undertaken at baseline and every 2 weeks through the study. Subjects completed 3 daily home diaries during the week before each visit.

Subjects randomised to istradefylline experienced a significant decrease in 'off' time compared with subjects randomised to placebo. Istradefylline recipients experienced a (mean ± standard error) reduction in the proportion of the awake day spent in the 'off' state of 7.1% ± 2.0% compared with an increase of 2.2% ± 2.7% in the placebo group ($p = 0.008$). Both istradefylline dose groups experienced a significant reduction in percent 'off' time compared with placebo. Similarly, subjects assigned to istradefylline experienced a reduction in 'off' time of 1.2 ± 0.3 hours compared with an increase of 0.5 ± 0.5 hours in the placebo group ($p = 0.004$). Thus, compared with placebo, istradefylline reduced 'off' time by 1.7 hours. This difference began to emerge at week 8 and was maximal at week 12, probably as a result of the escalating dose design of the study.

There were no significant differences in ratings of dyskinesia severity between the istradefylline and placebo groups. However, there was an increase in 'on' time with dyskinesia in the istradefylline group. It is difficult to determine the functional impact of this change because the diaries used in this study did not include such an assessment. Diaries that include the designation of troublesome and nontroublesome dyskinesia can help evaluate this effect and were therefore used in subsequent studies.[49]

Istradefylline was generally well tolerated. The most common adverse events in the istradefylline group were nausea (istradefylline 27.8% vs placebo 6.9%), aggravation of dyskinesia (16.7% vs 13.8%), dizziness (13.0% vs 3.4%) and vomiting (9.3% vs 6.9%). Nausea was mostly mild in intensity and usually resolved within 10 days.

This exploratory study suggested that istradefylline reduces 'off' time in patients with motor fluctu-

ations on levodopa and indicated that this should be the primary outcome variable in definitive studies of advanced PD patients. Severity of dyskinesia was not increased, although time 'on' with dyskinesia was increased.[8]

Two additional studies evaluating istradefylline as adjunctive therapy (US-005 and US-006) have been reported in abstract form.[9,50] These were 12-week, randomised, double-blind, placebo controlled studies of istradefylline 20, 40, or 60 mg/day in patients on levodopa with at least 2 hours of 'off' time on baseline diaries. In the 005 study, subjects were randomised to istradefylline 40 mg/day ($n = 130$) or placebo ($n = 66$); in the 006 study, subjects were randomised to istradefylline 20 mg/day ($n = 163$), istradefylline 60 mg/day ($n = 155$) or placebo ($n = 77$). In the 005 study, subjects assigned to istradefylline 40 mg/day experienced a (mean ± SD) reduction in 'off' time of 1.8 ± 2.8 hours compared with a reduction of 0.6 ± 2.7 hours in the placebo group ($p = 0.006$). Thus, compared with placebo, istradefylline provided a reduction of 'off' time of 1.2 hours. In the 006 study, there was a strong trend for greater reduction of percentage 'off' time in subjects assigned to istradefylline compared with placebo. The reductions in percent 'off' time were $7.9\% \pm 15.5\%$ and $8.1\% \pm 14.2\%$ in the istradefylline 20 mg/day and 60 mg/day groups, respectively, and $4.3\% \pm 17.2\%$ in the placebo group ($p < 0.1$). When baseline percent 'off' time was included as a covariate, both istradefylline doses were found to provide a significant reduction in percent 'off' time compared with placebo ($p = 0.026$ and $p = 0.024$). 'On' time with nontroublesome dyskinesia increased with istradefylline in both studies but 'on' time with troublesome dyskinesia did not. The most common adverse events were increased dyskinesia (20 mg/day, 23.9%; 40 mg/day, 30.2%; and 60 mg/day, 21.5% vs 005 placebo 5.2%, and 006 placebo 14.3%) and nausea (20 mg/day, 10.4%; 40 mg/day, 10.9%; and 60 mg/day, 19.0% vs 005 placebo 9.1%, and 006 placebo 6.5%).[9,50]

Thus, clinical trials of istradefylline to date have provided rather consistent outcomes. In patients with motor fluctuations on levodopa, istradefylline reduces 'off' time by approximately 1.2 to 1.7 hours compared with placebo. In contrast to animal studies, there is some increase in dyskinesia but this increase appears to be an increase in nontroublesome dyskinesia and, therefore, may not have a negative impact. The most common adverse effect is nausea, occurring in 1–13% more patients receiving istradefylline than placebo. Nausea is generally mild and usually resolves within 10 days. Based on these clinical trials, istradefylline appears to be on track for approval as an adjunct to levodopa to reduce 'off' time in advanced PD patients.

4. Conclusion

In animal models, adenosine A_{2A} receptor antagonists, either as monotherapy or as adjuncts to levodopa, have been demonstrated to improve parkinsonian signs without causing or worsening dyskinesia. This suggests that they may be useful as monotherapy in early PD to provide benefit without inducing dyskinesia and as adjuncts to levodopa in advanced disease to improve antiparkinsonian response without worsening dyskinesia. A_{2A} receptor antagonists will be tested in early PD patients to assess antiparkinsonian efficacy and their ability to avoid or delay dyskinesia. In advanced PD, istradefylline (KW-6002) has been shown in preliminary studies to reduce 'off' time, with only a modest increase in dyskinesia. It is anticipated that other A_{2A} receptor antagonists will soon enter clinical testing. In addition to symptomatic benefits, A_{2A} receptor antagonists have been demonstrated to protect dopamine neurons *in vivo* from a variety of toxic insults. This suggests that they might have the potential to slow progression of PD and are candidates for clinical trials evaluating neuroprotection.

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A Crucial Role for Forebrain Adenosine A_{2A} Receptors in Amphetamine Sensitization

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Adenosine A_{2A} receptors (A_{2A}Rs) are well positioned to influence the maladaptive CNS responses to repeated dopaminergic stimulation in psychostimulant addiction. Expression of A_{2A}Rs in brain is largely restricted to the nucleus accumbens and striatum, where molecular adaptations mediate chronic effects of psychostimulants such as behavioral sensitization. Using a novel forebrain-specific conditional (Cre/*loxP* system) knockout of the A_{2A}R in coordination with classical pharmacological approaches, we investigated the involvement of brain A_{2A}Rs in amphetamine-induced behavioral sensitization. Tissue-specific, functional disruption of the receptor was confirmed by autoradiography, PCR, and the loss of A_{2A} antagonist-induced motor stimulation. Daily treatment with amphetamine for 1 week markedly enhanced locomotor responses on day 8 in control mice and the sensitization remained robust after a week of washout. Their conditional knockout littermates however showed no sensitization to amphetamine on day 8 and only a modest sensitization following the washout. Pharmacological blockade of adenosine A_{2A}Rs also was able to block the development (but not the expression) of sensitization in multiple mouse strains. Thus activation of brain A_{2A}Rs plays a critical role in developing augmented psychomotor responses to repeated psychostimulant exposure.
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Keywords: adenosine; A_{2A} receptors; brain; conditional knockout; sensitization; amphetamine

