AWARD NUMBER: W81XWH-04-1-0881

TITLE: Caffeine, Adenosine Receptors and Estrogen in Toxin Models of Parkinson's Disease

PRINCIPAL INVESTIGATOR: Michael A. Schwarzschild, M.D., Ph.D.

CONTRACTING ORGANIZATION: Massachusetts General Hospital Boston, MA 02114

REPORT DATE: October 2009

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1 October 2009	Annual	1 Oct 2008 – 30 Sep 2009

4. TITLE AND SUBTITLE

Caffeine, Adenosine Receptors and Estrogen in Toxin Models of Parkinson's Disease

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0881

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Michael A. Schwarzschild, M.D., Ph.D.

E-Mail: michaels@helix.mgh.harvard.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Massachusetts General Hospital
Boston, MA 02114

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

During the reporting period continued progress has been made toward our original Specific Aims (1-3) through approved Modified Aims (M1 and M2) of our project, "Caffeine, adenosine receptors and estrogen 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 environmental agents (e.g., pesticides, caffeine, estrogen) that are leading candidate modulators of PD risk. In Year 5 we obtained and reported evidence that the adenosine receptor blocker caffeine as well as specific genetic depletion of the A2A subtype of adenosine receptor are capable of conferring protection against neuron degeneration in chronic pesticide and genetic mouse models of PD. Moreover, we have demonstrated and published that adenosine A2A receptors expressed in forebrain neuron are critical for neuron degeneration in toxin model of PD. We also identified CSF levels of urate (an environmentally and genetically determined antioxidant) is a predictor of progression of PD.

15. SUBJECT TERMS
adenosine, caffeine, estrogen, neuroprotection, neurotoxin, Parkinson's disease

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	115	USAMRMC

Table of Contents

Appendices

Introduction

Identifying the mechanisms by which caffeine and more specific A_{2A} antagonists protect dopaminergic neurons in multiple 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, estrogen and most recently the caffeine-related purine urate) 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***)

Modifications for Yr 4-5

Specific Aim M1: to definitively determine whether brain A_{2A}Rs (from either forebrain neurons or astrocytes) are required for caffeine's protective effect in chronic pesticides model of PD. [~280 mice]

Hypothesis: *Caffeine acts through blockade of brain A_{2A} receptors to protect dopaminergic neurons in chronic pesticides (paraquat/maneb) model of PD.*

Experiment #M1a: **Effect of caffeine in Pq/Mb model using forebrain-neuron specific A_{2A} KO mice**. Homozygous floxed A_{2A}R littermates with or without *CAMKII Cre* (from matings of homozygous floxed A_{2A} mice, one with Cre and the other without) will be treated chronically with caffeine or saline 10 min before paraquat and maneb or saline i.p. twice weekly for 8 weeks. Dopaminergic neuron degeneration will be measured one week after the last treatment by stereological cell counts of tyrosine hydroxylase-immunoreactive (TH-IR) cells in the substantia. Parallel assessment of striatal dopaminergic neuron injury will be assessed by ^{3}H-mazindol autoradiography for the striatal DAT binding. Glia (astrocytes and microglia) activation will also be measured using immunohistochemistry.
(**Months 1-12)**

Experiment #M1b: **Effect of caffeine in Pq/Mb model using astrocyte-directed A_{2A} KO mice**. Homozygous floxed A_{2A}R littermates with or without *GFAP Cre* will be treated with caffeine or saline followed by Pq/Mb (or saline/saline) injections biweekly for 8 weeks as in Exp #1. Genotyping and assessments of dopaminergic neuron injury and degeneration will be conducted as above.

(**Months 6-18)**

Specific Aim M2: to determine whether focal depletion of adenosine A_{2A} receptor in substantia nigra or striatum is sufficient to attenuate dopaminergic neuron toxicity. [~360 mice]

Hypothesis: *Elimination of A_{2A} receptors in striatum (but not substantia nigra or cortex) using a viral* cre/loxP *conditional knockout system will protect dopaminergic nigrostriatal neurons from MPTP toxicity.*

Experiment #M2: **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 (or wild type control 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 dopaminergic neuron counts will be assessed as in Exp #4
(**Months 1-24:**

***striatal target*:**	**months 1-12**
***nigral target*:**	**months 7-18**
***cortical target*:**	**months 13-24)**

Statement of Relevance (from original 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 during Year 5 on Specific Aims and experiments as laid out in our approved modification of the Statement of Work (SOW [in blue]) is described here in detail.

STATEMENT OF WORK (focus on main areas of progress in Yr 5)

Specific Aim M1: to definitively determine whether brain A_{2A}Rs (from either forebrain neurons or astrocytes) are required for caffeine's protective effect in chronic pesticides model of PD.

[Please see manuscript and abstract publication in Appendix A.]

- We have obtained and now published (Appendix A) evidence that forebrain A_{2A} receptors are in fact required for dopaminergic neuron toxicity in a chronic toxin model of PD. Working with collaborators at the University of Cagliari we have compared the doapminergic neuron toxicity produced by repeated daily administration of MPTP toxin in mice in which the adenosine A_{2A} receptor is conditionally 'knocked out' (cKO) of postnatal forebrain (striatal and cortical) **neurons**, compared to their control littermates with normal A_{2A} receptor expression. The findings attenuated toxicity and inflammation support the possibility that specific adenosine A_{2A} receptors -- currently in clinical (phase II/III) development as symptomatic therapy for later stage PD patients -- may have disease-modifying (neuroprotective) benefits if admininistered in earlier in the course of PD.

  Using the same Cre/*loxP* cKO system, we similarly compared the toxicity produced by repeated daily administration of MPTP toxin in mice in which the adenosine A_{2A} receptor is conditionally 'knocked out' of **astrocytes** compared to their control littermates with normal A_{2A} receptor expression. The finding that these astrocytic A_{2A} receptor cKO mice were not protected as observed in the neuronal A_{2A} cKO, suggest neuronal A_{2A} receptors could fully account for the hypothesized benefits of A_{2A} antagonists against neurodegeneration in PD. The astrocyte A_{2A} cKO data were removed from the final published manuscript pending a more complete validation of this cKO reagent (e.g., showning specific delepletion of A2A receptors from astrocytes in this line, given that our findings with this line showed no phenotype in this PD model).
- Also in Year 5, and in pursuit of the original SA #1, we have completed studies (see our submitted manuscript in Appendix E) in the double adenosine A_1 and A_{2A} receptor KO mice we developed under this project, in which the the individual and combined roles of these receptors have been probed in a mouse model of levodopa-induced dyskinesia (LID) in PD. Because the model entails a unilateral lesion to dopaminergic nigrostriatal projection using the locally (intrastrially) applied neurotoxin 6-hydroxydopamine, an important determination was the extent of the lesion (assessed as the amount of striatal dopamine loss relative to the contralateral unlesioned striatum) in the presence or absene of either or both of these adenosine receptors. We report that using this toxin neither the single or double KOs altered the lesion. The demonstration that the receptors were not playing a critical role in this form of dopaminergic neuron injury, allowed for the

main interpretations of this experiment to focus on the role of the A_1 and A_{2A} receptors in LID. Surprisingly, we found that the A_1 as well as the A_{2A} receptor was required for the full development of LID in this mouse model of PD. This finding prompted to assess the effect of caffeine, a non-specific adenosine (mixed A_1-A_{2A}) receptor antagonist, and we found that like the A_1-A_{2A} double KO, the mixed A_1-A_{2A} antagonist also reduced LID. The result suggests that early adjunctive use of adenosine antagonists (including caffeine) with levodopa, could help prevent the development of disabling LID in patients with PD.

- Also in Year 5 we have further investigated and preliminarily reported (Appendix D) how depletion of adenonsine A_{2A} receptors (in A_{2A} knockout mice) may mimic the neuroportective effect of caffeine in another chronic (genetic) mouse model of PD. Here we used a lifelong exposure to an 'endogenous toxin' of transgenically expressed mutant human α–synuclein (*hw-αSYN*). Mutations in the human α–synuclein gene (A53T and A30P) have been associated with autosomal dominant familial PD. Transgenic mice expressing a human α–synuclein gene with both of these mutations have been shown to develop age-related reductions in dopaminergic nigral neuron numbers, striatal dopamine (DA) and motor activity.

  We investigated whether deletion of the A_{2A} receptor (in A_{2A}[-/-] mice) would afford protection against the age-related spontaneous reduction of striatal dopamine in the double-mutant human α-synuclein (*hm²-αSYN*) mice. Heterozygous A_{2A}[+/-] mice were mated with A_{2A}[+/-] mice that were also transgenic for the wild-type (*hw-αSYN*) or *hm²-αSYN* form of the human synuclein gene under control of a 9-kb rat tyrosine hydroxylase promoter – all in a congenic C57Bl/6 background. Offspring were sacrificed at 20-24 months of age and striatal DA and metabolites were measured by HPLC-ECD. As expected in the presence of a functional A_{2A} receptor, DA content was significantly lower (by 35%) in hm²-αSYN mice compared to either hw-αSYN or non-transgenic (NT) mice. By contrast, in A_{2A}[-/-] mice (lacking functional A receptor) the hm²-αSYN transgene did not reduce striatal DA levels compared to either control. Data for the dopamine metabolite DOPAC showed a similar trend, as did dopamine data when split out by gender.

  In Yr 5, we completed stereological nigral TH-IR cell counts (Appendix D) clearly corroborating/confirming our observations with DA content above. Thus the A_{2A} receptor appears to be required for neurotoxicity in an α–synuclein mouse model of PD, supporting the neuroprotective potential of caffeine and more specific A_{2A} antagonists in the chronic dopaminergic neuron degeneration that characterizes PD.

Specific Aim M2: to determine whether focal depletion of adenosine A_{2A} receptor in substantia nigra or striatum is sufficient to attenuate dopaminergic neuron toxicity.

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...

Activity in Year 5 on SA M2 has primarily been in the expansion of our line of *floxed A_{2A}* mice which is necessary to conduct the virally mediated focal eliminations of A_{2A} receptors as planned. A period of reduced fertility/low offspring yields slowed the expansion. However after increasing the numbers of breeding pairs, the colony size has increased substantially in late 2009, with virally targeted Cre/*loxP* cKOs of A_{2A} receptors in different brain regions now scheduled for January 2010. Other activity on the project in Year 5 has been the adoption in our laboratory of several measures of oxidative damage (nitrotyrosine and 4-hydroxy-2-nonenal [HNE], measure by immunohistochemistry or Western blotting) to be assessed in parallel with measures of dopanergic neuron injury and loss.

Also in Year 5 with this support of this award our laboratory has demonstrated that CSF levels of urate – a major antioxidant as well as the end product of adenosine metabolism in humans – along with those in serum are predictive clinical progression in patients with typical early PD, with publication of the work (Appendix B) seen as a major advance in PD research. These findings have helped accelerate development of a clinical trial (http://clinicaltrials.gov/ct2/show/NCT00833690) of inosine (deamination product of adenosine and precursor of urate) to test its safety as a means to elevate urate in PD (http://www.michaeljfox.org/newsEvents_mjffInTheNews_pressReleases_article.cfm?ID=244).

Key Research Accomplishments (in Year 5)

- Achievement of key components of Aim M1, in demonstrating/publishing that targeted depletion of forebrain A_{2A} receptors reduces dopaminergic neuron degeneration in a sub-chronic toxin model of PD. The findings strengthen the translational rationale for conducting clinical trials with adenosine A_{2A} antagonists (either caffeine or more specific blockers) as a potential neuroprotective strategy.
- Identification and publication that higher CSF levels of the adenosine end product urate as predictor of favorable outcomes in PD. The findings are being rapidly translated into a major new clinical trial targeting urate elevation as a novel neuroprotective strategy in PD.
- Completion of preparations to conduct the key experiments under Aim M2 in which virally delivered cre gene will produce a focal conditional knockout of A_{2A} receptors to further localize the mechanism by which A_{2A} antagonists can confer neuroprotection in PD models.

Reportable Outcomes

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

- Carta AR, Kachroo A, Schintu N, Xu K, Schwarzschild MA, Wardas J, Morelli M. (2009) Inactivation of neuronal forebrain A_{2A} receptors protects dopaminergic

neurons in a mouse model of Parkinson's disease. *J Neurochem*. Oct 8. [Epub ahead of print] [See **Appendix A**.]

- Ascherio A, LeWitt PA, Xu K, Eberly S, Watts A, Matson WR, Marras C, Kieburtz K, Rudolph, A, Bogdanov MB, Schwid SR, Tennis M, Tanner CM, Beal MF, Lang AE, Oakes D, Fahn S, Shoulson I, & Schwarzschild MA on behalf of the PSG DATATOP investigators. (2009) Urate as a predictor of rate of clinical decline in Parkinson disease. *Arch. Neurol*. (published online Oct 12, 2009). [See **Appendix B**.]

- McFarland NR, Fan Z, Xu K, Schwarzschild MA, Feany MB, Hyman BT, McLean PJ. (2009) Alpha-synuclein S129 phosphorylation mutants do not alter nigrostriatal toxicity in a rat model of Parkinson disease. *J Neuropathol Exp Neurol*. 68:515-524. [See **Appendix C**.]

- Kachroo A, Richfield EK, Schwarzschild MA. 2009. Adenosine A2A receptor gene deletion confers protection of nigral neurons in an alpha-synuclein model of Parkinson's disease. 2008 Neuroscience Meeting. Chicago, IL: Society for Neuroscience. Abstract # 142.5. [See **Appendix D**.]

Conclusions/Modifications/Plans

Central hypothesis: Multiple environmental protectants and toxins interact to influence of the health of the dopaminergic neurons lost in Parkinson's disease. Our progress under this award supports the central hypothesis, particularly with respect to caffeine-estrogen interactions in models of PD. In Year 6 we expect to focus on the completion of Aim M2 to localize the A_{2A} receptors involved in dopaminergic neuron injury in models of PD. We also expect to expand the use of the conditional knockout reagents developed under this award to other environmentally relevant purine targets in PD, including adenosine metabolites like urate, which we have identified as a major new therapeutic target for neuroprotection in PD. Both our caffeine and urate findings hold promise for practical applications in the reduction of risk of developing PD or its decline.

Carta AR, Kachroo A, Schintu N, Xu K, Schwarzschild MA, Wardas J, Morelli M. (2009) Inactivation of neuronal forebrain A2A receptors protects dopaminergic neurons in a mouse model of Parkinson's disease. J Neurochem. Oct 8. [Epub ahead of print]

Appendix A

Inactivation of neuronal forebrain A2A receptors protects dopaminergic neurons in a mouse model of Parkinson's disease.

Journal:	*Journal of Neurochemistry*
Manuscript ID:	JNC-E-2009-0277.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	
Complete List of Authors:	Carta, Anna; University of Cagliari, Toxicology Kachroo, Anil; Massachusetts General Hospital, Institute for Neurodegenerative Disease Institute for Neurodegenerative Disase Schwarzschild, Michael; Massachusetts General Hospital, Institute for Neurodegenerative Disease Morelli, Micaela; University of Cagliari, Department of Toxicology; CNR, Institute for Neuroscience
Keywords:	adenosine, MPTP, astroglia, microglia, tyrosine hydroxylase, substantia nigra

Appendix A

Editor, Journal of Neurochemistry

Prof Tony Turner

Inactivation of neuronal forebrain A_{2A} receptors protects dopaminergic neurons in a mouse model of Parkinson's disease.

Anna R Carta[1], Anil Kachroo[2*], Nicoletta Schintu[1*], Kui Xu[2], Michael A. Schwarzschild[2], Micaela Morelli[1,3].

[1]Department of Toxicology, University of Cagliari, via Ospedale 72, 09124 Cagliari, Italy. Tel 0039-3804335298. acarta@unica.it; nicoletta.schintu@gmail.com; morelli@unica.it.

[2]Mass General Institute for Neurodegenerative Disease, Massachusetts General Hospital, Boston, MA 02129, USA. Tel 617-724-9611. AKACHROO@PARTNERS.ORG; xu@helix.mgh.harvard.edu; MichaelS@helix.mgh.harvard.edu.

[3]CNR Institute for Neuroscience, Cagliari, Italy.

*Authors equally contributed to this work.

Corresponding authors: Anna R Carta, PhD. Department of Toxicology, University of Cagliari, via Ospedale 72, 09124 Cagliari, Italy. Tel: 0039-3804335298; fax: 0039-0706758665; acarta@unica.it

Appendix A

Adenosine A_{2A} receptors antagonists produce neuroprotective effects in animal models of Parkinson's disease (PD). Since neuroinflammation is involved in PD pathogenesis, both neuronal and glial A_{2A} receptors might participate to neuroprotection. We employed complementary pharmacologic and genetic approaches to A_{2A} receptor inactivation, in a multiple 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD, to investigate the cellular basis of neuroprotection by A_{2A} antagonism. MPTP·HCl (20 mg/kg daily for 4 days)was administered in mice treated with the A_{2A} antagonist SCH58261, or in conditional knockout mice lacking A_{2A} receptors on forebrain neurons (fbnA_{2A}KO mice). MPTP induced partial loss of dopamine neurons in substantia nigra pars-compacta (SNc) and striatum (Str), associated with increased astroglial and microglial immunoreactivity in these areas. Astroglia was similarly activated one, three and seven days after MPTP administration, whereas maximal microglial reactivity was detected on day one, returning to baseline seven days after MPTP administration. SCH58261 attenuated dopamine cell loss and gliosis in SNc and Str. Selective depletion of A_{2A} receptors in fbnA_{2A}KO mice completely prevented MPTP-induced dopamine neuron degeneration and gliosis in SNc, and partially counteracted gliosis in Str. Results provide evidence of a primary role played by neuronal A_{2A} receptors in neuroprotective effects of A_{2A} antagonists in a multiple MPTP injections model of PD. With the symptomatic antiparkinsonian potential of several A_{2A} receptor antagonists being pursued in clinical trials, the present study adds to the rationale for broader clinical benefit and use of these drugs early in the treatment of PD.