INTRODUCTION

The behavioral effects of psychostimulants such as amphetamine and cocaine are mediated by their indirect activation of dopamine receptors in the nucleus accumbens and caudate-putamen (Koob, 1996). Psychostimulants are thought to be addictive due to neuronal and molecular adaptations both within and outside the mesoaccumbens circuitry (Vanderschuren and Kalivas 2000). Behavioral sensitization, which is described as a progressive augmentation of responses to repeated drug administration, is an expression of neuroadaptations. In humans, sensitization to psychostimulant drugs may appear as craving behavior or paranoia (Robinson and Berridge 1993; Kalivas *et al*, 1998). Critical roles have been established for dopaminergic and subsequently glutamatergic transmission in psychostimulant-induced behavioral sensitization (Wolf, 1998). Recently, the adenosine A_{2A} receptor (A_{2A}R) has also emerged as a potential modulator of psychostimulant sensitization and as an attractive therapeutic target. A_{2A}Rs are ideally located to modulate neuronal pathways involved in psychostimulant sensitization, given that their brain expression is largely restricted to the nucleus accumbens, striatum, and olfactory tubercle (Rosin *et al*, 2003). Furthermore, A_{2A} adenosinergic and D_2 dopaminergic systems interact at the membrane (Ferre *et al*, 1994; Canals *et al*, 2003), intracellular (Morelli *et al*, 1995), and behavioral levels (Ferre *et al*, 1993; Fenu *et al*, 1997). In addition, activation of A_{2A}Rs enhances the release of several brain neurotransmitters including dopamine and glutamate, which contribute to the development of psychostimulant behavioral sensitization (Quarta *et al*, 2004).

Recent pharmacological data provide direct evidence for an important role of adenosine A_{2A}Rs in the long-term adaptive responses to repeated dopaminergic stimulation both in rats (Bove *et al*, 2002; Bibbiani *et al*, 2003) and non-human primates (Bibbiani *et al*, 2003). In addition, using A_{2A}R knockout (A_{2A} KO) mice, our laboratory has shown that behavioral sensitization to repeated treatment either with L-dopa in hemiparkinsonian mice or with amphetamine in unlesioned mice does not develop in the absence of

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the $A_{2A}R$ (Fredduzzi *et al*, 2002; Chen *et al*, 2003). However, a facilitative role of $A_{2A}Rs$ in sensitization is controversial and other reports have shown that the A_{2A} agonist CGS21680 attenuates the development of behavioral sensitization induced by methamphetamine (Shimazoe *et al*, 2000). This discrepancy between genetic and pharmacological studies of $A_{2A}Rs$ functions may reflect particular limitations of either of these approaches, for example, developmental or chronic inactivation of the $A_{2A}R$ in the KO model, or exogenous *vs* endogenous modulation of the adenosinergic system using a pharmacological agonist.

In order to clarify the role for $A_{2A}Rs$ in behavioral adaptations induced by repeated psychostimulant exposure, we used a brain-specific conditional KO of the $A_{2A}R$ in coordination with a classical pharmacological approach. The genetic model allows us to specifically explore the $A_{2A}R$ in forebrain areas and to avoid possible compensatory developmental responses of other genes, given the fact that the deletion of the $A_{2A}R$ is postnatal. Moreover, we took advantage of newly available A_{2A} antagonists, which offer improved specificity despite persistent problems of solubility and stability, to test the role of the $A_{2A}R$ in the development of amphetamine sensitization and its discrete phases (induction and expression).

MATERIALS AND METHODS

Generation and Genotyping of Postnatal Forebrain-Specific A_{2A} Conditional KO Mice (Cre/*loxP* System)

The *L7ag13* line of *CaMKIIα-cre* transgenic mice (in a C57Bl/6 background) expresses the Cre recombinase under the direction of the *CaMKIIα* gene promoter in postnatal neurons of the striatum as well as other forebrain structures and in germline cells (Dragatsis *et al*, 2000; Dragatsis and Zeitlin 2000; Morozov *et al*, 2003) and was kindly provided by WT Dauer, A Morozov, R Hen, and ER Kandel. Mice with a 'floxed' adenosine $A_{2A}R$ gene were generated by insertion of *loxP* sequences within the introns flanking a critical exon (2) of the $A_{2A}R$ gene (YJ Day and J Linden, unpublished results). Homozygous floxed ($A_{2A}{}^{flox/flox}$) mice (F5 generation in a mixed 129-Steel and C57Bl/6 genetic background) were crossed with *L7ag13 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 were compared in an initial autoradiographic and pharmacological assessment (Figure 1a, c, and d). A_{2A} genotypes for several brain regions and peripheral tissues from (4-month-old) adult $A_{2A}{}^{flox/+}$ (and *cre*(−) $A_{2A}{}^{flox/+}$) littermates were compared (Figure 1b) to test for tissue-specific expression of the *cre* transgene and consequent Cre-mediated recombination of the floxed A_{2A} allele.

Initial efforts to expand the numbers of *cre*(+) $A_{2A}{}^{flox/flox}$ and *cre*(−) $A_{2A}{}^{flox/flox}$ littermates were complicated by unexpected germline *cre* transgene expression and recombination events in some female *cre*(+) $A_{2A}{}^{flox/+}$ breeder mice (in addition to those expected in male *cre*(+) $A_{2A}{}^{flox/+}$ mice; Dragatsis *et al*, 2000; Dragatsis and Zeitlin 2000; Zakharenko *et al*, 2003). This newly identified *CaMKIIα* promoter-driven recombination in female as well as male gametes may reflect differences in Cre-mediated recombination in germline cells seen with different floxed genes—presumably due to differences in local chromatin structure effects on *loxP* site accessibility (Morozov *et al*, 2003). Thus, the expected $A_{2A}{}^{flox/flox}$ offspring resulting from crossing a female *cre*(+) $A_{2A}{}^{flox/+}$ with male *cre*(−) $A_{2A}{}^{flox/+}$ mice were found to have one recombined (−) as well as one floxed A_{2A} allele (ie to be $A_{2A}{}^{flox/-}$). The *cre*(+) $A_{2A}{}^{flox/-}$ mice among these offspring were then crossed with their *cre*(−) $A_{2A}{}^{flox/-}$ littermates in multiple matings to produce the 16 male $A_{2A}{}^{flox/-}$ (half that were *cre*(+) and half that were *cre*(−)) employed in this study of amphetamine sensitization (Figure 2).

Genotyping for the presence of the *cre* transgene and separately for the presence of the wild-type (WT), floxed, or recombined (inactivated) alleles of the $A_{2A}R$ gene was conducted by polymerase chain reaction (PCR) analysis of tail DNA unless otherwise indicated. The three-probe PCR strategy employed for A_{2A} genotyping is schematized in Figure 1b, and is based on the location of *loxP* inserts within the gene (Day and Linden, unpublished results).