Keywords: adenosine, MPTP, microglia, astroglia, tyrosine hydroxylase

Abbreviated title: Neuronal A_{2A} receptors and neuroprotection

Appendix A

Since the initial demonstration that caffeine can attenuate MPTP-induced toxicity, a neuroprotective role of A_{2A} receptor blockade has been suggested in several experimental models of Parkinson's disease (PD) (Chen et al., 2001; Schwarzschild et al., 2006). In rodents different A_{2A} receptor antagonists have been shown to counteract the loss of dopaminergic neurons in the substantia nigra pars compacta (SNc), as well as dopamine depletion in the striatum (Str), induced by acute administration of systemic MPTP or acute infusion of 6-hydroxydopamine (6-OHDA) in the medial forebrain bundle (Chen et al., 2001; 2002; Ikeda et al., 2002; Pierri et al., 2005). Moreover, loss of striatal dopamine induced by acute MPTP was attenuated by the global genetic deletion of A_{2A} receptors in A_{2A} knockout (KO) mice (Chen et al., 2001). In contrast to acute models, neuroprotection by A_{2A} antagonists in the neurotoxicity induced by multiple MPTP administration was never evaluated. On the other hand, previous studies have shown that acute or multiple injections MPTP delivery result in different histopathological features and different modes of cell death (Schmidt and Fergen, 2001). Interstingly, A_{2A} receptor antagonism or gene KO has been found to be neuroprotective in different models of neurodegeneration, such as Alzheimer's disease, Huntington's disease and cerebral ischemia (Chen et al., 1999; Dall'Igna et al., 2003; Fink et al., 2004; Melani et al., 2003).

A_{2A} receptors are enriched in the Str, where they are located either postsynaptically in striatopallidal neurons, or presynaptically in nerve terminals (Rebola et al., 2005; Rosin et al., 1998; Schiffman et al., 1991; Svenningsson et al., 1999). Moreover, A_{2A} receptors are expressed at low level in other forebrain structures, such as cortex and hippocampus, whereas little or no A_{2A} receptor immunoreactivity has been detected in dopaminergic neurons in the SNc (Rosin et al., 1998). Besides neurons, non-neuronal cell types such as microglia and astroglia also express A_{2A} receptors (Fiebich et al., 1996; Saura et al., 2005).

The mechanism through which A_{2A} receptor antagonists achieve neuroprotection in PD models has not been elucidated. Given the cellular distribution of A_{2A} receptors in different cell types in brain,

neuroprotection by A_{2A} receptor blockade may be achieved through an action on receptors located either on neurons or on glial cells. Attenuation of gliosis by A_{2A} receptor antagonists in the Str and SNc of mice acutely treated with MPTP, suggests that reduction of neuroinflammation may be involved (Ikeda et al., 2002; Pierri et al., 2005). Moreover, it was recently suggested that A_{2A} receptors located on glial cells may play a role in neuroprotection mediated by A_{2A} antagonists against an acute MPTP-induced striatal dopamine depletion (Yu et al, 2008).

In order to get insight into the mechanism by which A_{2A} receptor antagonists induce neuroprotection, the present study evaluated 1) the neuroprotective activity of an A_{2A} antagonist in a mouse model of PD obtained by a multiple injections MPTP delivery; 2) the role of neuronal A_{2A} receptors on this effect and 3) whether neuronal A_{2A} receptor blockade might affect glial response to MPTP. To this aim, we treated mice with MPTP plus the A_{2A} antagonist SCH58261, or administered MPTP to genetically manipulated mice selectively lacking A_{2A} receptors in forebrain neurons (fbnA_{2A}KO mice) (Bastia et al., 2005; Shen et al., 2008). Neuronal damage was evaluated in the SNc and Str using tyrosine hydroxylase (TH) immunohistochemistry, whereas the inflammatory response in the SNc and Str was studied through analysis of glial fibrillary acidic protein (GFAP) and CD11b immunoreactivity as markers of astroglial and microglial cells, respectively.

The present study provides evidence of a primary role played by forebrain A_{2A} receptors in the neuroprotection that A_{2A} receptor antagonists confer against dopaminergic neuron degeneration and glial activation induced by repeated MPTP.

Methods

Animals and treatments: Male C57BL/6J mice (Charles River, Italy) were used for experiments involving pharmacological treatments with the A_{2A} receptor antagonist. Mice were housed 5 per cage,

with a 12:12 hrs light/dark cycle and with food and water *ad libitum*. Experiments were conducted in accordance with the guidelines for care and use of experimental animals of the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the National Institutes of Health.

Pharmacological treatment: Male C57BL/6J mice (25-30 gm, 3 months old), received a multiple injections treatment with vehicle (N= 15), MPTP·HCl (20 mg/kg i.p.) once a day for 4 days (N = 18), or the A_{2A} antagonist SCH58261 (0.5 mg/kg i.p.) twice a day plus MPTP (20 mg/kg i.p.) once a day for 4 days (N = 23). SCH58261 was injected half an hour before MPTP administration. After MPTP treatment discontinuation SCH58261 treatment continued once a day, until sacrifice, which occurred 1, 3 or 7 days after MPTP treatment. Injections were made at 8 a.m. and 8 p.m.

Postnatal forebrain neuron conditional A_{2A}KO mice were generated using the Cre/*loxP* system based on the specificity of *CaMKII*α promoter (Bastia et al, 2005, Yu et al, 2008; see also Additional Informations (A.I.) for online version). FbnA_{2A}KO mice and littermate controls were treated with repeated MPTP (n=18 or n=15) or vehicle (n=12 or n=10) as described above. Experiments with the A_{2A} antagonist (see below) showed that loss of TH-immunoreactive cells was stable 1, 3 and 7 days after MPTP treatment, and that CD11b immunoreactivity was greatest 1 and 3 days post-MPTP treatment. Based on these considerations, neuroprotection and glial reactivity parameters in transgenic mice were evaluated 3 days after MPTP treatment.

Drugs: MPTP·HCl (Sigma-Aldrich, Italy or USA) was dissolved in 0.9% saline in a volume of 0.1 ml/10 g; SCH58261 (kindly provided Prof Baraldi, Ferrara) was suspended in 0.5% of methylcellulose.

Immunohistochemistry: Animals were anesthetized with chloral hydrate (400 mg/kg i.p.) or Avertin (0.1 ml/10 g i.p.) prior to transcardial perfusion with 20 ml of saline and 60 ml of 4% paraformaldehyde. Brains were removed and post-fixed for 2 hour. Adjacent coronal sections (50 µm) from the Str and SNc were cut on a vibratome and stored at -20°C in an antifreeze medium until use

(Schintu et al, 2009). For TH, GFAP and CD11b immunostaining, adjacent sections were processed as described (Schintu et al, 2009; See also A.I.).

Analysis and statistics: Images were digitized (videocamera Pixelink PL-A686) under constant light conditions to standardize the measurements. Immunostained sections containing left and right SNc, were captured at 10X magnification (the entire SNc, corresponding to three frames, was digitized for the analysis). Immunostained sections of bilateral striata were captured at 20X magnification. One portion from the dorsolateral Str and one from the ventromedial Str (520 µm x 380 µm) were analysed. For each animal, three sections corresponding to rostral (within -2.90/-3.20 mm from bregma), medial (-3.20/-3.50) and caudal (-3.50/-3.80) SNc levels, and three sections corresponding to rostral (within 1.20/0.90 mm from bregma), medial (0.90/0.60) and caudal (0.60/0.30) Str (accordingly to Mouse Brain Atlas, Paxinos and Franklin, 2001) were analysed for each protein marker evaluated in the study.

TH and GFAP analyses: since the number of cells was different in the three SNc and Str levels analysed, for each mouse the number of TH- or GFAP-positive cells/level was first normalized with respect to the vehicle. Individual values from the 3 levels were then averaged to generate a mean. Adjacent SNc sections were stained with cresyl violet, in order to confirm cell loss in this area.

The SNc from three mice randomly selected from each experimental group were evaluated by unbiased stereological counting and the mean of TH-ir neurons per mm^3 ± SEM was calculated (see A.I).

CD11b analysis: images were digitized in a grey scale, and CD11b immunostaining was evaluated with the analysis program Scion Image. A threshold, the value of which was set above the mean value ± S.E.M. of the background, was applied for background-correction. Inside each frame, the area occupied by grey values above the threshold was automatically calculated. For each level of SNc or Str, the

Appendix A

obtained value was first normalized with respect to vehicle; the individual values from the 3 levels were then averaged to generate a mean.

Results from mice treated with MPTP plus SCH58261, or from fbnA_{2A}KO mice were statistically compared with a two-factors ANOVA, followed by Tukey's *post hoc* test, for comparison between experimental groups.

Results

A_{2A} receptor antagonist prevents dopaminergic cell loss in the SNc.

In C57BL/6J mice, MPTP treatment induced a partial dopaminergic neurons degeneration in the SNc (Fig 1A, B and Tab 1). Counting of cresyl violet stained cells confirmed this result (Fig 1A, Vehicle: 100 ± 1.5% versus MPTP: 67.8 ± 4.3%; as measured three days after last injection, relative to vehicle controls). Dopaminergic neurons loss was statistically significant 1 day after MPTP treatment (N= 5) and remained significant after 3 (N=5) and 7 days timepoints (N=8). Combined treatment with A_{2A} antagonist SCH58261 plus MPTP, attenuated TH-positive neurons loss in the SNc at 1 (N= 5), 3 (N=5) and 7 days (N=13) (Fig 1 A, B and Tab 1), as confirmed by cresyl violet staining (MPTP + SCH58261: 98.6 ± 1.3%). Two-factors ANOVA showed a significant effect of treatment (see Table 2 in A.I. for corresponding F and P values). In the Str, MPTP-induced decrease in TH-immunoreactivity was significantly attenuated by treatment with the A_{2A}R antagonist, as measured three days after treatment (Fig 2).

A_{2A} receptor antagonist inhibits astroglia and microglia activation.

In brain sections from vehicle-treated mice, few GFAP-positive cells (Fig 3), and a low CD11b immunostaining (Figs 3, 4), were detected in the SNc and Str. Repeated MPTP treatment induced an increase in GFAP and CD11b-positive cells in both the SNc and Str of C57BL/6J mice.

GFAP-positive cells displayed a highly branched morphology with tiny processes and a small body in control sections, and became hypertrophic in response to MPTP treatment (Fig 3A); moreover, CD11b-positive cells were ramified at baseline, but took on an ameboid aspect after MPTP treatment (Fig 4A), indicative of astroglial and microglial activation. In both the SNc and Str, GFAP immunolabelling was of similar intensity 1, 3 and 7 days post-MPTP treatment (Fig 3B). Combined SCH58261 plus MPTP treatment, completely prevented the increase in GFAP immunoreactivity in the SNc and partially prevented it in the Str, at 1, 3 and 7 days after MPTP treatment (Fig 3B). Two-factors ANOVA for GFAP analysis showed a significant effect of the treatment at all time points analyzed in the SNc and in the Str (see Table 2 in A.I.).

CD11b immunolabelling was highest 1 day after MPTP, gradually declining to basal levels after 7 days in both SNc and Str (Fig 4A, B). Combined SCH58261 plus MPTP treatment completely prevented the increase in CD11b immunoreactivity in the SNc 1 and 3 days after MPTP treatment (Fig 4A,B). In the Str, SCH58261 partially prevented MPTP-induced increase in CD11b at 1 day, and totally prevented it at 3 days after MPTP treatment. Two-factors ANOVA and *post hoc* analysis for CD11b revealed a significant effect of treatment, time, and a treatment/time interaction, 1 and 3 days after MPTP administration in the SNc and in the Str (se Table 2 in A.I.).

Dopaminergic cell loss is prevented in fbnA_{2A}KO mice.

In fbnA_{2A}WT mice, repeated MPTP treatment induced a significant loss of dopamine neurons in the SNc (Fig 5A, B and Tab 1). This result was confirmed by a similar reduction in cresyl violet stained cells (fbnWT V: 100 ± 1.21; fbnWT MPTP: 71.60 ± 2.73) (see right insert in fig 5A). In contrast, in

Appendix A

fbnA$_{2A}$KO mice, repeated MPTP treatment did not result in a decrease in TH-immunolabelling in the SNc, as confirmed by analysis of cresyl violet staining (fbnA$_{2A}$KO V: 99.12 ± 1.41; fbnA$_{2A}$KO MPTP: 99.71 ± 4.40) (Fig 5A, B and Tab 1). Two-factors ANOVA revealed a significant effect of treatment and genotype (see Table 2 in A.I. for corresponding F and P values). In the Str of fbnA $_{2A}$KO mice repeated MPTP reduced TH immunolabelling to a lesser extent as compared with fbnA$_{2A}$WT controls (Fig 2).

Astroglial and microglial activation is attenuated in fbnA$_{2A}$KO mice.

GFAP immunoreactivity in the SNc and Str was significantly enhanced in MPTP-treated fbnA$_{2A}$WT mice (Fig 6A, B). In contrast, in fbnA$_{2A}$KO mice, MPTP-induced increase in GFAP immunoreactivity was totally prevented in the SNc, and partially prevented in Str (Fig 6A, B). In both brain regions two-factors ANOVA revealed a significant effect of treatment, genotype and a significant treatment/genotype interaction (see Table 2 in A.I.).

CD11b immunolabelling in the SNc and Str was significantly enhanced in MPTP-treated fbnA$_{2A}$WT mice (Fig 7A, B). In fbnA$_{2A}$KO mice CD11b activation was totally prevented in the SNc and Str (Fig 7A, B). Two-factors ANOVA revealed a significant effect of treatment, genotype and a treatment/genotype interaction (see Table 2 in A.I.).

Discussion

Antagonism of adenosine A$_{2A}$ receptors or their selective deletion in forebrain neurons produced similar protection of TH-positive nigral neurons in a multiple MPTP injections mouse model of PD. These complementary pharmacological and genetic means of A$_{2A}$ receptor disruption also attenuated the neurotoxin-triggered activations of astroglia and microglia along the nigrostriatal pathway.

Together these data provide evidence that therapeutically accessible A_{2A} receptors located on forebrain neurons play a critical role in nigral dopaminergic neuron degeneration and inflammatory processes in the multiple MPTP injections mouse model of PD.

Pharmacological blockade of A_{2A} receptors prevents MPTP-induced dopaminergic neuron degeneration and glial activation

Systemic administration of the A_{2A} receptor antagonist SCH58261 prevented the degeneration of nigrostriatal TH-positive neurons induced by repeated MPTP exposure in mice. Changes in number of TH-positive neurons correlated with changes in Nissl-stained (cresyl violet-positive) cells, indicating that MPTP treatment resulted in actual loss of dopaminergic neurons, which were rescued by SCH58261.

A neuroprotective effect of A_{2A} receptor antagonists was previously observed upon acute administration of high MPTP doses in mice (Chen et al., 2001; Ikeda et al., 2002; Pierri et al., 2005; Yu et al, 2008). Here we report that neuroprotection with A_{2A} antagonism can also be achieved upon multiple low-doses MPTP exposure. A number of studies have provided evidence that a repeated daily MPTP administration protocol similar to the one used here, presents histopathological features that more closely reproduce the human PD neuropathology, including apoptotic death of dopaminergic neurons (Jackson-Lewis et al., 1995; Tatton & Kish, 1997). Therefore, the present study further substantiates the neuroprotective potential of A_{2A} antagonism in PD. To this regard, it is noteworthy that A_{2A}R antagonists were shown to inhibit apoptotic neuronal death in hippocampal neurons (Silva et al, 2007).

Based on their differential location in the Str or other brain regions, A_{2A} receptors may hold different levels of expression and intracellular signalling, reflecting A_{2A} receptor multiple functions (Kull et al, 2000; Pedata et al, 2003; Rebola et al, 2005; Rosin et al, 2003; Shen et al, 2008). According to such

varied roles of the A_{2A} receptor, diverse effects have been attributed to A_{2A} antagonists, ranging from symptomatic antiparkinsonian actions to neuroprotection in various neurodegenerative conditions (Alfinito et al., 2003; Blum et al., 2003; Chen et al., 1999; Melani et al., 2003; Monopoli et al., 1998; Popoli et al., 2002). Motor effects of A_{2A} receptor antagonists are likely mediated by A_{2A} receptors located on striatal neurons projecting to globus pallidus, whereas several mechanisms have been hypothesized for their neuroprotective effects, involving either neuronal or glial A_{2A} receptors, though no single mechanism has yet been proven to prevail (Carta et al., 2003; Chen et al., 2001; Huang et al, 2006; Melani et al., 2003; Popoli et al., 1995; Pedata et al., 2003; Schwarzschild et al, 2006; Yu et al, 2008). Noticeably, in the present study neuroprotection by SCH58261 was achieved at doses similar to those effective in other neurodegenerative conditions, but several times lower than doses displaying a symptomatic efficacy in PD (Chen et al., 2001; Dall'Igna et al., 2003; Melani et al., 2003; Pinna et al., 2007), supporting the concept that different mechanisms might account for A_{2A}-mediated neuroprotection or symptomatic effects.

SCH58261 fully prevented astroglia and microglia activation in the SNc, while only partially inhibiting astroglia and microglia reactivity in the striatum, in line with a partial protection of dopaminergic terminals. Noteworthy, A_{2A} receptor antagonism prevented both astroglia and microglia activation in the SNc and Str at all time-points evaluated, in accordance with blockade of neurodegeneration.

Though the mechanism through which A_{2A} receptor blockade produces neuroprotective effects in PD models is unclear, the modulation of neuroinflammation has been proposed as a likely target for neuroprotection (Hunot and Hirsch, 2003). Several findings have suggested that neuroinflammation may play an active role in the pathogenesis of neurodegeneration in PD, since focal inflammation has been described in in the SNc of PD patients and MPTP-treated primates (Barcia et al., 2004; McGeer et al., 1988). Intriguingly, blockade of microglia reactivity in mice rescued dopamine neurons from acute MPTP toxicity (Wu et al., 2002). Moreover, in mice acutely treated with MPTP, dopamine neuron

neuroprotection by pre-treatment with an A_{2A} antagonist was associated with an attenuation of astroglia and microglia activation in SNc and Str (Ikeda et al., 2002; Pierri et al., 2005), consistent with a causal relation between the two events.

Selective deletion of neuron specific forebrain (fbn) A_{2A} receptors prevents MPTP-induced dopamine neuron degeneration and glial activation.

Previous studies evaluating A_{2A} receptor-mediated neuroprotection have hypothesised several mechanisms that might underlie this process. In order to determine the role of neuronal *versus* glial A_{2A} receptors in neuroprotection of dopamine neurons, we exploited genetically modified mice with selective depletion of A_{2A} receptors from forebrain neurons (Bastia et al., 2005; Shen et al, 2008). Importantly, in the fbnA_{2A}KO mice, deletion of A_{2A} receptor is not only spatially restricted (to forebrain A_{2A} receptor) but is also temporally limited to postnatal A_{2A} receptors (Bastia et al., 2005; Yu et al., 2008), thus avoiding potential confounds of compensatory responses to A_{2A} receptor gene disruption during development as might occur in constitutive A_{2A} knockout mice.

Our results revealed that selective deletion of A_{2A} receptors from forebrain neurons totally prevented dopaminergic neuron loss in the SNc following multiple MPTP injections, while partially preventing damage to striatal dopaminergic terminals. A_{2A}R deletion provided a greater protection of SNc neurons than the A_{2A}R antagonist, as expected from a permanent as compared to a temporal pharmacological blockade of the receptor, in line with the reported half-life of SCH58261 of 2-3 h.

A_{2A} receptors are located both pre- and post-synaptically in striatal and cortical neurons, and are expressed in microglial as well as astroglial cells (Cunha, 2001; Kust et al., 1999; Nishizaki et al, 2004; Rebola et al., 2005; Rosin et al., 2003). Positive modulation of postsynaptic signalling as well as of pre-synaptic release of neurotransmitters as glutamate and acetylcholine by A_{2A} receptors have been described (Fredholm et al., 2003; Fuxe et al., 2003; Marchi et al., 2002; Popoli et al., 1995; Schiffmann et al., 2007; Schwarzschild et al, 2006). In addition, A_{2A} receptors interfere with glia-mediated

synthesis and release of neurotoxic factors such as COX-2, prostaglandins, nitric oxide and glutamate, which have been hypothesised to play central roles in inflammatory processes and neuronal damage (Fiebich et al., 1996; Li et al., 2001; Saura et al., 2005).

The present study, by showing that selective deletion of A_{2A} receptors from forebrain neurons protects dopaminergic neurons from MPTP toxicity, endorses a primary role of neuronal receptors in mediating neuroprotection in this multiple injections MPTP model of PD. Since very low levels of A_{2A} receptors are expressed by dopaminergic neurons in the SNc, it is unlikely that a direct action at this level might mediate neuroprotection from MPTP toxicity in the SNc. Rather, an indirect effect at the pre-synaptic level, through an inhibition of A_{2A}-mediated glutamate release, which contribute to neuronal damage, could be envisaged (Aguirre et al., 2005; Battaglia et al., 2004; Monopoli et al., 1998; Popoli et al., 2002). Interestingly, recent studies have reported a thight cross-talk between adenosine and GDNF receptors, resulting in a fine modulation of glutamate and dopamine release (Gomes et al, 2006; 2009). In the Str, A_{2A}R blockade would impair GDNF-stimulated increase of corticostriatal glutamate release, thus providing a beneficial effect on neurodegeneration. In addition, A_{2A} receptor antagonism on striatopallidal or subthalamic (STN) neurons might be protective from MPTP toxicity by modulating excessive activation of subthalamic nucleus, thereby reducing excitotoxic glutamate efflux to SNc neurons (Wallace et al., 2007).

A study by Yu and colleagues reported that fbnA_{2A}KO mice from the same line as used here, were not protected from striatal dopamine loss in response to acute MPTP exposure (in a single dose or multiple doses over 4 hr; Yu et al., 2008). These results open to several compelling interpretations. First, the cellular basis of A_{2A} receptor-dependence of MPTP toxicity might vary depending on the duration of toxin exposure, in line with the different type of neurotoxicity produced by acute as compared to subchronic MPTP. Moreover, it should be taken into account the different parameters used to evaluate nigrostriatal neurons damage. The drop of striatal dopamine levels assessed by Yu et al. may reflect

functional injury to dopaminergic terminals, whereas the measure of dopaminergic nigral neuron employed in our study may reflect an underlying neurodegenerative process in this area. All together results support the concept that A_{2A} receptors display complex actions related to the duration of insult, cellular elements and brain areas targeted by neurodegenerative processes.

Lack of a glial reaction in fbnA_{2A}KO mice, as compared to the robust astroglia and microglia activation in MPTP-treated control mice, indicates that deletion of neuronal A_{2A} receptors may indirectly inhibit the inflammatory response. Glutamate is a main contributor to the complex neuron-glia cross-talk engaged by pathological events, which trigger both microglia and astroglia activation. For instance, by an action on NMDA receptors, glutamate release stimulates mitogen-activated protein kinases (MAPKs). Neuronal as well as glial p38 MAPK activation has been involved in cell suffering and apoptotic death, being activated and inducing several inflammatory mediators (Gianfriddo et al., 2004; Irving et al., 2000; Kawasaki et al., 1997; Piao et al., 2003). Therefore, A_{2A} antagonists indirectly through a reduction of glutamate release might counteract glial reactivity and neuroinflammation in both SNc and Str (Melani et al., 2003, 2006). Activated glial cells, by the release of several toxic species as cytokines, free radicals, glutamate, is known to contribute to neuronal damage, and has been suggested to sustain a self-amplifying cycle which perpetuates MPTP toxicity (Hunot and Hirsh, 2003). Hence, interruption of such a detrimental vicious cycle might indirectly contribute to A_{2A} receptor-dependent neuroprotection. Accordingly results suggest that in our model, operational glial A_{2A} receptors did not contribute to MPTP-toxicity when A_{2A} forebrain neuronal receptors where deleted. All together, our results suggest that A_{2A} antagonists, by blocking neuronal A_{2A} receptors, might act upstream in the cascade of toxic events and lead to an attenuation of dopamine neuron degeneration in PD.

Acknowledgments:

Appendix A

This study was supported by Ministero dell'Università e della Ricerca Scientifica e Tecnologica, project FIRB [grant number RBNE03YA3L-2005] and National Institute of Health [grant numbers ES010804, S54978] and U.S. Army Medical Research Acquisition Activity [grant number W81XWH-04-1-0881].

The authors thank Prof P.G. Baraldi and Dr M.A. Tabrizi, Department of Pharmaceutical Sciences, University of Ferrara, for providing SCH58261, David H. Gutmann and M. Livia Bajenaru for providing *GFAP-cre* mice, and Yuehang Xu for expert technical assistance.

References

Aguirre JA, Kehr J, Yoshitake T, Liu FL, Rivera A, Fernandez-Espinola S, Andbjer B, Leo G, Medhurst AD, Agnati LF, Fuxe K (2005) Protection but maintained dysfunction of nigral dopaminergic nerve cell bodies and striatal dopaminergic terminals in MPTP-lesioned mice after acute treatment with the mGluR5 antagonist MPEP. *Brain Res* **1033**, 216-20.

Alfinito PD, Wang SP, Manzino L, Rijhsinghani S, Zeevalk GD, Sonsalla PK (2003) Adenosinergic protection of dopaminergic and GABAergic neurons against mitochondrial inhibition through receptors located in the substantia nigra and striatum, respectively. *J Neurosci* **23**, 10982-87.

Barcia C, Sánchez Bahillo A, Fernández-Villalba E, Bautista V, Poza Y Poza M, Fernández-Barreiro A, Hirsch EC, Herrero MT (2004) Evidence of active microglia in substantia nigra pars compacta of parkinsonian monkeys 1 year after MPTP exposure. *Glia* **46**, 402-09.

Appendix A

Bastia E, Xu YH, Scibelli AC, Day YJ, Linden J, Chen JF, Schwarzschild MA (2005) A crucial role for forebrain adenosine A(2A) receptors in amphetamine sensitization. *Neuropsychopharmacology* **30**, 891-900.

Battaglia G, Busceti CL, Molinaro G, Biagioni F, Storto M, Fornai F, Nicoletti F and Bruno V (2004) Endogenous activation of mGlu5 metabotropic glutamate receptors contributes to the development of nigro-striatal damage induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in mice. *J. Neurosci* **24**, 828–35.

Blum D, Galas MC, Pintor A, Brouillet E, Ledent C, Muller CE, Bantubungi K, Galluzzo M, Gall D, Cuvelier L, Rolland AS, Popoli P, Schiffmann SN (2003) A dual role of adenosine A2A receptors in 3-nitropropionic acid-induced striatal lesions: implications for the neuroprotective potential of A2A antagonists. *J Neurosci* **23**, 5361-69.

Carta AR, Pinna A, Tronci E, Morelli M (2003) Adenosine A2A and dopamine receptor interactions in basal ganglia of dopamine denervated rats. *Neurology* **61**, S39-43.

Chen JF, Huang Z, Ma J, Zhu J, Moratalla R, Standaert D, Moskowitz MA, Fink JS, Schwarzschild MA (1999) A(2A) adenosine receptor deficiency attenuates brain injury induced by transient focal ischemia in mice. *J Neurosci* **19**, 9192-200.

Chen JF, Xu K, Petzer JP, Staal R, Xu YH, Beilstein M, Sonsalla PK, Castagnoli K, Castagnoli N Jr, Schwarzschild MA (2001) Neuroprotection by caffeine and A(2A) adenosine receptor inactivation in a model of Parkinson's disease. *J Neurosci* **21**, RC143: 1-6.

Cunha RA (2001) Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. *Neurochem Int* **38**, 107-25.

Dall'Igna OP, Porciúncula LO, Souza DO, Cunha RA, Lara DR (2003) Neuroprotection by caffeine and adenosine A2A receptor blockade of beta-amyloid neurotoxicity. *Br J Pharmacol* **138**, 1207-09.

Fiebich BL, Biber K, Lieb K, van Calker D, Berger M, Bauer J, Gebicke-Haerter PJ (1996) Cyclooxygenase-2 expression in rat microglia is induced by adenosine A2a-receptors. *Glia* **18**,152-60.

Fink JS, Kalda A, Ryu H, Stack EC, Schwarzschild MA, Chen JF, Ferrante RJ (2004) Genetic and pharmacological inactivation of the adenosine A2A receptor attenuates 3-nitropropionic acid-induced striatal damage. *J Neurochem* **88**, 538-44.

Fredholm BB, Cunha RA, Svenningsson P (2003) Pharmacology of adenosine A2A receptors and therapeutic applications. *Curr Top Med Chem* **3**, 413-26.

Fuxe K, Agnati LF, Jacobsen K, Hillion J, Canals M, Torvinen M, Tinner-Staines B, Staines W, Rosin D, Terasmaa A, Popoli P, Leo G, Vergoni V, Lluis C, Ciruela F, Franco R, Ferré S (2003) Receptor heteromerization in adenosine A2A receptor signaling: relevance for striatal function and Parkinson's disease. *Neurology* **61**, S19-23.

Gianfriddo M, Melani A, Turchi D, Giovannini MG, Pedata F (2004) Adenosine and glutamate extracellular concentrations and mitogen-activated protein kinases in the striatum of Huntington

transgenic mice. Selective antagonism of adenosine A2A receptors reduces transmitter outflow. *Neurobiol Dis* **17**, 77-88.

Gomes CA, Vaz SH, Ribeiro JA, Sebastião AM (2006) Glial cell line-derived neurotrophic factor (GDNF) enhances dopamine release from striatal nerve endings in an adenosine A2A receptor-dependent manner. *Brain Res* **1113**, 129-36.

Gomes CA, Simões PF, Canas PM, Quiroz C, Sebastião AM, Ferré S, Cunha RA, Ribeiro JA (2009). GDNF control of the glutamatergic cortico-striatal pathway requires tonic activation of adenosine A receptors. *J Neurochem* **108**, 1208-19.

Huang QY, Wei C, Yu L, Coelho JE, Shen HY, Kalda A, Linden J, Chen JF (2006) Adenosine A2A receptors in bone marrow-derived cells but not in forebrain neurons are important contributors to 3-nitropropionic acid-induced striatal damage as revealed by cell-type-selective inactivation. J Neurosci **26**, 11371-8.

Hunot S, Hirsch EC. Neuroinflammatory processes in Parkinson's disease (2003) *Ann Neurol* **53**, S49-58.

Ikeda K, Kurokawa M, Aoyama S, Kuwana Y (2002) Neuroprotection by adenosine A2A receptor blockade in experimental models of Parkinson's disease. *J Neurochem* **80**, 262-70.

Irving EA, Barone FC, Reith AD, Hadingham SJ, Parsons AA (2000) Differential activation of MAPK/ERK and p38/SAPK in neurones and glia following focal cerebral ischaemia in the rat. *Brain Res Mol Brain Res* **77**, 65-75.

Jackson-Lewis V, Jakowec M, Burke RE, Przedborski S (1995) Time course and morphology of dopaminergic neuronal death caused by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Neurodegeneration* **4**, 257-69.

Kawasaki H, Morooka T, Shimohama S, Kimura J, Hirano T, Gotoh Y, Nishida E (1997) Activation and involvement of p38 mitogen-activated protein kinase in glutamate-induced apoptosis in rat cerebellar granule cells. *J Biol Chem* **272**, 18518-21.

Kull B, Svenningsson P, Fredholm BB. (2000) Adenosine A(2A) receptors are colocalized with and activate g(olf) in rat striatum. *Mol Pharmacol* **58**, 771-7.

Küst BM, Biber K, van Calker D, Gebicke-Haerter PJ (1999) Regulation of K+ channel mRNA expression by stimulation of adenosine A2a-receptors in cultured rat microglia. *Glia* **25**, 120-30.

Kwon CH, Zhu X, Zhang J, Knoop LL, Tharp R, Smeyne RJ, Eberhart CG, Burger PC, Baker SJ (2001) Pten regulates neuronal soma size: a mouse model of Lhermitte-Duclos disease. *Nat Genet* **29**, 404-411.

Li XX, Nomura T, Aihara H, Nishizaki T (2001) Adenosine enhances glial glutamate efflux via A2a adenosine receptors. *Life Sci* **68**, 1343-50.

Appendix A

Marchi M, Raiteri L, Risso F, Vallarino A, Bonfanti A, Monopoli A, Ongini E, Raiteri M (2002) Effects of adenosine A1 and A2A receptor activation on the evoked release of glutamate from rat cerebrocortical synaptosomes. *Br J Pharmacol* **136**, 434-40.

McGeer PL, Itagaki S, Boyes BE, McGeer EG (1988) Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. *Neurology* **38**, 1285-91.

Melani A, Pantoni L, Bordoni F, Gianfriddo M, Bianchi L, Vannucchi MG, Bertorelli R, Monopoli A, Pedata F (2003) The selective A2A receptor antagonist SCH 58261 reduces striatal transmitter outflow, turning behavior and ischemic brain damage induced by permanent focal ischemia in the rat. *Brain Res* **959**, 243-50.

Melani A, Gianfriddo M, Vannucchi MG, Cipriani S, Baraldi PG, Giovannini MG, Pedata F (2006) The selective A2A receptor antagonist SCH 58261 protects from neurological deficit, brain damage and activation of p38 MAPK in rat focal cerebral ischemia. *Brain Res* **1073-1074**, 470-80.

Nishizaki T. (2004) ATP- and adenosine-mediated signaling in the central nervous system: adenosine stimulates glutamate release from astrocytes via A2a adenosine receptors. *J Pharmacol Sci* **94**,100-2.

Paxinos G, Franklin KBJ (2001) *The Mouse Brain in Stereotaxic Coordinates, 2nd edition*. Academic Press, San Diego

Pedata F, Pugliese AM, Melani A, Gianfriddo M (2003) A2A receptors in neuroprotection of dopaminergic neurons. *Neurology* **61**, S49-50.

Piao CS, Yu YM, Han PL, Lee JK (2003) Dynamic expression of p38beta MAPK in neurons and astrocytes after transient focal ischemia. Brain Res 976:120-24.

Pierri M, Vaudano E, Sager T, Englund U. KW-6002 protects from MPTP induced dopaminergic toxicity in the mouse. (2005) *Neuropharmacology* **48**, 517-24.

Pinna A, Pontis S, Borsini F, Morelli M (2007) Adenosine A2A receptor antagonists improve deficits in initiation of movement and sensory motor integration in the unilateral 6-hydroxydopamine rat model of Parkinson's disease. *Synapse* **61**, 606-14.

Popoli P, Pintor A, Domenici MR, Frank C, Tebano MT, Pèzzola A, Scarchilli L, Quarta D, Reggio R, Malchiodi-Albedi F, Falchi M, Massotti M (2002) Blockade of striatal adenosine A2A receptor reduces, through a presynaptic mechanism, quinolinic acid-induced excitotoxicity: possible relevance to neuroprotective interventions in neurodegenerative diseases of the striatum. *J Neurosci* **22**, 1967-75.

Popoli P, Betto P, Reggio R, Ricciarello G (1995) Adenosine A2A receptor stimulation enhances striatal extracellular glutamate levels in rats. *Eur J Pharmacol* **287**, 215-17.

Rebola N, Canas PM, Oliveira CR, Cunha RA (2005) Different synaptic and subsynaptic localization of adenosine A2A receptors in the hippocampus and striatum of the rat. *Neuroscience* **132,** 893-903.

Rosin DL, Hettinger BD, Lee A, Linden J (2003) Anatomy of adenosine A2A receptors in brain: morphological substrates for integration of striatal function. *Neurology* **61**, S12-18.

Rosin DL, Robeva A, Woodard RL, Guyenet PG, Linden J (1998) Immunohistochemical localization of adenosine A2A receptors in the rat central nervous system. *J Comp Neurol* **401**, 163-86.

Saura J, Angulo E, Ejarque A, Casadó V, Tusell JM, Moratalla R, Chen JF, Schwarzschild MA, Lluis C, Franco R, Serratosa J (2005) Adenosine A2A receptor stimulation potentiates nitric oxide release by activated microglia. *J Neurochem* **95**, 919-29.

Schiffmann SN, Fisone G, Moresco R, Cunha RA, Ferré S (2007) Adenosine A2A receptors and basal ganglia physiology. *Prog Neurobiol* **83**, 277-92.

Schiffmann SN, Jacobs O, Vanderhaeghen JJ (1991) Striatal restricted adenosine A2 receptor (RDC8) is expressed by enkephalin but not by substance P neurons: an in situ hybridization histochemistry study. *J Neurochem* **57**, 1062-7.

Schmidt N and Ferger B (2001) Neurochemical findings in the MPTP model of Parkinson's disease. *J Neural Transm* **108**, 1263–1282.