Animals and Drug Treatments

All experiments were performed in accordance with the Massachusetts General Hospital and NIH guidelines on the ethical use of animals. Both colony and commercial (129-Steel and C57Bl/6) mice (Charles River Laboratories) were habituated to the testing environment for 120 min prior to behavioral testing. In the dose–response studies (Figure 3a), mice were treated intraperitoneally (i.p.) with the A_{2A} antagonist SCH58261, KW-6002, or vehicle (10% DMSO, 15% ethoxylated castor oil, 75% distilled water). In the amphetamine-induced sensitization studies (Figure 3b–d), as schematized in Figure 4, mice received daily injections (in their test cage) of amphetamine (2.5 mg/kg, i.p.) combined with vehicle, SCH58261 (0.03 mg/kg, i.p.), or KW-6002 (0.03 mg/kg, i.p.) for 8 consecutive days. A_{2A} antagonists were injected 1–2 min before amphetamine. Locomotor activity was recorded (using an automated open field system) for 120 min following drug injection on days 1 and 8 and 1 and 2 weeks after the cessation of the treatment (days 15 and 22). In the induction study of amphetamine sensitization (Figure 5a), drug treatments are identical to those of the amphetamine sensitization studies up to day 15. On day 22, the locomotor activity was monitored upon challenge with amphetamine (2.5 mg/kg, i.p.) alone in all mice. In the expression study (Figure 5b), all mice were treated for 8 consecutive days with amphetamine alone (2.5 mg/kg, i.p.). A week after the last treatment (day 15), mice were treated either with amphetamine (2.5 mg/kg, i.p.) alone or paired with SCH58261 (0.03 mg/kg, i.p.), and their locomotor activity was recorded on days 1, 8, and 15. When using the A_{2A} conditional KO mice in the amphetamine sensitization study (Figure 2), animals were treated with amphetamine (2.5 mg/kg, i.p.) alone.

Locomotor and Fine Motor Activity

Horizontal locomotor and fine motor activity were primarily assessed by an automated recording system (San Diego Instruments) in standard polypropylene cages (15 × 25 cm)

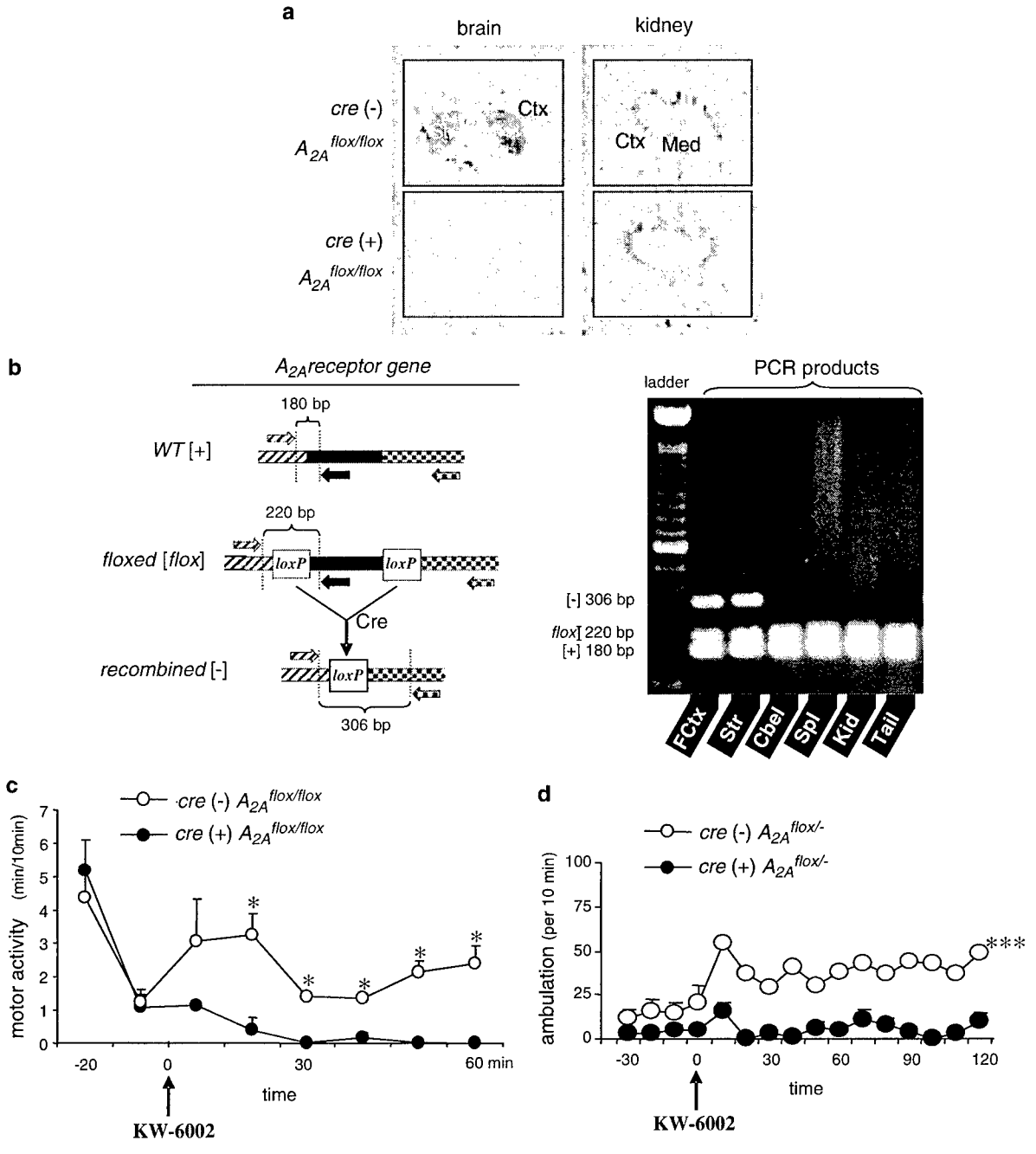

Figure 1 Tissue-specific, functional disruption of the adenosine A_{2A}R. (a) A_{2A}R autoradiography (^{3}H-CGS21680 binding) of coronal brain and sagittal kidney sections from a 3-month-old *cre*(−) $A_{2A}^{flox/flox}$ control mouse and its *cre*(+) $A_{2A}^{flox/flox}$ conditional KO littermate qualitatively shows striatum-specific depletion of A_{2A}Rs. st: striatum; ctx: cortex; med: medulla. (b) Assessing A_{2A}R genotypes of CNS and peripheral tissues at 4 months in *cre*(+) $A_{2A}^{flox/+}$ mice using oligonucleotide primers designed (as schematized on the left) to produce distinct PCR products for WT [+], floxed, and recombined [−] A_{2A} alleles. Frontal cortex (FCtx), striatum (Str), cerebellum (Cbel), spleen (Spn), kidney (Kid), and Tail. (c) Motor response to KW-6002 (3 mg/kg, i.p.) in *cre*(+) $A_{2A}^{flox/flox}$ conditional KO and their littermate controls. *$p<0.001$, $n=3$. (d) Locomotor response to KW-6002 (3 mg/kg, i.p.) in *cre*(+) $A_{2A}^{flox/-}$ conditional KO mice and their littermate controls. ***$p<0.001$, $n=8$.

placed into adjustable frames equipped with five infrared photocell beams that traverse each cage in a plane above the floor. Locomotor activity ('ambulation') was measured as number of sequential breaks in two adjacent beams, and fine motor activity was measured as number of sequential breaks in a single beam. For the functional characterization of *cre*(+) $A_{2A}^{flox/flox}$ mice (Figure 1c), a manual blinded recording method for locomotor activity (time spent in horizontal motion during each 10 min period) was used.

Receptor Autoradiography

Qualitative receptor autoradiography for detecting A_{2A}Rs using the specific ligand ^{3}H-CGS21680 (46.0 Ci/mmol; NEN,

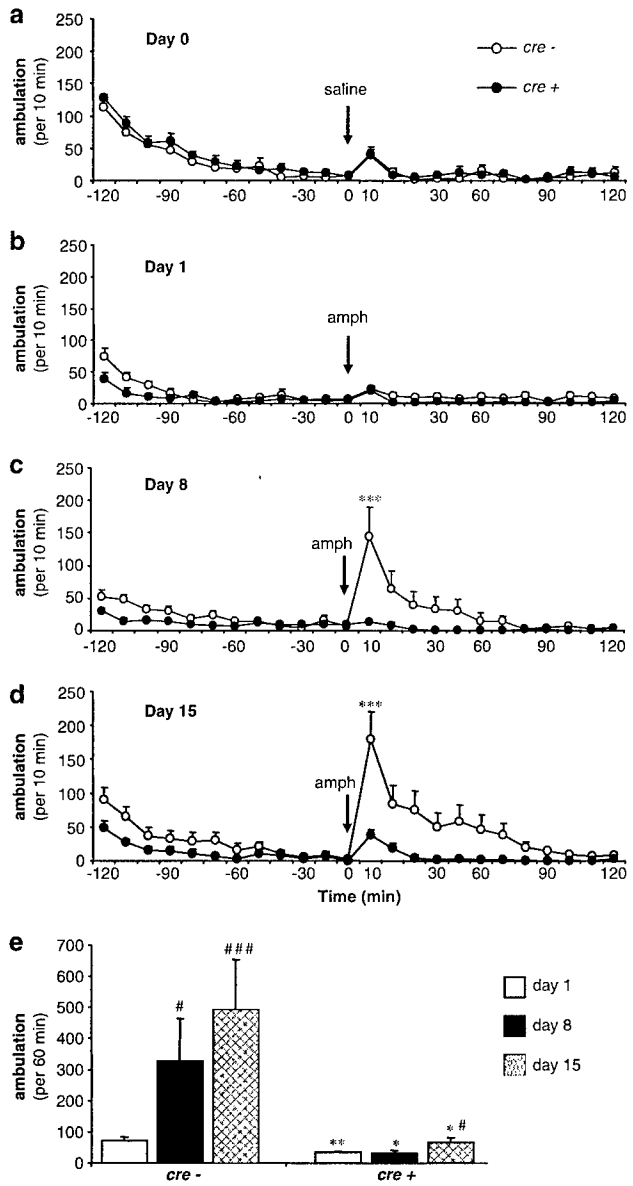

Figure 2 Absence of amphetamine-induced behavioral sensitization in postnatal brain-specific A_{2A} KO mice. Conditional A_{2A} KO (*cre*(+)) mice and littermate (*cre*(−)) controls were habituated to test cages and injected with saline (day 0, panel a), treated with amphetamine (2.5 mg/kg, i.p.) daily for 8 days, and then rechallenged with the same dose of amphetamine after a week-long washout period. Locomotor responses to amphetamine were recorded on the first day of treatment (panel b), the 8th day of daily treatment (panel c), and upon rechallenge on day 15 (panel d). ***$p<0.001$. Cumulative locomotion for the 60 min after amphetamine injection is compared between genotypes for days 1, 8, and 15 in panel e ($^{\dagger}p<0.05$, $^{\dagger\dagger\dagger}p<0.001$ *vs* day 1 of *cre*(−); *$p<0.05$, **$p<0.01$ *vs* the same day of treatment of *cre*(−); $^{\ddagger}p<0.05$ *vs* day 1 of *cre*(+); $n=7$–8).

Boston, MA) was performed as described previously (Chen *et al*, 1999). Coronal brain and tissue sections were preincubated at room temperature with 50 mM Tris-HCl buffer, pH 7.5, and 1 U of adenosine deaminase for 30 min and then incubated with the Tris buffer containing 5 nM ^{3}H-CGS21680 for 60 min. To define nonspecific binding, 2.5 μM of 2-chloroadenosine was coincubated in adjacent sections.

Statistical Analysis

All data are expressed as mean ± SEM. Statistical analyses were performed using Prism3 software. The effects of genotype and chronic treatment (treatment days 1, 8, and 15) were analyzed by two-way ANOVA followed by post-test using the Bonferroni method. For all the other behavioral studies, one-way ANOVA followed by Dunnett's test was applied.

RESULTS

Complete, Specific Inactivation of Adult Brain A_{2A}Rs in Conditional A_{2A} KO Mice

To clarify the neurobiology of A_{2A}Rs, we generated conditional A_{2A} KO mice using a forebrain-specific Cre/*loxP* system (Morozov *et al*, 2003). Transgenic mice expressing the Cre recombinase under the direction of the *CaMKIIα* gene promoter in postnatal forebrain neurons (including those of the striatum) were crossed with mice whose *$A_{2A}R$* gene contained *loxP* excision sequences inserted on either side of (ie flanking) a critical exon, yielding so-called 'floxed' *$A_{2A}R$* alleles. Successful forebrain-specific recombination was confirmed by autoradiographic, genetic, and behavioral assessments.

Receptor autoradiography with the A_{2A} agonist ^{3}H-CGS21680 demonstrates characteristic ligand binding to A_{2A}Rs in the striatum of adult nontransgenic (ie *cre*(−)) $A_{2A}{}^{flox/flox}$ mice but complete absence of detectable binding in the striatum of *cre*(+) $A_{2A}{}^{flox/flox}$ littermates (Figure 1a). By contrast, A_{2A}R binding sites in the kidneys (specifically in the renal medulla, where A_{2A}R expression is known to be enriched; Weaver and Reppert 1992) of the same mice appear indistinguishable in the *cre*(+) and *cre*(−) ($A_{2A}{}^{flox/flox}$) mice (Figure 1a). Together with genetic evidence against Cre-mediated recombination in all other peripheral somatic tissues tested (heart, spleen, tail; Figure 1b and data not shown) in *CaMKIIα-cre*(+) mice, these anatomical findings demonstrate the brain specificity of this conditional A_{2A} KO approach. It should be noted however that disruption of the *$A_{2A}R$* gene also occurred in some gonadal cells (due to variable germline expression of *cre* as expected with the *CaMKIIα* promoter; Dragatsis *et al*, 2000; Morozov *et al*, 2003), complicating breeding strategies to generate *cre*(+) $A_{2A}{}^{flox/flox}$ mice (see Materials and methods).