Schintu N, Frau L, Ibba M, Garau A, Carboni E and Carta AR (2009) Progressive dopaminergic degeneration in the chronic MPTPp mouse model of Parkinson's disease. *Neurotox Res* **16**, 127-39.

Schwarzschild MA, Agnati L, Fuxe K, Chen JF, Morelli M (2006) Targeting adenosine A2A receptors in Parkinson's disease. *Trends Neurosci* **29**, 647-54.

Shen HY, Coelho JE, Ohtsuka N, Canas PM, Day YJ, Huang QY, Rebola N, Yu L, Boison D, Cunha RA, Linden J, Tsien JZ, Chen JF (2008) A critical role of the adenosine A2A receptor in extrastriatal neurons in modulating psychomotor activity as revealed by opposite phenotypes of striatum and forebrain A2A receptor knock-outs. *J Neurosci* **28**, 2970-5.

Silva CG, Porciúncula LO, Canas PM, Oliveira CR, Cunha RA (2007) Blockade of adenosine A(2A) receptors prevents staurosporine-induced apoptosis of rat hippocampal neurons. *Neurobiol Dis* **27**, 182-89.

Svenningsson P, Le Moine C, Fisone G, Fredholm BB (1999) Distribution, biochemistry and function of striatal adenosine A2A receptors. *Prog Neurobiol* **59**, 355-96.

Tatton NA, Kish SJ (1997) In situ detection of apoptotic nuclei in the substantia nigra compacta of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice using terminal deoxynucleotidyl transferase labelling and acridine orange staining. *Neuroscience* **77**, 1037-48.

Wallace BA, Ashkan K, Heise CE, Foote KD, Torres N, Mitrofanis J, Benabid AL (2007) Survival of midbrain dopaminergic cells after lesion or deep brain stimulation of the subthalamic nucleus in MPTP-treated monkeys. *Brain* **130**, 2129-45.

Yu L, Shen HY, Coelho JE, Araújo IM, Huang QY, Day YJ, Rebola N, Canas PM, Rapp EK, Ferrara J, Taylor D, Müller CE, Linden J, Cunha RA, Chen JF (2008) Adenosine A2A receptor antagonists exert motor and neuroprotective effects by distinct cellular mechanisms. *Ann Neurol* **63**, 338-46.

Wu DC, Jackson-Lewis V, Vila M, Tieu K, Teismann P, Vadseth C, Choi DK, Ischiropoulos H, Przedborski S (2002) Blockade of microglial activation is neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson disease. *J Neurosci* **22**, 1763-71.

Figures legend

Fig 1: **Adenosine A_{2A} receptor antagonist SCH58261 prevents dopaminergic cell loss in the SNc.** (A) shows representative sections immunostained for TH from SNc of mice sacrificed 3 days after MPTP treatment. Left insert shows TH-positive cells at higher magnification, right insert shows cresyl violet-stained sections; scale bar: 50 µm. Mice were treated with MPTP-HCl (20 mg/kg once a day for 4 days), plus SCH58261 (0.5 mg/kg) or vehicle (twice a day during MPTP treatment and once daily thereafter until sacrifice), and sacrificed 1 , 3, 7 days after MPTP treatment. (B) shows analysis of TH immunostaining at 1, 3, 7 days after MPTP, reported as a percentage of TH-positive cells as compared to vehicle-treated mice. * indicates $p<0.001$ versus vehicle; # indicates $p<0.001$ versus MPTP group, by Tukey's *post hoc* test.

Fig 2: **Adenosine A_{2A} receptor antagonist SCH58261 attenuates degeneration of dopaminergic terminals in the Str.** (A) representative sections immunostained for TH, from the Str of mice

sacrificed 3 days after MPTP treatment. (B) Results from pharmacological blockade with SCH58261 or genetic $A_{2A}R$ depletion are presented. In the left column, - and + indicate the administration of vehicle or SCH58261to MPTP-treated mice. In the right column + and – indicate fbnA_{2A}WT and fbnA_{2A}KO mice, respectively.

Fig 3: **Adenosine A_{2A} receptor antagonist SCH58261 counteracts astroglia activation in the SNc and Str.** (A) shows representative sections immunostained for GFAP, from SNc (upper images) and Str (lower images) of mice sacrificed 3 days after MPTP treatment. Mice were treated as described in Fig 1. (B) shows analysis of GFAP immunostaining 1, 3, 7 days after MPTP, reported as percentage of GFAP-positive cells as compared to vehicle-treated mice in the SNc (left graph) and in the Str (right graph). * indicates $p<0.001$ versus corresponding vehicle and MPTP+SCH58261 groups; # indicates $p<0.001$ versus corresponding MPTP group, by Tukey's *post hoc* test.

Fig 4: **Adenosine A_{2A} receptor antagonist SCH58261 counteracts microglia activation.** (A) Representative images from the SNc immunostained for CD11b as a marker of microglia activation. Mice were treated as described in Fig 1 and sacrificed 1, 3, 7 days after MPTP treatment. (B) CD11b analysis in SNc and Str was performed in grey-scale digitized images. The area occupied by grey values above a threshold was calculated and expressed as square pixels and as percentage of vehicle-treated mice. Tukey's post hoc test: * $p<0.001$ versus vehicle and MPTP+SCH58261 group; # $p<0.001$ versus MPTP group; ^, ^^ $p<0.05$, $p<0.001$ versus the indicated time point.

Fig 5: **fbnA_{2A} KO mice are protected against MPTP-induced loss of dopaminergic cells in the SNc.** (A) shows representative sections from SNc immunostained for TH. Inserts show higher magnification of TH-labelled (left) and cresyl violet-labelled (right) cells. Mice were treated with MPTP (20 mg/kg once a day for 4 days)or vehicle. (B) shows analysis of TH immunostaining in

fbnA_{2A}KO mice, reported as a percentage of TH-positive cells as compared to vehicle-treated mice. Tukey's post hoc test: * $p<0.05$ versus vehicle group; # $p<0.05$ versus WT MPTP group.

Fig 6: **Astroglia activation is attenuated in SNc and Str of fbnA_{2A}KO mice.** (A) representative images from the SNc immunostained for GFAP, as a marker of astroglial cells. (B) Graphs show the analysis of GFAP immunostaining in SNc and Str, in fbnWT and fbnA_{2A}KO mice treated with vehicle or MPTP. Tukey's post hoc test: * $p<0.001$ versus vehicle group; # $p<0.001$ versus WT MPTP group.

Fig 7: **Microglia activation is prevented in SNc and Str of fbnA_{2A}KO mice.** (A) representative images from the SNc immunostained for CD11b, as a marker of microglia activation. (B) Graphs show the analysis of CD11b immunostaining in SNc and Str, in fbnWT and fbnA_{2A}KO mice treated with vehicle or MPTP. Tukey's post hoc test: * $p<0.001$ versus vehicle group; # $p<0.001$ versus WT MPTP group.

Table 1: unbiased evaluation of TH-IR neurons by stereological analysis in the SNc of mice following pharmacological blockade of A_{2A}Rs with the antagonist SCH58261 or A_{2A}R genetic depletion. * $p< 0.01$ versus vehicle group. ^ $p<0.01$ versus the corresponding MPTP treated group.

Appendix A

Tab 1. Stereological evaluation of TH-IR neurons in the substantia nigra pars compacta

Treatment	*N*	Density of TH-IR neurons/mm^3
pharmacological blockade of $A_{2A}R$		
Vehicle	3	29725.89 ± 439.56
MPTP	3	15211.79 ± 1300.06*
SCH + MPTP	3	23003.66 ± 495.76* ^
fbn$A_{2A}R$ depletion		
WT vehicle	3	27447.34 ± 1365.08
WT MPTP	3	14278.85 ± 2493.30*
KO vehicle	3	23654.36 ± 2277.74
KO MPTP	3	24267.96 ± 2277.83^

Appendix A

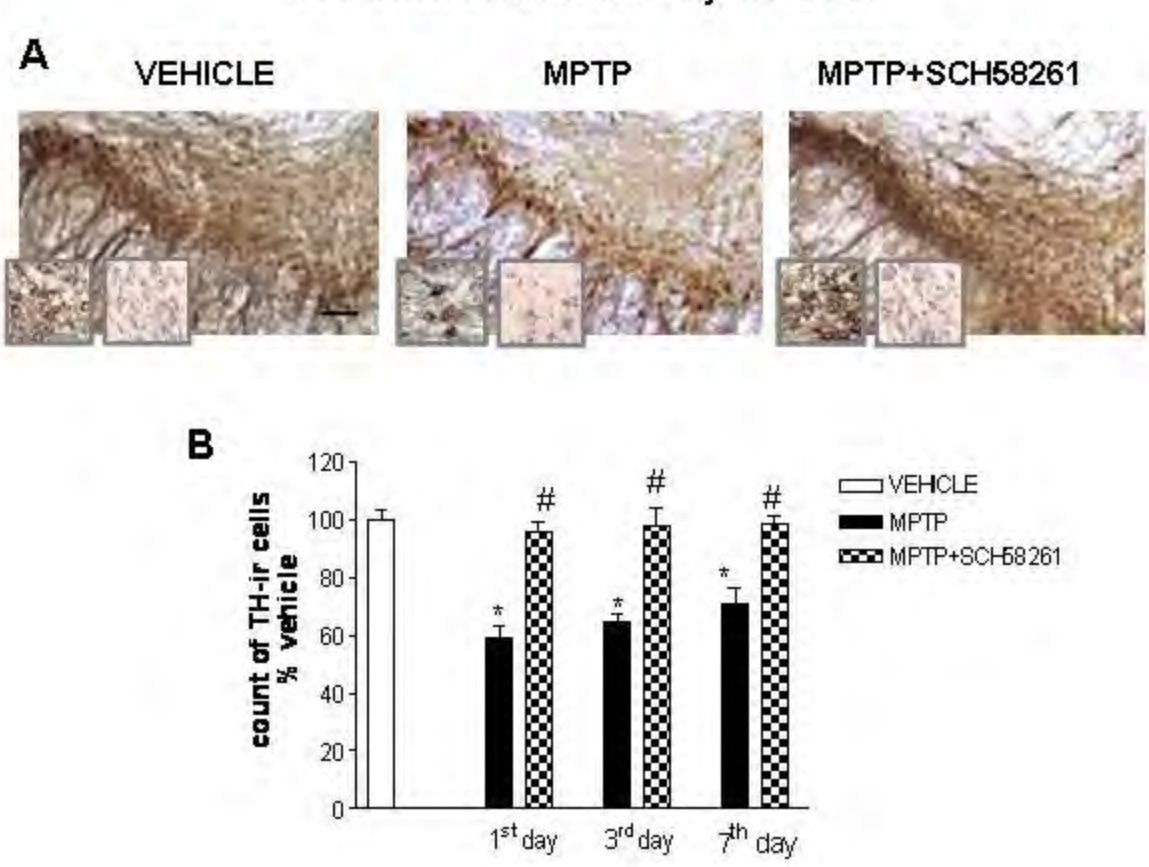
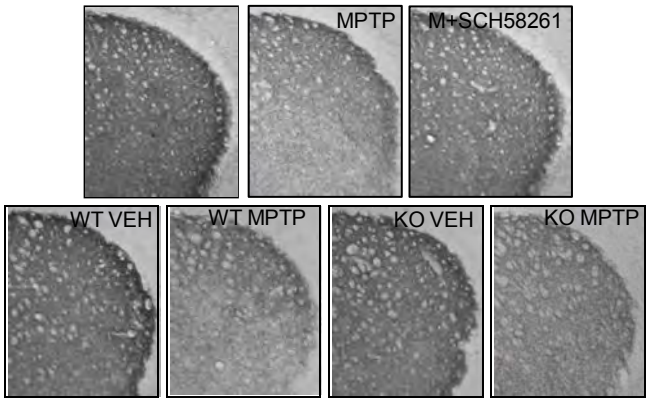


Fig 1

Appendix A

A



B

	$A_{2A}R$ antagonism		$fbnA_{2A}R$	
	-	+	+	-
MPTP	73.26 ± 5.85^	90.34 ± 6.81	71.29 ± 7.7^	87.52 ± 10.69

Fig 2

Appendix A

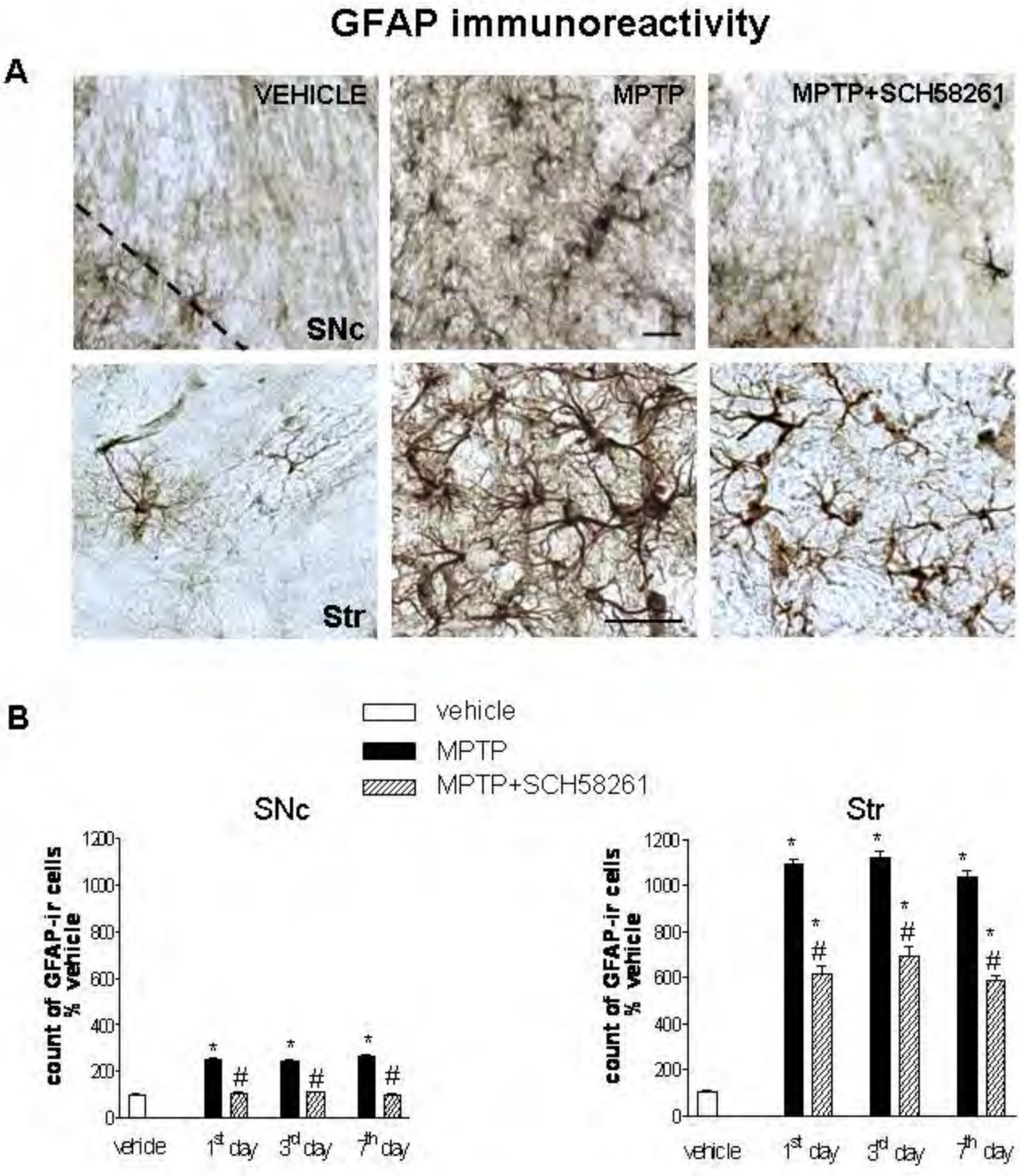


Fig.3

Appendix A

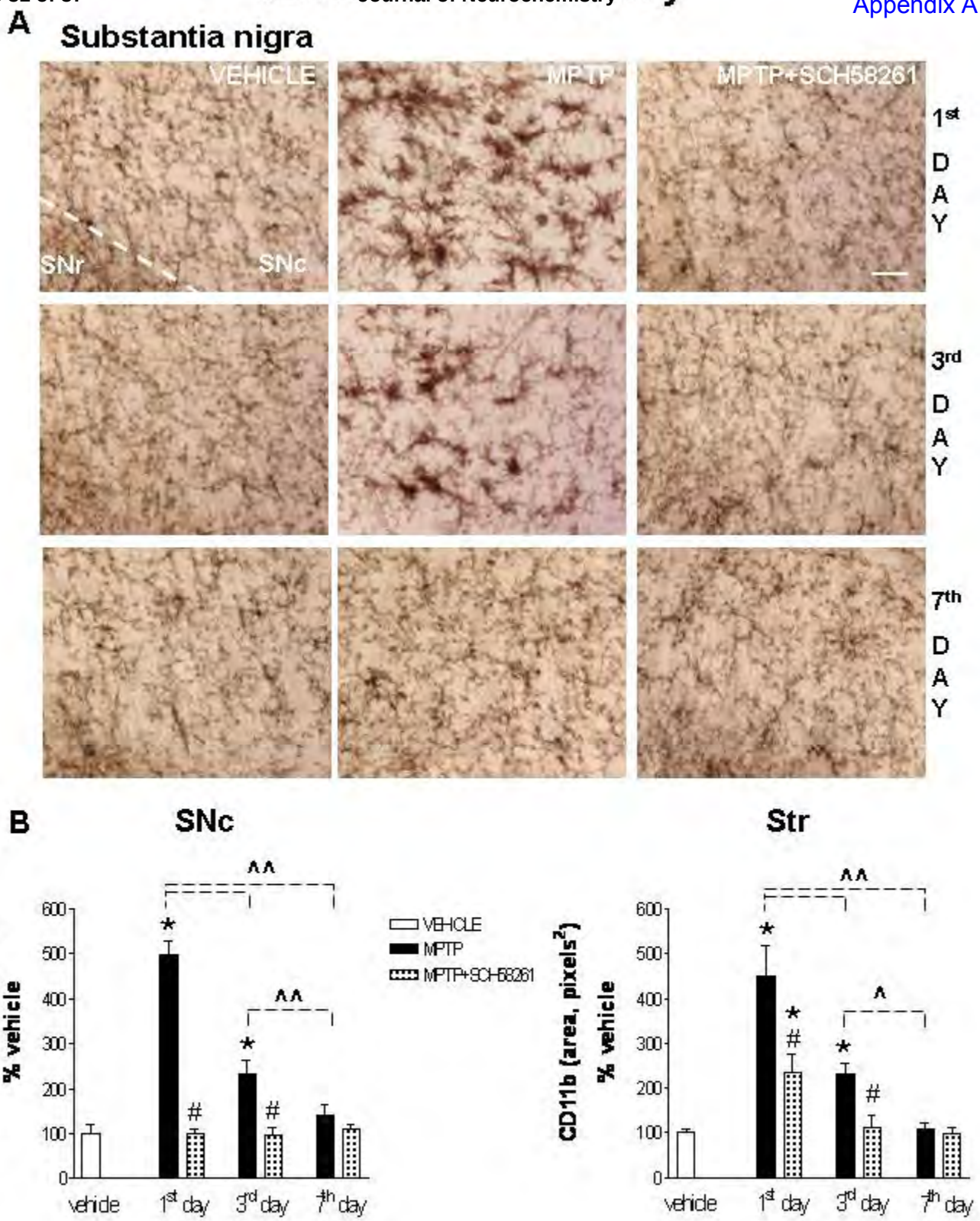


Fig 4

Appendix A

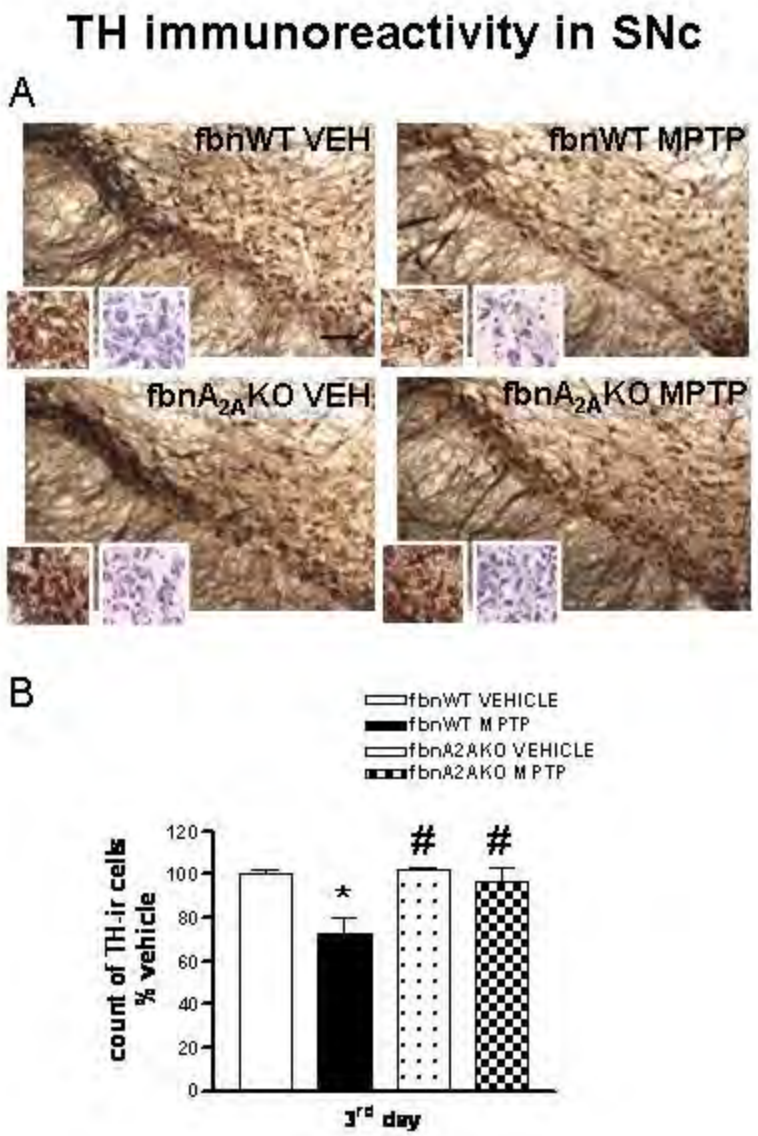


Fig 5

Appendix A

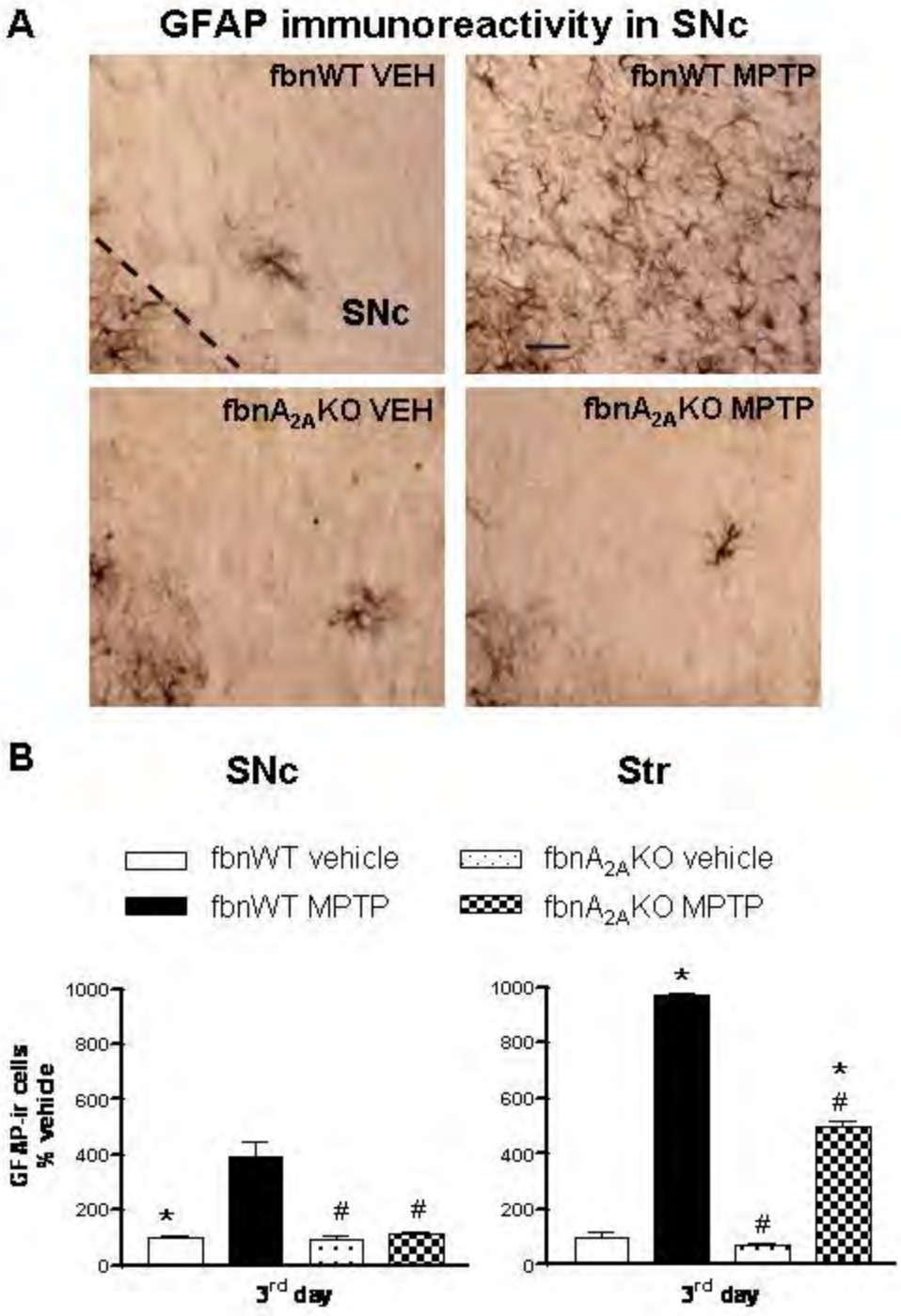


Fig 6

Appendix A

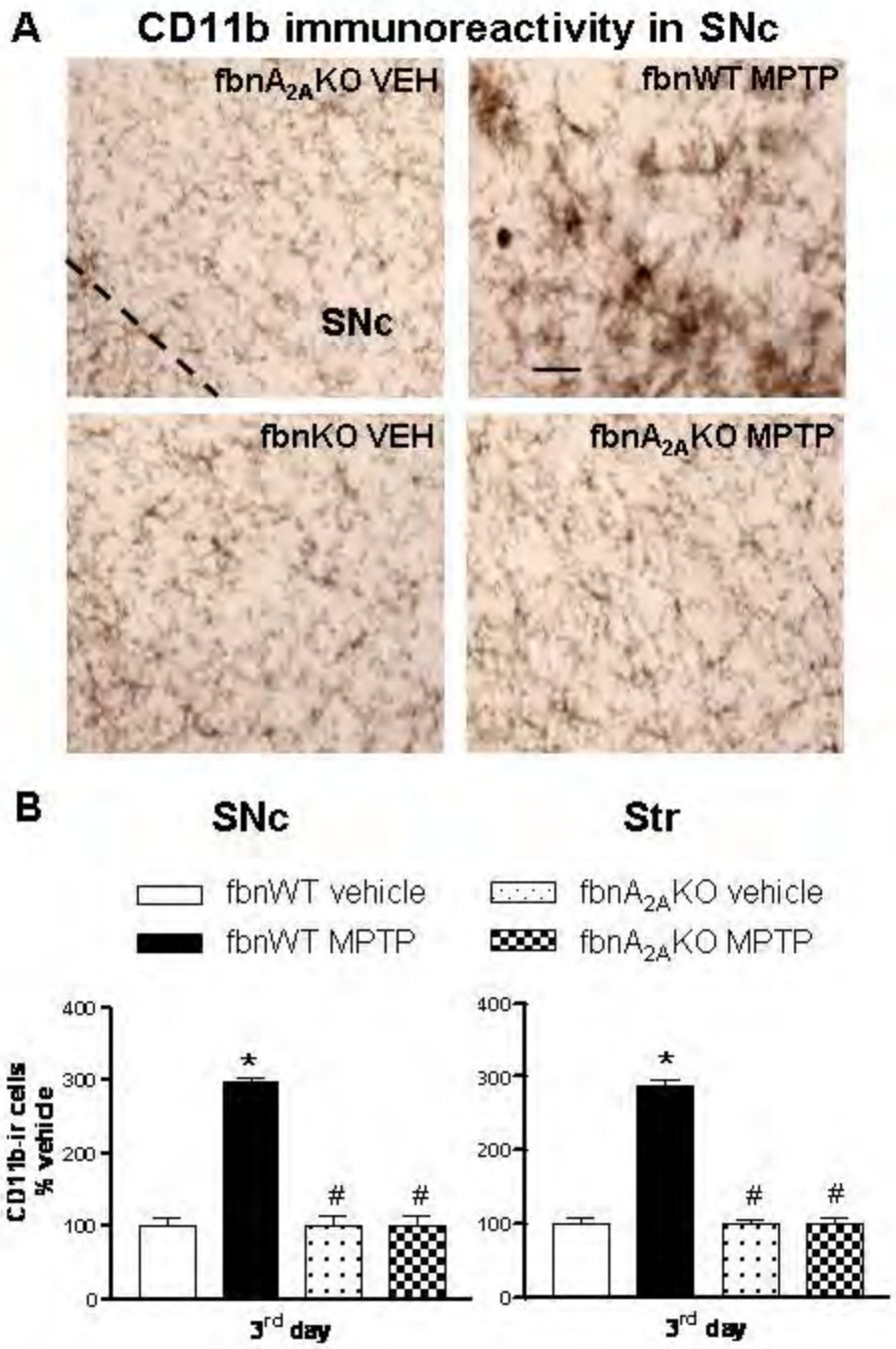


Fig 7

Appendix A

Additional Informations for online version only:

Table 2. F and P values from two factors ANOVA statistics

Experiment	Marker	Structure	Main effect of treatment		Main effect of time/genotype		Treatment-time/genotype interaction	
			F(2,47)	***P***	***F(2,47)***	***P***	***F(4,47)***	***P***
Pharmacological blockade of $A_{2A}R$	TH	SNc	90.05	4.47E-18	1.53	n.s.	0.79	n.s.
	GFAP	SNc	826.99	0	0.67	n.s.	3.76	n.s.
		Str	1665.32	0	6.62	0.0025	2.17	n.s.
	CD11b	SNc	107.49	1.87E-18	35.12	4.01E-10	33.60	2.27E-13
		Str	41.01	1.73E-11	36.79	9.56E-11	15.38	2.06E-08
			F(1,26)	***P***	***F(1,26)***	***P***	***F(1,26)***	***P***
fbn$A_{2A}R$ depletion	TH	SNc	5.17	0.032	7.92	0.0092	3.35	n.s.
	GFAP	SNc	20.05	0.00013	23.29	5.32E-05	18.83	0.00019
		Str	248.94	7.85E-15	1634.94	5.29E-25	192.23	1.6E-13
	CD11b	SNc	14.14	0.0013	18.37	0.00040	33.00	1.55E-05
		Str	106.10	1.15E-09	111.57	7.37E-10	108.56	9.39E-10

Table 2 (A.I. for online version): F and *P* values from two-way ANOVA relative to experiments with A_{2A} receptor antagonist (pharmacological blockade of $A_{2A}R$), or in genetically manipulated mice (fbn$A_{2A}R$ depletion). n.s.: not significant.

Appendix A

***Generation of fbnA_{2A}KO mice*:** Postnatal forebrain neuron conditional A_{2A}KO mice were generated using the Cre/*loxP* system based on the specificity of *CaMKII*α promoter. Near congenic (N6, C57Bl/6) male $A_{2A}^{flox/flox}$ mice (i.e., homozygous for the floxed allele of the $A_{2A}R$ gene) with or without a *CaMKII*α*-cre* transgene were used for this study. Tissue-specific postnatal recombination and disruption of the floxed $A_{2A}R$ alleles driven by *CaMKII*α*-cre* expression in forebrain neurons (i.e., fbn A_{2A}KO) were confirmed by PCR and Western blot as described previously (Bastia et al, 2005, Yu et al, 2008).

***Immunohistochemistry*:** For TH, GFAP and CD11b immunostaining, sections were incubated for 1 hr with 5% normal goat serum as blocking agent. The sections were then incubated overnight in primary antibodies (polyclonal rabbit anti-TH, 1:1000, Biomol, United Kingdom; monoclonal mouse anti-GFAP, 1:400, Sigma-Aldrich, Italy; monoclonal rat anti-mouse CD11b, 1:1000, Serotec, United Kingdom). Subsequently the sections were incubated in biotinylated secondary antibodies (goat anti-rabbit IgG for TH, goat anti-mouse IgG for GFAP and goat anti-rat IgG for CD11b, all purchased from Vector, United Kingdom). For visualization, the Avidin-peroxidase protocol (ABC, Vector) was applied, using 3,3'-diaminobenzidine (Sigma-Aldrich, Italy) as the chromogen. Sections were mounted into chrome-alum-gelatin-coated slides dehydrated and coverslipped.

***Stereological counting of TH-immunoreactive neurons*:** TH-immunoreactive neurons were counted on both hemispheres. All stereological counting was performed using a Leica microscope (DMLB; Leica, Denmark) equipped with a camera (Basler Vision Technologies, Germany) and a stage connected to an *xyz* stepper (PRIOR ProScan) and the newCAST Visiopharm (Denmark) software. The SNc region was outlined at low magnification (5X) for area estimation. The number of TH-immunoreactive neurons was calculated under 63X magnification using randomized meander sampling

Appendix A

and optical dissector methods. The cut thickness of sections was 50 μm and the optical dissector height was 12 μm. The top (13 μm) and bottom (25 μm) layers that shrunk during staining procedure were discarded. The sampling area covered 100% of the region of interest. The counting frame (8302,8 μm^2) applied the exclusion and inclusion lines and unbiased counting was performed by an experimenter blinded to the treatment. Results are presented as the mean of TH-immunoreactive neurons per mm^3 ± SEM, calculated using the following formula:

D = 3Q / V sampling

D = density of TH-ir cells per mm^3

V sampling = area of the region of interest x dissector height

Q = total count of TH-ir neurons.

ORIGINAL CONTRIBUTION

Urate as a Predictor of the Rate of Clinical Decline in Parkinson Disease

ARCHIVES EXPRESS

Alberto Ascherio, MD, DrPH; Peter A. LeWitt, MD; Kui Xu, MD, PhD; Shirley Eberly, MS; Arthur Watts, BS; Wayne R. Matson, PhD; Connie Marras, MD; Karl Kieburtz, MD; Alice Rudolph, PhD; Mikhail B. Bogdanov, PhD; Steven R. Schwid, MD†; Marsha Tennis, RN; Caroline M. Tanner, MD, PhD; M. Flint Beal, MD; Anthony E. Lang, MD; David Oakes, PhD; Stanley Fahn, MD; Ira Shoulson, MD; Michael A. Schwarzschild, MD, PhD; for the Parkinson Study Group DATATOP Investigators

Background: The risk of Parkinson disease (PD) and its rate of progression may decline with increasing concentration of blood urate, a major antioxidant.

Objective: To determine whether serum and cerebrospinal fluid concentrations of urate predict clinical progression in patients with PD.

Design, Setting, and Participants: Eight hundred subjects with early PD enrolled in the Deprenyl and Tocopherol Antioxidative Therapy of Parkinsonism (DATATOP) trial. The pretreatment urate concentration was measured in serum for 774 subjects and in cerebrospinal fluid for 713 subjects.

Main Outcome Measures: Treatment-, age-, and sex-adjusted hazard ratios (HRs) for clinical disability requiring levodopa therapy, the prespecified primary end point of the original DATATOP trial.