In addition, within the CNS, the predicted further restriction of *$A_{2A}R$* gene recombination to the forebrain was confirmed by the genetic (PCR) analysis, showing prominent recombination of the floxed *$A_{2A}R$* allele in the frontal cortex and striatum but no detectable recombination in the cerebellum (as well as peripheral tissues) (Figure 1b). Note that the relatively small amount of residual floxed (nonrecombined) *$A_{2A}R$* allele likely reflects the lack of *cre* expression in glial cells, since forebrain neurons rather than glia are primarily targeted in the *CaMKIIα-cre*(+) mice used here (Dragatsis and Zeitlin 2000; Morozov *et al*, 2003). On the other hand, incomplete recombination in striatal neurons could be excluded as a contributor to the residual floxed *$A_{2A}R$* gene in the adult striatum.

In initially assessing the functional effects of eliminating A_{2A}Rs in adult striatal neurons, we examined behavioral

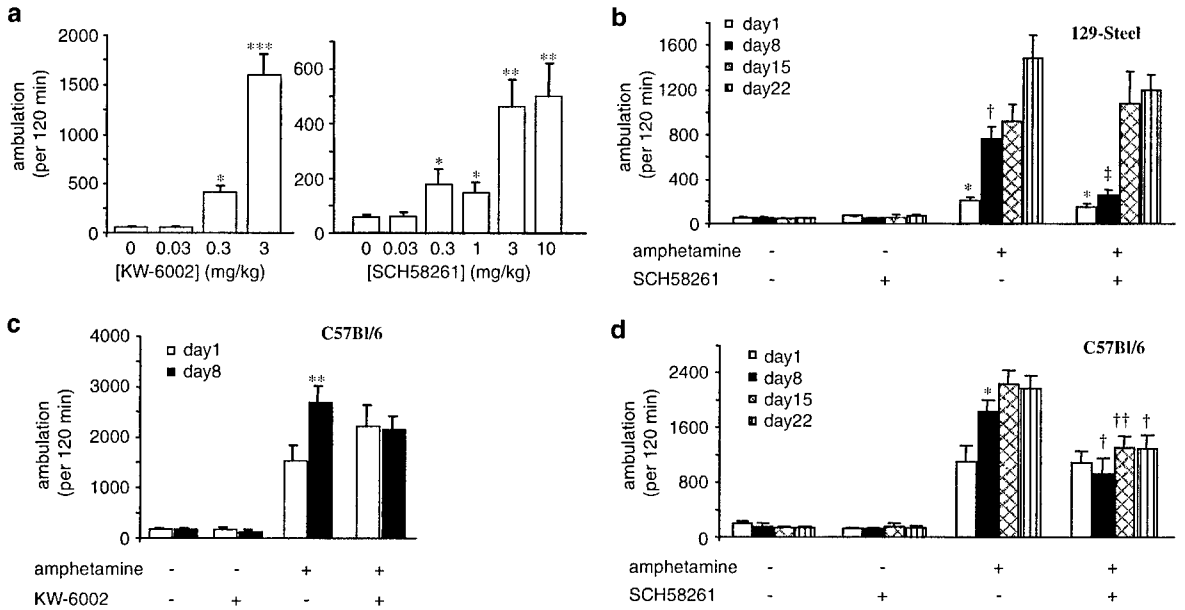

Figure 3 A_{2A} antagonists attenuate amphetamine-induced behavioral sensitization. (a) In 129-Steel mice, A_{2A} antagonists enhance locomotor activity at concentrations ≥ 0.3 mg/kg ($n = 8$–10). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. (b) 129-Steel mice ($n = 10$–18) were treated with amphetamine (2.5 mg/kg or saline) and SCH58261 (0.03 mg/kg or vehicle) daily for 8 days. After a washout period of 1 week, they were rechallenged (with the same treatments) on day 15 and again on day 22. $^{*}p < 0.01$ *vs* day 1 of the vehicle-treated mice; $^{\dagger}p < 0.01$ *vs* amphetamine day 1; $^{\ddagger}p < 0.05$ *vs* amphetamine at the same day of treatment. (c) C57Bl/6 mice were treated with amphetamine (2.5 mg/kg) and KW-6002 (0.03 mg/kg) daily for 8 days ($n = 8$). $^{**}p < 0.01$ *vs* amphetamine at day 1. (d) C57Bl/6 mice ($n = 7$–9) were treated with amphetamine (2.5 mg/kg) and SCH58261 (0.03 mg/kg) as in panel b. $^{*}p < 0.05$ *vs* amphetamine at day 1; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ *vs* amphetamine treatment at the same day.

responses to the selective A_{2A} antagonist KW-6002 (3 mg/kg, i.p.) in conditional A_{2A} KO mice. Although basal locomotor activity did not differ between conditional A_{2A} KO (*cre*(+) $A_{2A}^{flox/flox}$ or *cre*(+) $A_{2A}^{flox/-}$) mice and their respective control (*cre*(−)) littermates, KW-6002 induced locomotion only in the *cre*(−) controls (Figure 1c and d). In control mice from the *CaMKIIα-cre* line (expressing the *cre* transgene in the forebrain without a floxed A_{2A} gene target), KW-6002 stimulated locomotion to the same extent as in their nontransgenic littermates, ruling out the possibility that the absence of A_{2A} antagonist-induced locomotion in the conditional KO is simply due to the expression of *cre* alone. It is to be noted that these locomotor data provide the strongest evidence yet that KW-6002 and other relatively specific A_{2A} antagonists enhance movement in Parkinson's disease patients (Bara-Jimenez *et al*, 2003; Hauser *et al*, 2003) as well as laboratory animals (Jenner 2003) through blockade of neuronal CNS A_{2A}Rs rather than non-neuronal or peripheral A_{2A}Rs. Together, these behavioral, anatomical, and genetic features of the Cre/*loxP* conditional A_{2A} KO approach confirm that it selectively eliminates the A_{2A}R from the forebrain of adult mice.