Results: The HR of progressing to the primary end point decreased with increasing serum urate concentrations (HR for highest vs lowest quintile=0.64; 95% confidence interval [CI], 0.44-0.94; HR for a 1-SD increase=0.82; 95% CI, 0.73-0.93). In analyses stratified by ct-tocopherol treatment (2000 IU/d), a decrease in the HR for the primary end point was seen only among subjects not treated with ct-tocopherol (HR for a 1-SD increase=0.75; 95% CI, 0.62-0.89; vs HR for those treated=0.90; 95% CI, 0.75-1.08). Results were similar for the rate of change in the Unified Parkinson's Disease Rating Scale score. Cerebrospinal fluid urate concentration was also inversely related to both the primary end point (HR for highest vs lowest quintile=0.65; 95% CI, 0.44-0.96; HR for a 1-SD increase=0.89; 95% CI, 0.79-1.02) and the rate of change in the Unified Parkinson's Disease Rating Scale score. As with serum urate concentration, these associations were present only among subjects not treated with ct-tocopherol.

Conclusions: Higher serum and cerebrospinal fluid urate concentrations at baseline were associated with slower rates of clinical decline. The findings strengthen the link between urate concentration and PD and the rationale for considering central nervous system urate concentration elevation as a potential strategy to slow PD progression.

Arch Neurol. 2009;66(12):(doi:10.1001/archneurol.2009.247)

Author Affiliations are listed at the end of this article.
Group Information: A list of the Parkinson Study Group DATATOP Investigators was published in *N Engl J Med*. 1993;328(3):176-183.
†Deceased.

In humans, urate is a major antioxidant as well as the end product of purine metabolism.[1,2] Its high concentrations in cerebrospinal fluid (CSF) and blood have been attributed to a mutation in the urate oxidase gene occurring late in hominid evolution.[3] Oxidative damage is suspected to contribute to the neurodegenerative process in Parkinson disease (PD),[4,5] and antioxidants like urate may provide an endogenous defense against the development and progression of PD.

Prospective epidemiological studies have demonstrated that healthy individuals with higher blood urate concentrations are at reduced risk for developing PD.[6-9] Similarly, a lower risk of PD has also been reported among individuals consuming diets that increase serum urate concentration[10] and among those with a history of gout.[11,12] Recently, we found that higher urate blood concentrations in patients in the early stages of PD predict a slower rate of disease progression, assessed by both clinical and neuroimaging measures.[13] These studies suggest that urate concentration measured systemically may serve as a robust predictor of the brain neurodegeneration that leads to the initiation and progression of PD.

The studies also raise the possibility that central nervous system urate directly protects against the neuronal degeneration underlying clinical deterioration in PD. Cerebrospinal fluid may more closely reflect

the microenvironment of degenerating neurons than does blood.[14] Accordingly, we used the clinical database from a completed multicenter, randomized, placebo-controlled trial (the Deprenyl and Tocopherol Antioxidative Therapy of Parkinsonism [DATATOP] trial)[15,16] to test the hypothesis that higher urate concentrations in both CSF and blood specimens from patients with PD predict a slower rate of clinical disease progression.

METHODS

STUDY DESIGN

The DATATOP study was a 2-year, double-blind, randomized trial originally designed to test the hypothesis that long-term treatment of early PD with the monoamine oxidase type B inhibitor deprenyl (selegiline hydrochloride) and/or the antioxidant dt-tocopherol would extend the time until the emergence of disability requiring therapy with levodopa.[15] The 800 participants were enrolled between September 1987 and November 1988 at 28 sites across the United States and Canada.

STUDY POPULATION

Subjects enrolled in the study had typical and early PD (Hoehn and Yahr stages 1 and 2) of less than 5 years' duration and were excluded if they used symptomatic PD medication or had severe tremor, serious dementia (Mini-Mental State Examination score <22), or depression (Hamilton Scale for Depression score ≥16). Subjects were reviewed and examined by neurologists who were PD specialists. After baseline evaluation, study participants were randomized according to a 2×2 factorial design to 1 of 4 treatment assignments: deprenyl (10 mg/d) and dt-tocopherol placebo, dt-tocopherol (2000 IU/d) and deprenyl placebo, active deprenyl and active dt-tocopherol, or double placebo.[17]

SERUM AND CSF URATE CONCENTRATIONS AND COVARIATES

Urate concentration was measured in serum samples collected at the baseline visit prior to treatment assignment. Serum was shipped without freezing to a central commercial clinical laboratory (SciCor, Indianapolis, Indiana) for immediate enzymatic assay of urate concentrations, which were available for 774 of the 800 enrolled subjects. Values maintained in a digitized database were not analyzed with respect to disease progression outcome measures until their retrieval in May 2006 specifically for this purpose.

Cerebrospinal fluid was collected at baseline after overnight bed rest from 730 subjects (ie, 91.2% of enrollees, with technical difficulties in performing lumbar punctures precluding the collection from the others)[18] and at the end of the study in 486 subjects. Specimens were rapidly frozen for storage at −70°C after first splitting all CSF collection tubes into aliquots with or without metabisulfite preservative added.[18] Baseline and final CSF urate concentrations were measured in 1991 by high-performance liquid chromatography with electrochemical detection from collection tubes containing the 18th to 20th milliliter of lumbar CSF flow in 2 selected subsets totaling 290 subjects who had provided both baseline and final CSF collections.[19] The values of CSF urate concentrations at baseline correlated well with those at the end of treatment or follow-up in both subsets (Spearman coefficient=0.69; $P<.001$), a result that supports the reproducibility of the assay as well as relatively stable within-person CSF urate concentrations. For the present analyses, in 2008 we obtained CSF aliquots from the same collection tubes (containing no metabisulfite preservative) and repeated the measurement of urate concentrations by high-performance liquid chromatography with electrochemical detection. For these assays, 50μM dt-methyldopa served as an internal standard. Baseline CSF urate concentrations could be determined in 713 participants (ie, 97.7% of those from whom a baseline CSF sample was obtained and stored). Although mean CSF urate concentrations were lower than those measured in 1991, a good correlation was found between original urate concentrations and those measured in 2008 among the 277 individuals in both sets (Spearman coefficient=0.72; $P<.001$). Furthermore, baseline serum urate concentrations correlated more strongly with baseline CSF urate concentrations measured in 2008 (r=0.73) than in 1991 (r=0.58). These results provide evidence of the stability of urate in these samples and of the accuracy of CSF urate concentration measurements.

CLINICAL EVALUATION AND OUTCOMES

Following the baseline visit and initiation of study drugs, subjects were scheduled for visits every 3 months until 24 months had elapsed.[17] At each visit the site investigator evaluated the subject for disability sufficient to require dopaminergic therapy, the primary end point for the study, and for the secondary response variables, including the Unified Parkinson's Disease Rating Scale (UPDRS) score (sum of the motor, cognitive, and activity of daily living subscale scores).[17] Because the UPDRS score is modified by the dopaminergic treatment instituted at the primary end point, the annualized rate of change in the UPDRS score was determined based on change from baseline to the primary end point (or the final visit if the primary end point was not reached) for each subject and was calculated as follows: [(total UPDRS score at the last assessment before initiation of dopaminergic treatment − total UPDRS score at baseline)/number of days between the 2 assessments]×365 d/y. The vital status and date of death of participants in the DATATOP trial were collected in 2001 to 2002 as previously described.[20] The shortest time elapsed between enrollment and vital status update was 13 years. Information was available for 768 subjects with baseline serum urate concentration measurement.

STATISTICAL ANALYSIS

In the original trial, the hazard ratios (HRs) for the primary end point were 0.50 (95% confidence interval [CI], 0.41-0.62) among patients assigned to deprenyl and 0.91 (95% CI, 0.74-1.12) among patients assigned to dt-tocopherol.[17] Accordingly, all of the analyses were adjusted for assignment to deprenyl vs placebo.

Cox proportional hazards models were used to estimate the HRs of reaching the primary end point according to quintiles of baseline serum urate concentration, adjusting for sex, age (in 5-year groups), and treatment assignment (deprenyl vs placebo). Initial analyses were conducted using quintiles based on the combined urate concentration distribution in men and women. However, because this categorization resulted in a markedly skewed distribution within sex as expected, we also conducted analyses based on sex-specific quintiles. Tests for trend were conducted by including serum urate concentration as a continuous variable in the proportional hazards models. Potential confounding was assessed by adjusting the regression analyses for body mass index (BMI) (calculated as weight in kilograms divided by height in meters squared) and use of antihypertensive drugs or nonsteroidal anti-inflammatory drugs (use vs no use). With the exception of BMI, these adjustments did not affect the results. Therefore, only the treatment-, age-, and sex-adjusted results or the treatment-, age-, sex-, and BMI-

Table 1. Baseline Characteristics of Study Participants According to Quintiles of Baseline Serum Urate Concentration

Characteristic	Baseline Serum Urate Concentration Quintile 1	2	3	4	5	All Subjects
Serum urate concentration, mg/dL	<3.90	3.91-4.60	4.61-5.20	5.21-6.20	2:6.21	5.1[a]
Subjects, No.	162	140	149	165	158	774
Female, %	75.9	40.7	24.2	17.0	12.7	34.1
Age, median, y	62	63	61	63	63	62
BMI, mean	23.7	26.2	26.1	27.1	28.4	26.3
Current smokers, %	9.3	5.7	12.1	10.3	6.3	8.8
Using antihypertensive drugs for hypertension, %	6.8	7.1	7.4	8.5	15.7	9.2
Using thiazides, %	2.5	2.9	3.4	4.2	6.9	4.0
Using NSAIDs, %	22.2	22.1	20.1	27.3	17.6	21.9
Cardiac comorbidity, %	17.9	25.0	24.8	27.9	36.5	26.5
Time since onset per rater, y	1.9	2.0	2.0	2.0	1.9	2.0
Total UPDRS score, mean	25.1	25.7	25.4	26.4	24.5	25.5
UPDRS rest tremor score, mean	4.5	4.8	4.4	4.7	4.6	4.6
MMSE score, mean	28.8	28.9	29.0	28.8	28.8	28.9

Abbreviations: BMI, body mass index (calculated as weight in kilograms divided by height in meters squared); MMSE, Mini-Mental State Examination; NSAIDS, nonsteroidal anti-inflammatory drugs; UPDRS, Unified Parkinson's Disease Rating Scale.
[a]Value is expressed as the mean.

adjusted results are presented. Possible interactions were explored by including the cross-product of serum urate concentration (continuous variable) with age (continuous in years), sex, or deprenyl and ct-tocopherol treatments in the proportional hazards model. No interaction terms were significant, and only results that do not include these terms are reported. The results of these exploratory analyses, however, suggested a possible interaction between ct-tocopherol treatment and serum urate concentration. Because both ct-tocopherol and urate have antioxidant properties, this interaction has some biological plausibility. This interaction was examined further by estimating the HRs for the primary outcome in groups of subjects classified according to both their serum urate concentration and treatment group. The relationship between serum urate concentration and rate of change in the UPDRS score (between baseline and the last visit before reaching the primary end point) was assessed by linear regression with adjustment for treatment, age, and sex using both common quintiles of serum urate concentration and sex-specific quintiles. This analysis was complemented by a repeated-measures analysis using all available UPDRS score determinations. This analysis was conducted by fitting a linear mixed model with random intercept and slope and fixed effects for treatment, age, sex, urate concentration, and the interaction between urate concentration and ct-tocopherol treatment. The association between serum urate concentration and time from study enrollment until death was investigated using Cox proportional hazards models adjusted for treatment, age, sex, and smoking history (pack-years), with or without further adjustment for cardiac morbidity at baseline. Analyses for CSF urate concentration were conducted in the same manner.

RESULTS

SERUM URATE CONCENTRATION

Serum urate concentration at baseline was available for 774 of the 800 subjects (96.8%; 510 men and 264 women) enrolled in the trial. Selected characteristics of these subjects are shown in **Table 1**. As expected, serum urate concentrations correlated positively with being male, BMI, use of thiazide diuretics, and hypertension. Use of calcium channel blockers was reported by only 17 patients and showed no relationship to serum urate concentration.

Overall, 369 (47.7%) of these participants progressed to disability sufficient to require levodopa therapy during follow-up. The HR of reaching this primary end point declined with increasing concentrations of serum urate (P for trend=.002) and was 36% lower among subjects in the top quintile as compared with those in the bottom quintile of serum urate concentration (HR=0.64; 95% CI, 0.44-0.94) (**Table 2**). This association was stronger in men than in women, although a test for interaction of urate concentration with sex was not significant (P=.54). Further, in both sexes, the HR for reaching the primary end point decreased with increasing BMI (P for trend=.05 in men, P for trend=.02 in women). After adjustment for BMI, the association between serum urate concentration and the primary clinical end point was partially attenuated; the HRs for a 1-SD increase in serum urate concentration were 0.89 in all subjects (P=.07), 0.85 in men (P=.04), and 1.01 in women (P=.94).

When subjects were classified simultaneously according to serum urate concentration and ct-tocopherol treatment, a decreasing HR for reaching the primary end point with increasing serum urate concentration was observed among untreated subjects (HR=0.75; 95% CI, 0.62-0.89; P=.001) but not among those treated (HR=0.90; 95% CI, 0.75-1.08; P=.24), consistent with comparisons of baseline urate concentration quintiles (**Figure 1**A) and unadjusted Kaplan-Meier analyses (eFigure; http://www.archneurol.com) in subgroups without or with ct-tocopherol treatment. Conversely, randomization to ct-tocopherol treatment appeared to lower the HR of reaching the primary end point among subjects in the lowest quintile of serum urate concentration (HR=0.59; 95% CI, 0.36-0.97) but not among those with a higher serum urate concentration (Figure 1A). Further analyses were conducted within sex. In men, the HRs for a 1-SD increase in serum urate concentration were 0.74 (95% CI, 0.59-0.92; P=.008) among subjects not receiving ct-tocopherol and

Table 2. Hazard Ratios for Reaching the Primary End Point According to Common Quintiles of Baseline Serum Urate Concentration or Corresponding to a 1-SD Increase in Serum Urate Concentration[a]

Serum Urate Concentration Quintile	Serum Urate Concentration, mg/dL	All Subjects (n=774) No.	HR (95% CI)	P Value	Men (n=510) No.	HR (95% CI)	P Value	Women (n=264) No.	HR (95% CI)	P Value
1	<3.90	162	1 [Reference]		39	1 [Reference]		123	1 [Reference]	
2	3.91-4.60	140	0.88 (0.62-1.25)	.47	83	0.88 (0.52-1.49)	.63	57	0.90 (0.53-1.51)	.68
3	4.61-5.20	149	1.04 (0.73-1.47)	.83	113	1.17 (0.71-1.91)	.54	36	0.86 (0.45-1.62)	.63
4	5.21-6.20	165	0.80 (0.55-1.15)	.23	137	0.78 (0.48-1.29)	.33	28	1.34 (0.68-2.64)	.40
5	2:6.21	158	0.64 (0.44-0.94)	.02	138	0.67 (0.41-1.11)	.12	20	0.58 (0.23-1.50)	.26
1-SD increase in serum urate concentration			0.82 (0.73-0.93)	.002		0.81 (0.70-0.94)	.005		0.89 (0.71-1.12)	.32

Abbreviations: CI, confidence interval; HR, hazard ratio.
[a] A 1-SD increase indicates an increase of 1.4 mg/dL. The HRs are adjusted for age, sex, and treatment group (deprenyl or placebo).

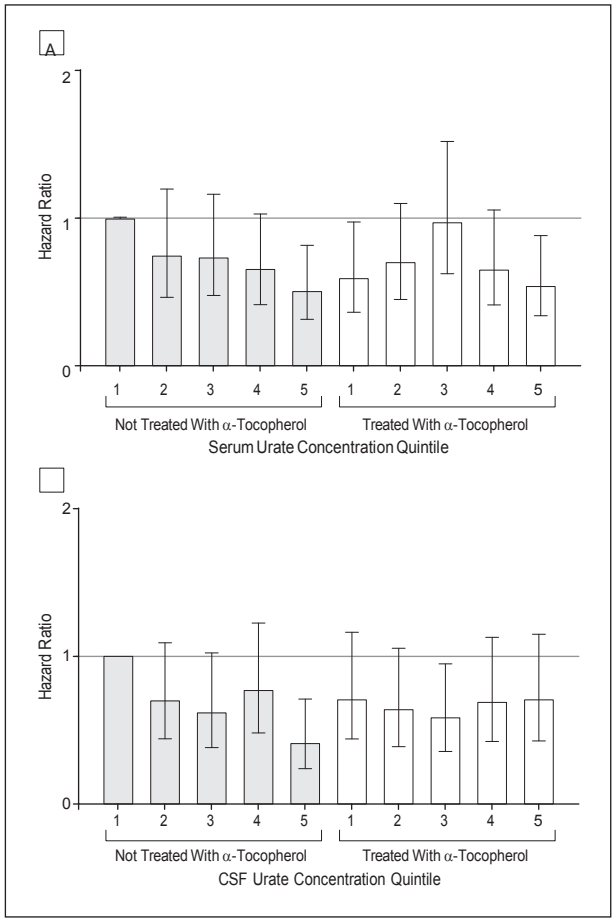


Figure 1. Hazard ratio for reaching the primary end point according to assignment to ct-tocopherol (vitamin E) and quintile of baseline serum (A) or cerebrospinal fluid (CSF) (B) urate concentration (referenced to placebo-treated subjects in the lowest quintile). Error bars indicate 95% confidence intervals for hazard ratios adjusted for age, sex, and treatment group (deprenyl or placebo).

0.88 (95% CI, 0.71-1.08; *P*=.21) among subjects receiving ct-tocopherol. In women, the corresponding HRs were 0.73 (95% CI, 0.52-1.02; *P*=.06) for subjects not receiving ct-tocopherol and 1.04 (95% CI, 0.69-1.59; *P*=.84) for subjects receiving ct-tocopherol. The interaction between ct-tocopherol and serum urate concentration was nonsignificant for men and for women (*P* for interaction=.55 in men, and *P* for interaction=.06 in women). No significant interaction was found between serum urate concentration and deprenyl treatment; a decreasing HR with increasing serum urate concentration was found in the placebo-placebo and deprenyl-placebo groups but not in the placebo–ct-tocopherol and deprenyl–ct-tocopherol groups (eTable 1).

The change in UPDRS score between baseline and either the time of reaching the primary end point or the end of follow-up was available for 760 of the 774 subjects with baseline serum urate concentrations. Overall, the rate of UPDRS score change declined with increasing serum urate concentration (*P* for trend=.03). As observed previously for the primary end point, results were more robust in men, although there was no statistically significant interaction with sex. Among men, the adjusted rate of UPDRS score change declined from 14.8 points per year for subjects in the lowest quintile of serum urate concentration to 8.9 points per year for those in the highest quintile (*P* for trend=.03); comparable results among women were 11.0 and 8.2 points per year, respectively (*P* for trend=.35). The relationship between serum urate concentration and the rate of UPDRS score change was modified by ct-tocopherol treatment (*P* for interaction=.009) (**Figure 2**A). In separate models, among subjects not assigned to receive ct-tocopherol, the rate of UPDRS score change was 9.8 points per year lower in the highest serum urate concentration quintile than in the lowest quintile (*P*=.003), whereas no difference was observed for subjects assigned to receive ct-tocopherol (0.5 points per year higher in the highest quintile as compared with the lowest quintile; *P*=.89). In analyses based on repeated measures, the overall association between higher urate levels at baseline and a slower rate of UPDRS score increase was even stronger (*P*=.001). There was also a significant interaction between urate concentration and ct-tocopherol treatment (*P*=.003), and consistent with results observed in the primary analyses, higher levels of serum urate were strongly associated with a slower rate of UPDRS score increase among patients not treated with ct-tocopherol (*P*=.001) but not in those treated with ct-tocopherol (*P*=.37). No significant interaction was found between serum urate concentration and deprenyl treatment (eTable 2).

Two hundred eleven men (41.4%) and 81 women (30.7%) were identified as having died after 13 years of

follow-up. In men and women combined, after adjustment for deprenyl treatment, age, sex, and pack-years of smoking (**Table 3**) and after adjustment for deprenyl treatment, age, sex, pack-years of smoking, and cardiac comorbidity at baseline (**Table 4**), serum urate concentration was not significantly associated with mortality. In men, however, the relationship between serum urate concentration and mortality was a U-shaped curve, with the lowest mortality in the fourth quintile of urate concentration. In women, a suggestion of increased mortality at any urate concentration higher than that in the lowest quintile was not substantiated statistically. No significant interactions between serum urate concentration and ct-tocopherol were found in analyses on mortality.

CSF URATE CONCENTRATION

Mean urate concentrations in CSF collected at baseline were higher in men (0.42 mg/dL) than in women (0.28 mg/dL) and, as expected, were substantially lower than in serum.[21] Despite the lower concentrations of CSF urate, a strong correlation was found between CSF and serum urate concentrations (r=0.73; P<.001).

The primary clinical end point of disability was reached by 342 of the 713 subjects (48%) for whom CSF urate concentrations were available. Overall, the HR of reaching the primary end point of disability was significantly lower among individuals with higher concentrations of CSF urate. The HR comparing subjects in the highest quintile of CSF urate concentration with those in the lowest quintile was 1.65 (95% CI, 0.44-0.96; P=.03); the HR associated with a 1-SD increase in CSF urate concentration was 0.89 (95% CI, 0.79-1.02; P=.09) (**Table 5**). Results were not significantly different by sex, although a strong interaction was found between ct-tocopherol assignment and CSF urate concentration (P for interaction=.009) (Figure 1B). As for serum urate concentration, a significant decrease in the HRs for the primary end point with increasing CSF urate concentration was observed only among subjects not receiving ct-tocopherol. The HR corresponding to a 1-SD increase in CSF urate concentration was 0.77 (95% CI, 0.62-0.96; P=.02) among men not treated with ct-tocopherol and 1.10 (95% CI, 0.90-1.34; P=.34) among those receiving ct-tocopherol. In women, the corresponding HRs were 0.64 (95% CI, 0.40-1.03; P=.07) for subjects not assigned to ct-tocopherol and 0.77 (95% CI, 0.43-1.37; P=.37) for subjects treated with ct-tocopherol. No significant interaction was found between CSF urate concentration and deprenyl treatment (eTable 3).

The change in UPDRS score between baseline and either the time of reaching the primary end point or the end of follow-up was available for 702 of the 713 subjects with baseline CSF urate concentrations. Overall, the rate of UPDRS score change was not related significantly to CSF urate concentration. As observed for serum urate concentration, however, the relationship between CSF urate concentration and the rate of UPDRS score change was modified by ct-tocopherol treatment (P for interaction=.04) (Figure 2B). Among subjects not treated with ct-tocopherol, the rate of UPDRS score change declined with increasing CSF urate concentrations (P for trend=.05). Conversely, randomization to ct-tocopherol treatment appeared to lower the rate of UPDRS score change among subjects in the lowest quintile of urate concentration measured either in CSF (Figure 2B) or in serum (Figure 2A) but not among those with higher urate concentrations. No significant interaction was found between CSF urate concentration and deprenyl treatment (eTable 4).

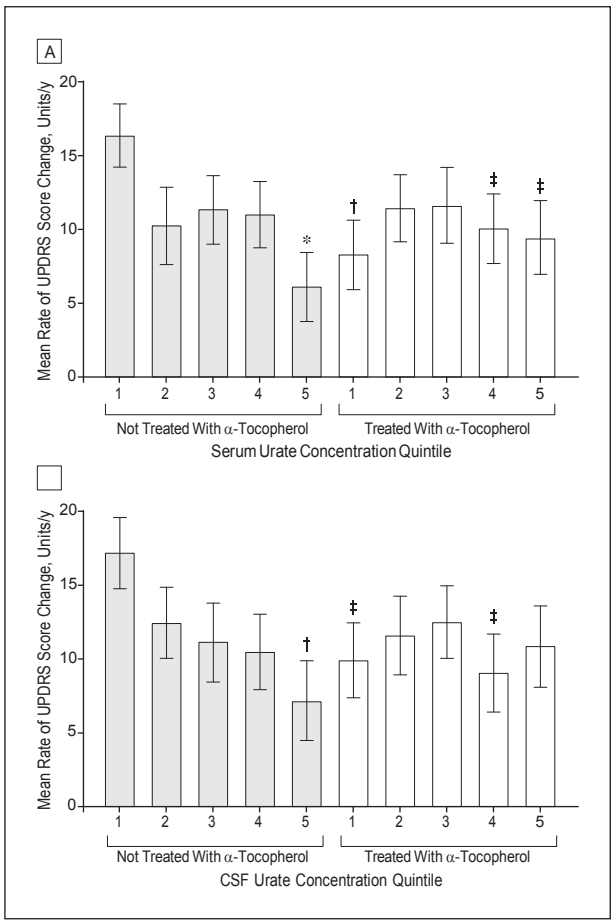


Figure 2. Mean annualized rate of Unified Parkinson's Disease Rating Scale (UPDRS) score change according to assignment to ct-tocopherol (vitamin E) and quintile of baseline serum (A) or cerebrospinal fluid (CSF) (B) urate concentration. Error bars indicate standard errors of the mean adjusted for age, sex, and treatment group (deprenyl or placebo). *Significantly different from the placebo-treated subjects in the lowest quintile (P<.001). †Significantly different from the placebo-treated subjects in the lowest quintile (P<.01). ‡Significantly different from the placebo-treated subjects in the lowest quintile (P<.05).

COMMENT

Among subjects with early PD participating in a large randomized trial, we found that both serum and CSF urate concentrations measured at baseline were inversely related to clinical progression of PD. The internal consistency of the results across the primary and secondary end points supports their validity. These findings, like data from a similar early PD trial (the Parkinson Research Examination of CEP-1347 Trial [PRECEPT] study),[13] demonstrate a robust link between blood urate concentrations and the rate of clinical progression in PD. In addition, the association of CSF urate concentration with disease progression

Table 3. Hazard Ratios for Death From Any Cause According to Common Quintiles of Baseline Serum Urate Concentration, Adjusted for Age, Sex, Treatment Group (Deprenyl or Placebo), and Pack-Years of Smoking

Serum Urate Concentration Quintile	All Subjects (n=768)		Men (n=504)		Women (n=264)	
	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
1	1 [Reference]		1 [Reference]		1 [Reference]	
2	1.17 (0.77-1.78)	.47	0.66 (0.38-1.15)	.14	1.68 (0.90-3.11)	.10
3	1.20 (0.78-1.83)	.41	0.66 (0.38-1.12)	.12	1.30 (0.64-2.66)	.47
4	1.11 (0.72-1.72)	.62	0.60 (0.35-1.02)	.06	1.88 (0.89-3.97)	.10
5	1.48 (0.96-2.27)	.06	0.89 (0.53-1.50)	.67	1.96 (0.89-4.33)	.10

Abbreviations: CI, confidence interval; HR, hazard ratio.

Table 4. Hazard Ratios for Death From Any Cause According to Common Quintiles of Baseline Serum Urate Concentration, Adjusted for Age, Sex, Treatment Group (Deprenyl or Placebo), Pack-Years of Smoking, and Cardiac Comorbidity at Baseline

Serum Urate Concentration Quintile	All Subjects (n=768)		Men (n=504)		Women (n=264)	
	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
1	1 [Reference]		1 [Reference]		1 [Reference]	
2	1.12 (0.73-1.71)	.61	0.63 (0.36-1.10)	.10	1.66 (0.90-3.08)	.11
3	1.14 (0.74-1.74)	.56	0.63 (0.37-1.08)	.09	1.29 (0.63-2.64)	.49
4	1.05 (0.68-1.62)	.83	0.56 (0.33-0.95)	.03	1.85 (0.87-3.93)	.11
5	1.38 (0.89-2.12)	.15	0.83 (0.49-1.39)	.47	1.89 (0.83-4.30)	.13

Abbreviations: CI, confidence interval; HR, hazard ratio.

Table 5. Hazard Ratios for Reaching the Primary End Point According to Common Quintiles of Baseline Cerebrospinal Fluid Urate Concentration or Corresponding to a 1-SD Increase in Cerebrospinal Fluid Urate Concentration[a]

CSF Urate Concentration Quintile	CSF Urate Concentration, mg/dL	All Subjects			Men			Women		
		No.	HR (95% CI)	*P* Value	No.	HR (95% CI)	*P* Value	No.	HR (95% CI)	*P* Value
1	<0.23	143	1 [Reference]		38	1 [Reference]		105	1 [Reference]	
2	0.24-0.32	143	0.78 (0.55-1.10)	.16	81	1.05 (0.61-1.81)	.85	62	0.65 (0.38-1.11)	.11
3	0.32-0.39	144	0.70 (0.48-1.01)	.06	109	0.96 (0.57-1.63)	.89	35	0.51 (0.26-1.03)	.06
4	0.39-0.50	142	0.84 (0.58-1.22)	.36	117	1.08 (0.64-1.80)	.78	25	0.72 (0.34-1.55)	.40
5	2:0.51	141	0.65 (0.44-0.96)	.03	128	0.85 (0.51-1.43)	.54	13	0.47 (0.16-1.39)	.17
1-SD increase in CSF urate concentration			0.89 (0.79-1.02)	.09		0.93 (0.80-1.07)	.28		0.79 (0.57-1.10)	.17

Abbreviations: CI, confidence interval; CSF, cerebrospinal fluid; HR, hazard ratio.
[a] A 1-SD increase indicates an increase of 0.16 mg/dL. The HRs are adjusted for age, sex, and treatment group (deprenyl or placebo).

strengthens the possibility that brain urate concentration (or its determinants) might protect against the neurodegeneration of PD. Taken together, these data establish urate as the first molecular predictor of clinical progression in PD and provide a rationale for investigating the possibility that a therapeutic increase of urate in patients with PD might act favorably to slow the disease course. Interestingly, the inverse relationship between urate concentration and clinical progression was not observed among patients randomized to ct-tocopherol at a dosage of 2000 IU/d, suggesting that there may be an interaction between these antioxidants.

There is strong evidence that oxidative stress and nitrative stress are major pathogenetic mechanisms in PD.[13,22,23] Urate is an effective antioxidant,[1] peroxynitrite scavenger,[24-27] iron chelator,[28] and ascorbate stabilizer.[29] In cellular models of PD neurodegeneration, urate can reduce oxidative stress, mitochondrial dysfunction, and cell death occurring spontaneously in culture or induced by the pesticide rotenone, 1-methyl-4-phenylpyridinium, glutamate, and iron ions.[30-32] Although urate appears to have the potential for neuroprotection, it is possible that the predictive association between urate concentration and PD progression reflects instead the effect of a urate precursor, such as adenosine or inosine, or another determinant of systemic and CSF urate concentrations.

As compared with serum urate concentration, the weaker association of CSF urate concentration to clinical progression of PD may seem at odds with the hypothesis that urate (or its metabolic precursors) exerts a beneficial effect through presence in the central nervous system. The CSF urate concentrations, how-

ever, display a strong caudorostral gradient from the lumbar space, with lumbar region values approximately 50% higher than those arising at the cisterna magna (brainstem) level.[33,34] Although we consistently used CSF aliquots obtained from the 18th to 20th milliliter of CSF flow, variations in CSF circulation patterns between patients[35]—along with freezer storage for 20 years—may have contributed to a reduction of the accuracy of this measure compared with assays of freshly collected serum samples. In addition to technical variability, substantial biological differences between the urate in CSF sampled from the subarachnoid space and that in the degenerating neurons themselves may lessen the strength of a CSF urate concentration–clinical progression correlation in PD.

The finding that the inverse relationship between urate concentration and clinical progression of PD was modified by ct-tocopherol treatment was unforeseen because, as originally reported, no favorable effect of ct-tocopherol on PD progression was found among study participants in the DATATOP trial.[16] The mechanisms for a possible interaction between urate and ct-tocopherol remain uncertain. Although hydrophilic (eg, urate) and hydrophobic (eg, ct-tocopherol) antioxidants target different subcellular compartments, their functional interactions have been described.[36,37] Further, ct-tocopherol at doses commonly used in vitamin supplements may reduce concentrations of other endogenous antioxidants,[38,39] and ct-tocopherol at high doses may have pro-oxidant rather than antioxidant effects.[40,41] Alternatively, a simple competitive interaction or "ceiling effect" may have contributed to the observed lack of ct-tocopherol benefits among patients with PD with higher urate concentrations as well as to the loss of the inverse association between urate concentration and PD progression among those receiving supplemental ct-tocopherol. Regardless of the mechanism for a possible interaction between ct-tocopherol and urate, our results raise the possibility that such an interaction may have obscured a protective effect of ct-tocopherol among those subjects with low baseline concentrations of urate in the DATATOP trial. Further investigations are therefore needed to consider the possibility that ct-tocopherol supplementation may be beneficial in individuals with low urate concentrations.

Serum urate may also affect the progression of cognitive impairment in that higher concentrations seem to be associated with slower rates of cognitive decline and lower risk of dementia.[42-44] As in the present study, among participants in a randomized trial, this association was observed in patients treated with placebo but not in those treated with ct-tocopherol.[43] Higher serum urate concentration has also been linked to a lower rate of worsening in Huntington disease.[45] Although each of these neurodegenerative disorders manifests differently from PD, the relationships between urate concentration and these disorders may be indicative of a more general influence of urate (or its precursors) on neuronal cell death.

The main results of this study are strikingly consistent with those recently reported from the PRECEPT study.[13] Although the overall inverse relationship between serum urate concentration and the clinical progression of PD was greater in the PRECEPT study than in the DATATOP study, results among subjects in the DATATOP study not assigned to ct-tocopherol were virtually identical to those observed in the PRECEPT study (which did not include an ct-tocopherol treatment arm). In both trials, HRs for risk of disability progression showed a decline in patients whose values were higher than the median concentration but still within the normal range of serum urate concentrations. Moreover, in both trials, the concentration-dependent inverse relationship was robust in men but weak and nonsignificant among women. This consistent difference between men and women could result in part from a biological effect of sex on urate mechanisms in PD[46] or could simply reflect the small number of women with urate concentrations high enough to slow disease progression if urate were protective.

A potentially therapeutic effect of elevating serum urate concentration warrants consideration. Urate levels can be elevated by dietary means, including increased intake of fructose[47-49] or purines,[50] or by pharmacological means. The latter may include administration of the purine metabolite and urate precursor inosine, which is being investigated as a therapy for multiple sclerosis.[26,27] The potential benefit of elevating urate concentration in individuals with PD, however, has to be weighed against possible adverse effects, which may include an increased risk of hypertension, coronary heart disease, and stroke[6,51-53] in addition to the known risks of gout and urolithiasis. Available data are therefore insufficient to support a therapeutic recommendation.

The discovery of a urate link to PD progression was achieved through additional analyses of 2 rigorously conducted clinical trials whose databases were made available to test an unforeseen hypothesis months[54] or decades[18,19] after conclusion of the primary investigations. These latent insights highlight a broader opportunity to achieve further advances through explorations of the growing repository of high-quality data collected from neuroprotection trials of PD and other neurodegenerative disorders.

Accepted for Publication: May 27, 2009.
Published Online: October 12, 2009 (doi:10.1001/archneurol.2009.247).
Author Affiliations: Departments of Nutrition and Epidemiology, Harvard School of Public Health (Dr Ascherio), Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School (Dr Ascherio), and Department of Neurology, MassGeneral Institute for Neurodegenerative Disease, Massachusetts General Hospital (Drs Xu and Schwarzschild and Ms Tennis), Boston, and Bedford Veterans Administration Medical Center, Bedford (Drs Matson and Bogdanov); Department of Neurology, Henry Ford Hospital and Wayne State University School of Medicine, Detroit, Michigan (Dr LeWitt); Departments of Biostatistics (Ms Eberly, Mr Watts, and Dr Oakes) and Neurology (Drs Kieburtz, Rudolph, Schwid, and Shoulson), University of Rochester, Rochester, and Department of Neurology and Neuroscience, Cornell University (Dr Beal) and Department of Neurology, Columbia University (Dr Fahn), New York, New York; The Morton and Gloria Shulman Movement Disorders Centre, Toronto West-

ern Hospital, University of Toronto, Toronto, Ontario, Canada (Drs Marras and Lang); and Department of Clinical Research, Parkinson's Institute, Sunnyvale, California (Dr Tanner).

Correspondence: Michael A. Schwarzschild, MD, PhD, Department of Neurology, MassGeneral Institute for Neurodegenerative Disease, Massachusetts General Hospital, 11416th St, Boston, MA 02129 (michaels@helix.mgh.harvard.edu).