Amphetamine-Induced Sensitization Requires Brain A_{2A}R Activation

We compared the effects of brain A_{2A}R inactivation on locomotor responses to daily treatment with a low dose of amphetamine (2.5 mg/kg) in *cre*(+) $A_{2A}^{flox/-}$ (conditional KO) mice and their *cre*(−) $A_{2A}^{flox/-}$ (control) littermates. Although forebrain-specific A_{2A}R depletion had no effect on the locomotor response to a novel environment or to a habituating saline injection on day 0 (Figure 2a), locomotor activity after the first dose of amphetamine was slightly greater in control mice (on day 1; Figure 2b and e). This is consistent with a partial A_{2A}R dependence of the acute stimulant action of amphetamine, which we had observed with a global A_{2A}R KO line (Chen *et al*, 2000). Continued daily treatment with amphetamine markedly enhanced (sensitized) locomotor responses in control mice ($p < 0.05$ day 8 *vs* 1), whereas no sensitization to amphetamine occurred in their conditional KO littermates on day 8 (Figure 2c and e). Similarly, 1 week after discontinuation of daily amphetamine exposure (day 15), robust locomotor sensitization persisted in control ($p < 0.001$ *vs* day 1) but was not seen in conditional A_{2A} KO mice (Figure 2d and e), although at this point a slightly enhanced locomotor activity appeared to be present in the conditional KO group compared to day 1 ($p < 0.05$) (Figure 2e). Given this result and the fact that on day 1 the amphetamine response in the conditional A_{2A} KO was lower compared to the WT mice, we cannot exclude the possibility that the absence of sensitization on day 8 in the conditional A_{2A} KO is a reflection of subthreshold motor responses at the dose of amphetamine used in this study. We have however noted that the absence of sensitization we observed in global A_{2A} KO mice (Chen *et al*, 2003) was independent of the amphetamine dose used and was not attributable to a threshold effect on sensitization.

The possibility that the absence of sensitization in conditional A_{2A} KO mice is due to the expression of the *cre* gene rather than the selective deletion of the A_{2A}R was excluded by the finding that (*L7ag13*) control (A_{2A} WT) mice expressing *cre* (ie in the absence of a floxed $A_{2A}R$ gene)

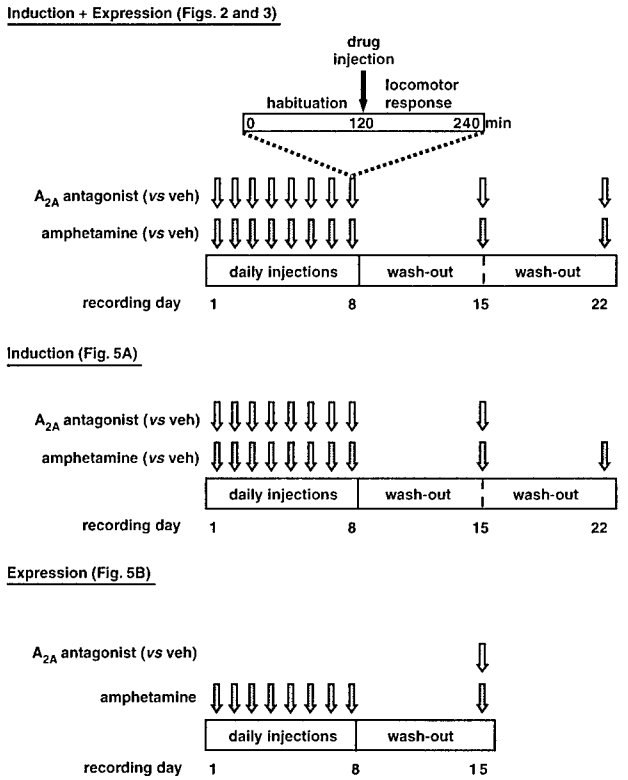

Figure 4 Schematics of paradigms for assessing $A_{2A}R$ involvement in different phases of amphetamine-induced locomotor sensitization.

showed the same level of amphetamine sensitization as their nontransgenic (ie *cre*(−), fully WT) littermates (ambulations for 120 min after the eighth amphetamine dose: 4154±844 and 3568±854, respectively). Since amphetamine is known to induce stereotyped stationary behaviors as well as locomotion, we considered the possibility that the lack of locomotor sensitization in conditional KO mice could be due to immobility associated with increased stereotypes in these mice. However, an enhancement of amphetamine-induced fine movement behavior in the control mice on days 8 and 15 ($p<0.05$) was also blocked in their A_{2A} conditional KO littermates—with increases from day 1 (182±40 repetitive single photobeam breaks) to day 8 (391±86) to day 15 (456±101) in *cre*(−) $A_{2A}^{flox/-}$ controls, *vs* no significant change from day 1 (123±22) to day 8 (220±69) to day 15 (251±69) in *cre*(+) $A_{2A}^{flox/-}$ mice. Together, these data demonstrate that brain A_{2A}Rs play an important role in amphetamine-induced sensitization of both locomotor and stereotyped behaviors.

A_{2A} Antagonists Attenuate Amphetamine-Induced Locomotor Sensitization

To investigate whether pharmacological blockade of A_{2A}Rs can also attenuate amphetamine sensitization, we first determined the dose of A_{2A} antagonist to be paired with amphetamine administration. To avoid the confound of the motor stimulant effects of adenosine A_{2A} antagonists (Popoli *et al*, 1998; El Yacoubi *et al*, 2000; Halldner *et al*, 2000), and given the evidence that doses of an A_{2A} antagonist below its threshold for motor stimulation are

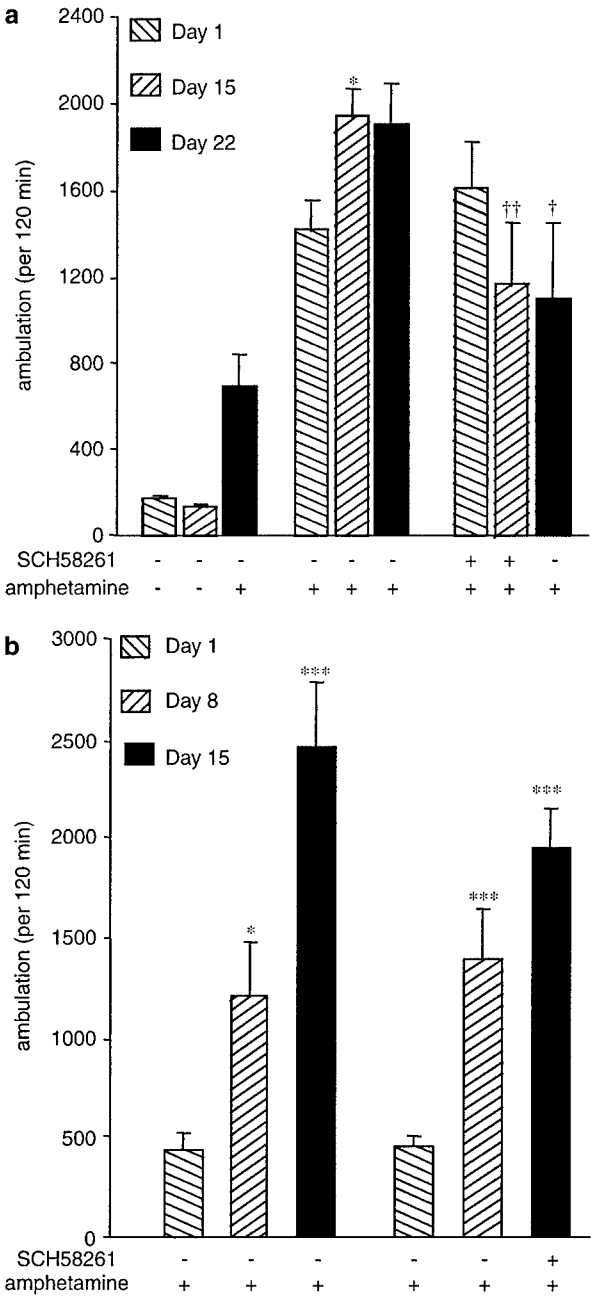

Figure 5 A_{2A}Rs are required for the induction of sensitization by amphetamine. (a) C57Bl/6 mice ($n=8$) were treated for 8 consecutive days with amphetamine (2.5 mg/kg) alone or paired to SCH58261 (0.03 mg/kg). On day 22, all mice were challenged with amphetamine (2.5 mg/kg) alone. $^{*}p<0.05$ *vs* amphetamine at day 1; $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ *vs* amphetamine treatment at the same day. (b) C57Bl/6 mice ($n=8$) were treated with amphetamine (2.5 mg/kg) daily for 8 days. On day 15, mice were challenged with amphetamine paired to SCH58261 (0.03 mg/kg) or vehicle. $^{*}p<0.05$, $^{***}p<0.001$ *vs* amphetamine at day 1. Note that although several fold variability in acute motor response to amphetamine is routinely observed between experiments (eg a *vs* b for day 1), the amphetamine-induced sensitization phenomenon occurs consistently.

capable of modulating striatal physiology (Monopoli *et al*, 1998; Popoli *et al*, 2002), we tested several doses of two different A_{2A} antagonists, SCH58261 and KW-6002. Both

dose-dependently stimulated locomotor activity, with 0.3 mg/kg being the lowest dose that stimulated locomotion (Figure 3a). Accordingly, we selected 0.03 mg/kg as a subthreshold dose (ie that does not enhance locomotor activity) for each antagonist to be coadministered with amphetamine in sensitization experiments.

Pairing daily amphetamine doses with an A_{2A} antagonist (SCH58261 or KW-6002) prevented locomotor sensitization on day 8 in both 129-Steel and C57Bl/6 strains of mice ($p<0.05$) (Figure 3b–d). Upon rechallenge with amphetamine plus SCH58261 on days 15 and 22, C57Bl/6 mice still did not show sensitization, while sensitization remained undiminished in the amphetamine plus vehicle group (Figure 3d). In contrast, 129-Steel mice developed a sensitized response upon delayed rechallenge with amphetamine plus SCH58261 on days 15 and 22 (Figure 3b). Fine movements were also monitored and they paralleled the results with locomotor activity (data not shown). These data suggest that A_{2A}R blockade prevents or delays the development of locomotor sensitization to amphetamine, and that A_{2A}Rs facilitate neuroadaptive changes induced by repeated dopaminergic stimulation.

A_{2A}Rs are Required for the Induction (but not Expression) of Amphetamine Sensitization

We also explored the effects of adenosine A_{2A}Rs on discrete phases of sensitization. In particular, we challenged with amphetamine alone mice that have been daily treated with amphetamine or amphetamine plus the A_{2A} antagonist SCH58261 (0.03 mg/kg). Upon amphetamine challenge, locomotor activity of amphetamine plus SCH58261 chronically treated mice was still not sensitized and remained significantly lower than that of the amphetamine chronically treated mice ($p<0.05$) (Figure 5a). We also addressed possible A_{2A}R involvement in the expression phase of amphetamine sensitization. After having induced sensitization with daily administration of amphetamine alone, mice were challenged either with amphetamine or amphetamine paired with SCH58261 (0.03 mg/kg). Amphetamine-treated mice as well as SCH58261-treated mice still showed sensitization ($p<0.001$ *vs* amphetamine day 1) (Figure 5b). The data suggest that A_{2A}Rs play a role in the induction or maintenance of amphetamine-induced sensitization rather than its expression.

DISCUSSION

We have shown that postnatal inactivation of brain adenosine A_{2A}Rs dramatically attenuates sensitized behavioral responses to repeated amphetamine administration using a conditional gene depletion technique in combination with classical pharmacology. We previously showed that a global KO of the A_{2A}R (ie in all cells and at all times from conception onward) prevents amphetamine sensitization. However, our earlier study could not distinguish between a developmental, chronic, or acute inactivation of the A_{2A}R as the basis for this phenotype. Given that the A_{2A}R is expressed in brain as early as E-15 in rats (Weaver, 1993), it was possible that altered development of dopaminergic, glutamatergic, GABAergic, or other CNS signaling systems in A_{2A} KO mice could lead to an alteration in their amphetamine-induced behavioral sensitization. Similarly, the global KO study could not distinguish between the effects of A_{2A}R inactivation in brain and its many effects in the periphery. The absence of amphetamine sensitization in the conditional KO in the present study argues strongly against compensatory developmental modifications by A_{2A}R depletion as the cause of altered amphetamine-induced sensitization because the particular (*L7ag13*) line of *CaMKIIa-cre* mice used here has been shown to reduce forebrain 'floxed' gene expression somewhere between postnatal days 6 and 60 (Dragatsis and Zeitlin, 2000). In addition, the conditional KO phenotype also largely excludes the possibility of non-CNS (or non-neuronal) A_{2A}Rs contributing to amphetamine sensitization, as we (Figure 1) and others (Dragatsis *et al*, 2000; Dragatsis and Zeitlin 2000) have found no evidence of Cre recombinase activity outside the brain (or neurons) in male *CaMKIIa-cre* mice except for that in testes, which is unlikely to be a major contributor to psychostimulant-induced sensitization.

Although the postnatal conditional KO strategy helps eliminate a role for developmental actions of A_{2A}Rs, the absence of forebrain A_{2A}Rs for weeks to months prior to repeated amphetamine administration in the *cre*(+), floxed *A_{2A}* mice precludes a distinction between effects of chronic receptor depletion and the effects of acute inactivation just at the times of the amphetamine exposure. To address A_{2A}R involvement in amphetamine sensitization with an even greater temporal resolution than afforded by the conditional KO, we turned to complementary pharmacological antagonists of the A_{2A}R that were administered acutely together with the individual amphetamine doses. Pairing of A_{2A} antagonists with amphetamine also prevented locomotor sensitization after eight daily drug injections in both the 129-Steel and C57Bl/6 mouse strains, but prevented persistent sensitization weeks later only in C57Bl/6 mice. The different durations of sensitization blockade in the two mouse strains might be related to their differences in the metabolism of the drugs as well as to different drug sensitivities in the CNS. It also might be possible that the phases of sensitization (eg induction and maintenance) are affected differently in different mouse strains. In any event, the recapitulation of the global and conditional A_{2A} KO phenotype of attenuated amphetamine sensitization in the antagonist-treated mice strengthens further the evidence against developmental or prolonged actions of the A_{2A}R as the basis for its facilitative role in psychostimulant sensitization. Thus, from the present findings, we can now conclude that postnatal forebrain A_{2A}Rs—probably on neurons—play a critical role in behavioral sensitization to repeated amphetamine administration.

The absence of behavioral sensitization to repeated amphetamine treatment in A_{2A} KO and antagonist-treated mice may reflect a broader phenotype of attenuated adaptive motor responses to intermittent dopaminergic stimulation. Fredduzzi *et al* (2002) showed that in unilaterally 6-OHDA-lesioned (global) A_{2A} KO mice, daily treatment with L-dopa did not produce progressively sensitized behaviors (contralateral rotations and grooming) compared to their WT littermates. In analogous pharmacological studies of A_{2A}R involvement in neuroplasticity induced by L-dopa in hemiparkinsonian rodents, Bibbiani

et al (2003) have recently shown that oral KW-6002 coadministered with L-dopa daily prevented the characteristic shortening of motor response to acute L-dopa challenge. Together, these studies raise the possibility that the maladaptive involuntary movements (known as dyskinesias) that develop after chronic L-dopa treatment in Parkinson's disease may be reduced or prevented by antagonist coadministration. This hypothesis was strongly supported by a study of parkinsonian non-human primates in which chronic oral administration of KW-6002 with a dopaminergic agonist completely prevented the delayed development of dyskinesias (Bibbiani *et al*, 2003). Furthermore, A_{2A}R involvement in neural adaptations may extend beyond those induced by direct dopaminergic stimulation. For example, El Yacoubi *et al* (2001) recently reported that classical genetic deletion of A_{2A}Rs also attenuates a withdrawal syndrome after chronic alcohol administration.

On the other hand, not all pharmacological studies have supported a facilitative role for A_{2A}Rs in the neural adaptations that underlie sensitization. Shimazoe *et al* (2000) found that the A_{2A} agonist CGS21680 attenuates sensitization to repeated methamphetamine administration in rats. Their use of a different psychostimulant drug and paradigm of sensitization, as well as an A_{2A} agonist (which may be less relevant to endogenous adenosine actions on CNS A_{2A}Rs than are A_{2A} antagonists) could account for the difference in results. Moreover, although Lundblad *et al* (2003) have confirmed that treatment of 6-OHDA-lesioned rats with an adenosine A_{2A} antagonist alone did not elicit any abnormal involuntary movements while relieving motor disabilities, they did not observe any effect of KW-6002 on the severity of dyskinesias when it was coadministered with L-dopa. Another study of unilaterally 6-OHDA-lesioned rats found that an A_{2A} antagonist reversed but did not prevent L-dopa-induced motor alterations (Bove *et al*, 2002). In general, all these studies have suggested an A_{2A}R role in behavioral sensitization despite some differences in the nature of its role. The present study greatly strengthens the evidence that in the case of the brain A_{2A}R, its role in psychostimulant sensitization is facilitative.

Our finding that pharmacological blockade of A_{2A}Rs can be as effective as their genetic depletion in preventing amphetamine sensitization adds to the therapeutic potential of A_{2A} antagonists for neuropsychiatric diseases. Several A_{2A} antagonists (eg KW-6002) are already in various phases of clinical trials as a novel symptomatic treatment for Parkinson's disease. Our findings support the possibility that brain A_{2A} blockade may help prevent or delay the development of maladaptive dyskinetic motor responses to chronic dopaminergic stimulation (Pinna *et al*, 2001; Fredduzzi *et al*, 2002; Bibbiani *et al*, 2003). Moreover, our data raise the possibility that A_{2A} antagonists could provide a rational pharmacological intervention for the treatment of addictive disorders. In support of A_{2A} antagonists as therapy in neuropsychiatric disorders is the efficacy of very low doses, which are subthreshold for stimulatory motor effects. The development of sensitization may result from a series of transient neural adaptations that occur with each drug administration, ultimately establishing enduring changes in the response of the brain to subsequent drug administration. Our results implicating CNS A_{2A}Rs in the development rather than the expression of amphetamine sensitization indicate not only that CNS A_{2A}Rs play a critical role in sensitized psychostimulant responses, but also that they could be targeted to prevent or delay the maladaptive neuroplasticity that contributes to the induction or maintenance phases of some addictive behaviors.

The neurochemical mechanisms by which basal ganglia A_{2A}Rs may facilitate behavioral sensitization are unknown. A_{2A}R inactivation may prevent behavioral sensitization by modulating presynaptic dopamine release (Zetterstrom and Fillenz 1990; Okada *et al*, 1996; Dassesse *et al*, 2001). Since there is no evidence of A_{2A}Rs expression on nigrostriatal neurons (Rosin *et al*, 2003), it has been suggested that A_{2A}R-mediated facilitation of dopamine release may arise indirectly, that is, through regulation of glutamate and GABA release (Sebastiao and Ribeiro, 1996; Wolf, 1998; Corsi *et al*, 1999). Alternatively, a direct postsynaptic interaction between A_{2A} and dopamine D_2 receptor may facilitate amphetamine sensitization. In addition, the interaction among A_{2A} and mGluR5 metabotropic glutamate receptors in the basal ganglia could also modulate psychostimulant-induced sensitization (Chiamulera *et al*, 2001). Changes in the expression of presumably postsynaptic A_{2A}Rs after repeated dopaminergic exposures might also play a functional role in the nucleus accumbens or dorsal striatum (Zeng *et al*, 2000; Calon *et al*, 2004; Tomiyama *et al*, 2004). Potential downstream postsynaptic mediators of sensitization that are also known to be regulated by the A_{2A}R include cytoplasmic signal transducers (eg dopamine- and cAMP-regulated phosphoprotein of 32 kDa or DARPP-32) and nuclear transcriptional targets (eg ΔFosB, enkephalin, and dynorphin gene expression in striatal neurons; Fienberg *et al*, 1998; Canals *et al*, 2003; Lundblad *et al*, 2003; Hakansson *et al*, 2004).

In summary, by complementing classical pharmacology with a powerful new conditional KO approach to brain receptor inactivation, we have demonstrated that antagonists of the A_{2A}R and its genetic disruption in the postnatal forebrain markedly attenuate behavioral sensitization to repeated amphetamine exposure. Furthermore, the findings indicate a critical if not requisite role for brain A_{2A}Rs in an early phase of psychostimulant-induced neuroplasticity. Thus, targeting the A_{2A}R in the basal ganglia may provide a novel therapeutic strategy to prevent or reduce maladaptive biochemical and behavioral responses to repeated drug administration in human psychostimulant addiction.

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