Author Contributions: Dr Schwarzschild had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. *Study concept and design:* Ascherio, Kieburtz, Shoulson, and Schwarzschild. *Acquisition of data:* LeWitt, Xu, Matson, Marras, Rudolph, Schwid, Tennis, Tanner, Beal, Lang, Fahn, Shoulson, and Schwarzschild. *Analysis and interpretation of data:* Ascherio, LeWitt, Xu, Eberly, Watts, Marras, Kieburtz, Bogdanov, Tennis, Tanner, Lang, Oakes, Fahn, Shoulson, and Schwarzschild. *Drafting of the manuscript:* LeWitt, Oakes, and Schwarzschild. *Critical revision of the manuscript for important intellectual content:* Ascherio, LeWitt, Xu, Eberly, Watts, Matson, Marras, Kieburtz, Rudolph, Bogdanov, Tennis, Tanner, Beal, Lang, Oakes, Fahn, Shoulson, and Schwarzschild. *Statistical analysis:* Eberly and Oakes. *Obtained funding:* Ascherio, LeWitt, Shoulson, and Schwarzschild. *Administrative, technical, and material support:* LeWitt, Xu, Watts, Matson, Kieburtz, Rudolph, Schwid, Fahn, Shoulson, and Schwarzschild. *Study supervision:* Beal and Schwarzschild.

Financial Disclosure: Dr LeWitt reports receiving lecture fees from Allergan, Boehringer Ingelheim, GlaxoSmithKline, Novartis, Schwarz Pharma (now UCB), Solstice Neurosciences, and Vernalis (now Ipsen), consulting fees from Asubio Pharmaceuticals, Boehringer Ingelheim, Brittania Pharmaceuticals, Eisai, Neurologix, Novartis, Orion, Prestwick Pharmaceuticals (now Biovail), Santhera Pharmaceuticals, Schering-Plough, Schwarz Pharma, Solvay, Spherics, Supernus Pharmaceuticals, Valeant Pharmaceuticals International, Vernalis, and XenoPort, and grant support from Boehringer Ingelheim, Chelsea Therapeutics, Novartis, Santhera Pharmaceuticals, Schering-Plough, and Schwarz Pharma.

Funding/Support: This work was supported by grants NS24778, NS27892, NS048517, NS054978, and NS060991 from the National Institutes of Health, by grant W81XWH-04-1-0881 from the US Department of Defense, and by the RJG Foundation, the Beeson Scholars/Hartford Collaborative Research program of the American Federation for Aging Research, and a data-mining research award from the Parkinson Disease Foundation and the Parkinson Study Group.

Role of the Sponsors: The funding organizations had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; and preparation, review, or approval of the manuscript.

Additional Information: The eFigure and eTables are available at http://www.archneurol.com.

Additional Contributions: Eilis O'Reilly, ScD, conducted an expert secondary review of statistical programs and reported results, Andrew McAleavey, AB, provided excellent technical assistance in neurochemical analyses, and Leslie Unger, BA, provided technical assistance in preparing the manuscript.

REFERENCES

1. Ames BN, Cathcart R, Schwiers E, Hochstein P. Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: a hypothesis. *Proc Natl Acad Sci U S A*. 1981;78(11):6858-6862.
2. Alho H, Leinonen JS, Erhola M, Lonnrot K, Aejmelaeus R. Assay of antioxidant capacity of human plasma and CSF in aging and disease. *Restor Neurol Neurosci*. 1998;12(2-3):159-165.
3. Johnson RJ, Titte S, Cade JR, Rideout BA, Oliver WJ. Uric acid, evolution and primitive cultures. *Semin Nephrol*. 2005;25(1):3-8.
4. Burkhardt CR, Weber HK. Parkinson's disease: a chronic, low-grade antioxidant deficiency? *Med Hypotheses*. 1994;43(2):111-114.
5. Beal MF. Mitochondria take center stage in aging and neurodegeneration. *Ann Neurol*. 2005;58(4):495-505.
6. Davis JW, Grandinetti A, Waslien CI, Ross GW, White LR, Morens DM. Observations on serum uric acid levels and the risk of idiopathic Parkinson's disease. *Am J Epidemiol*. 1996;144(5):480-484.
7. de Lau LM, Koudstaal PJ, Hofman A, Breteler MM. Serum uric acid levels and the risk of Parkinson disease. *Ann Neurol*. 2005;58(5):797-800.
8. Weisskopf MG, O'Reilly E, Chen H, Schwarzschild MA, Ascherio A. Plasma urate and risk of Parkinson's disease. *Am J Epidemiol*. 2007;166(5):561-567.
9. Chen H, Mosley TH, Alonso A, Huang X. Plasma urate and Parkinson's disease in the Atherosclerosis Risk in Communities (ARIC) study. *Am J Epidemiol*. 2009;169(9):1064-1069.
10. Gao X, Chen H, Choi HK, Curhan G, Schwarzschild MA, Ascherio A. Diet, urate, and Parkinson's disease risk in men. *Am J Epidemiol*. 2008;167(7):831-838.
11. Alonso A, Rodriguez LA, Logroscino G, Hernan MA. Gout and risk of Parkinson disease: a prospective study. *Neurology*. 2007;69(17):1696-1700.
12. De Vera M, Rahman MM, Rankin J, Kopec J, Gao X, Choi H. Gout and the risk of Parkinson's disease: a cohort study. *Arthritis Rheum*. 2008;59(11):1549-1554.
13. Schwarzschild MA, Schwid SR, Marek K, et al; Parkinson Study Group PRECEPT Investigators. Serum urate as a predictor of clinical and radiographic progression in Parkinson's disease. *Arch Neurol*. 2008;65(6):716-723.
14. Blennow K. Cerebrospinal fluid protein biomarkers for Alzheimer's disease. *NeuroRx*. 2004;1(2):213-225.
15. Parkinson Study Group. DATATOP: a multicenter controlled clinical trial in early Parkinson's disease. *Arch Neurol*. 1989;46(10):1052-1060.
16. Parkinson Study Group. Effects of tocopherol and deprenyl on the progression of disability in early Parkinson's disease. *N Engl J Med*. 1993;328(3):176-183.
17. Parkinson Study Group. Effect of deprenyl on the progression of disability in early Parkinson's disease. *N Engl J Med*. 1989;321(20):1364-1371.
18. Parkinson Study Group. Cerebrospinal fluid homovanillic acid in the DATATOP study on Parkinson's disease. *Arch Neurol*. 1995;52(3):237-245.
19. LeWitt PA, Galloway MP, Matson W, et al; Parkinson Study Group. Markers of dopamine metabolism in Parkinson's disease. *Neurology*. 1992;42(11):2111-2117.
20. Marras C, McDermott MP, Rochon PA, et al; Parkinson Study Group. Survival in Parkinson disease: thirteen-year follow-up of the DATATOP cohort. *Neurology*. 2005;64(1):87-93.
21. Niklasson F, Agren H. Brain energy metabolism and blood-brain barrier permeability in depressive patients: analyses of creatine, creatinine, urate, and albumin in CSF and blood. *Biol Psychiatry*. 1984;19(8):1183-1206.
22. Moore DJ, West AB, Dawson VL, Dawson TM. Molecular pathophysiology of Parkinson's disease. *Annu Rev Neurosci*. 2005;28:57-87.
23. Danielson SR, Andersen JK. Oxidative and nitrative protein modifications in Parkinson's disease. *Free Radic Biol Med*. 2008;44(10):1787-1794.
24. Squadrito GL, Cueto R, Splenser AE, et al. Reaction of uric acid with peroxynitrite and implications for the mechanism of neuroprotection by uric acid. *Arch Biochem Biophys*. 2000;376(2):333-337.
25. Whiteman M, Ketsawatsakul U, Halliwell B. A reassessment of the peroxynitrite scavenging activity of uric acid. *Ann N Y Acad Sci*. 2002;962:242-259.
26. Spitsin S, Hooper DC, Leist T, Streletz LJ, Mikheeva T, Koprowskil H. Inactivation of peroxynitrite in multiple sclerosis patients after oral administration of inosine may suggest possible approaches to therapy of the disease. *Mult Scler*. 2001;7(5):313-319.
27. Koprowski H, Spitsin SV, Hooper DC. Prospects for the treatment of multiple sclerosis by raising serum levels of uric acid, a scavenger of peroxynitrite. *Ann Neurol*. 2001;49(1):139.
28. Davies KJ, Sevanian A, Muakkassah-Kelly SF, Hochstein P. Uric acid-iron ion complexes: a new aspect of the antioxidant functions of uric acid. *Biochem J*. 1986;235(3):747-754.
29. Stocker R, Frei B. Endogenous antioxidant defences in human blood plasma. In:

Sies H, ed. *Oxidative Stress: Oxidants and Antioxidants.* San Diego, CA: Academic Press; 1991:213-243.
30. Duan W, Ladenheim B, Cutler RG, Kruman II, Cadet JL, Mattson MP. Dietary folate deficiency and elevated homocysteine levels endanger dopaminergic neurons in models of Parkinson's disease. *J Neurochem*. 2002;80(1):101-110.
31. Haberman F, Tang SC, Arumugam TV, et al. Soluble neuroprotective antioxidant uric acid analogs ameliorate ischemic brain injury in mice. *Neuromolecular Med*. 2007;9(4):315-323.
32. Guerreiro S, Ponceau A, Toulorge D, et al. Protection of midbrain dopaminergic neurons by the end-product of purine metabolism uric acid: potentiation by low-level depolarization. *J Neurochem*. 2009;109(4):1118-1128.
33. Niklasson F, Hetta J, Degrell I. Hypoxanthine, xanthine, urate and creatinine concentration gradients in cerebrospinal fluid. *Ups J Med Sci*. 1988;93(3):225-232.
34. Degrell I, Nagy E. Concentration gradients for HVA, 5-HIAA, ascorbic acid, and uric acid in cerebrospinal fluid. *Biol Psychiatry*. 1990;27(8):891-896.
35. Quencer RM, Post MJ, Hinks RS. Cine MR in the evaluation of normal and abnormal CSF flow: intracranial and intraspinal studies. *Neuroradiology*. 1990; 32(5):371-391.
36. Yeum KJ, Russell RM, Krinsky NI, Aldini G. Biomarkers of antioxidant capacity in the hydrophilic and lipophilic compartments of human plasma. *Arch Biochem Biophys*. 2004;430(1):97-103.
37. Niki E, Noguchi N, Tsuchihashi H, Gotoh N. Interaction among vitamin C, vitamin E, and beta-carotene. *Am J Clin Nutr*. 1995;62(6)(suppl):1322S-1326S.
38. Jiang Q, Christen S, Shigenaga MK, Ames BN. Gamma-tocopherol, the major form of vitamin E in the US diet, deserves more attention. *Am J Clin Nutr*. 2001; 74(6):714-722.
39. Huang HY, Appel LJ. Supplementation of diets with alpha-tocopherol reduces serum concentrations of gamma- and delta-tocopherol in humans. *J Nutr*. 2003; 133(10):3137-3140.
40. Bowry VW, Stocker R. Tocopherol-mediated peroxidation: the prooxidant effect of vitamin E on the radical-initiated oxidation of human low-density lipoprotein. *J Am Chem Soc*. 1993;115(14):6029-6044. doi:10.1021/ja00067a019.
41. Abudu N, Miller JJ, Attaelmannan M, Levinson SS. Vitamins in human arteriosclerosis with emphasis on vitamin C and vitamin E. *Clin Chim Acta*. 2004; 339(1-2):11-25.
42. Petersen RC, Thomas RG, Grundman M, et al; Alzheimer's Disease Cooperative Study Group. Vitamin E and donepezil for the treatment of mild cognitive impairment. *N Engl J Med*. 2005;352(23):2379-2388.
43. Irizarry MC, Raman R, Schwarzschild MA, et al. Plasma urate and progression of mild cognitive impairment. *Neurodegener Dis*. 2009;6(1-2):23-28.
44. Euser SM, Hofman A, Westendorp RG, Breteler MM. Serum uric acid and cognitive function and dementia. *Brain*. 2009;132(pt 2):377-382.
45. Auinger P, Kieburtz K, McDermott MP. The relationship between uric acid levels and Huntington's disease progression [abstract]. *Mov Disord*. 2008;23(suppl 1):S187.
46. Nilsen J, Brinton RD. Mitochondria as therapeutic targets of estrogen action in the central nervous system. *Curr Drug Targets CNS Neurol Disord*. 2004;3 (4):297-313.
47. Hallfrisch J. Metabolic effects of dietary fructose. *FASEB J*. 1990;4(9):2652-2660.
48. Gao X, Qi L, Qiao N, et al. Intake of added sugar and sugar-sweetened drink and serum uric acid concentration in US men and women. *Hypertension*. 2007; 50(2):306-312.
49. Choi JW, Ford ES, Gao X, Choi HK. Sugar-sweetened soft drinks, diet soft drinks, and serum uric acid level: the Third National Health and Nutrition Examination Survey. *Arthritis Rheum*. 2008;59(1):109-116.
50. Choi HK, Liu S, Curhan G. Intake of purine-rich foods, protein, and dairy products and relationship to serum levels of uric acid: the Third National Health and Nutrition Examination Survey. *Arthritis Rheum*. 2005;52(1):283-289.
51. Bos MJ, Koudstaal PJ, Hofman A, Witteman JC, Breteler MM. Uric acid is a risk factor for myocardial infarction and stroke: the Rotterdam study. *Stroke*. 2006; 37(6):1503-1507.
52. Wheeler JG, Juzwishin KD, Eiriksdottir G, Gudnason V, Danesh J. Serum uric acid and coronary heart disease in 9458 incident cases and 155 084 controls: prospective study and meta-analysis. *PLoS Med*. 2005;2(3):e76. doi:10.1371 /journal.pmed.0020076.
53. Forman JP, Choi H, Curhan GC. Plasma uric acid level and risk for incident hypertension among men. *J Am Soc Nephrol*. 2007;18(1):287-292.
54. Parkinson Study Group PRECEPT Investigators. Mixed lineage kinase inhibitor CEP-1347 fails to delay disability in early Parkinson disease. *Neurology*. 2007; 69(15):1480-1490.

Editorial Manager(tm) for Journal of Neuropathology and Experimental Neurology
Manuscript Draft

Manuscript Number: JNEN 08-271R2

Title: Alpha-synuclein S129 phosphorylation mutants do not alter nigrostriatal toxicity in a rat model of Parkinson disease

Article Type: Original Study

Keywords: Adeno-associated virus; dopamine; Lewy bodies; striatum; substantia nigra; tyrosine hydroxylase

Corresponding Author: Dr. Nikolaus R McFarland, M.D., Ph.D.

Corresponding Author's Institution: Massachusetts General Hospital

First Author: Nikolaus R McFarland, M.D., Ph.D.

Order of Authors: Nikolaus R McFarland, M.D., Ph.D.; Zhanyun Fan, MD; Kui Xu, PhD; Michael A Schwarzschild, MD, PhD; Mel B Feany, MD, PhD; Bradley T Hyman, MD, PhD; Pamela J McLean, PhD

Abstract: Lewy bodies are found in Parkinson disease and related disorders, and are extensively phosphorylated at Ser-129. Recent studies suggest that S129 phosphorylation may mediate α-synuclein aggregation and neurotoxicty, but have had conflicting results. We used recombinant adeno-associated virus (rAAV) to overexpress α-synuclein in the rat nigrostriatal system. Rats were injected with rAAV2/8 expressing either human wild type (wt) or mutant α-synuclein with S129 replaced by alanine (S129A) or aspartate (S129D). Contralateral substantia nigra injections containing empty vector served as control. Both wt and S129 mutants resulted in significant dopaminergic cell loss by 6 weeks, but only small decreases in nigrostriatal terminal density and tyrosine hydroxylase (TH) expression. There were no significant differences in dopaminergic cell loss, nigrostriatal terminal density, or TH expression between wt and S129 mutants. Furthermore, we did not observe any differences in α-synuclein aggregate formation or distribution among wt and either S129 mutant. These findings contrast with those from previous studies, and suggest that both S129 phosphorylation mutants result in dopaminergic neurotoxicity similar to wt. Further study is needed to clarify the effects of these S129 mutants and α-synuclein phosphorylation in mammalian systems.

Alpha-synuclein S129 phosphorylation mutants do not alter nigrostriatal toxicity in a rat model of Parkinson disease

[1]Nikolaus R. McFarland, MD, PhD,
[1]Zhanyun Fan, MD,
[1]Kui Xu, PhD,
[1] Michael A. Schwarzschild, MD, PhD,
[2]Mel B. Feany, MD, PhD,
[1]Bradley T. Hyman, MD, PhD and
[1]Pamela J. McLean, PhD

[1]MassGeneral Institute for Neurodegenerative Disease (MIND), Department of Neurology, Massachusetts General Hospital and Harvard Medical School, Charlestown, MA 02129
[2]Department of Pathology, Brigham and Women's Hospital, Harvard Medical School Boston, MA 02115

Send correspondence to: Nikolaus R. McFarland, MD, PhD
Building 114, 16th Street - 2400
Charlestown, MA 02129-4404
Tel: 617-726-2299 fax: 617-724-1480
nmcfarland@partners.org

Running Title

α-Synuclein toxicity unaffected by S129 mutants

Acknowledgements/Support

This work was supported by NIH NS038372A-08 (B.T.H.) and DOD W81XWH-04-1-0881 (M.A.S.). N.R.M. is supported by an American Parkinson Disease Association (APDA) and the Koch Jr Fellowship.

Abstract

Lewy bodies are found in Parkinson disease and related disorders, and are extensively phosphorylated at Ser-129. Recent studies suggest that S129 phosphorylation may mediate α-synuclein aggregation and neurotoxicity, but have had conflicting results. We used recombinant adeno-associated virus (rAAV) to overexpress α-synuclein in the rat nigrostriatal system. Rats were injected with rAAV2/8 expressing either human wild type (wt) or mutant α-synuclein with S129 replaced by alanine (S129A) or aspartate (S129D). Contralateral substantia nigra injections containing empty vector served as control. Both wt and S129 mutants resulted in significant dopaminergic cell loss by 6 weeks, but only small decreases in nigrostriatal terminal density and tyrosine hydroxylase (TH) expression. There were no significant differences in dopaminergic cell loss, nigrostriatal terminal density, or TH expression between wt and S129 mutants. Furthermore, we did not observe any differences in α-synuclein aggregate formation or distribution among wt and either S129 mutant. These findings contrast with those from previous studies, and suggest that both S129 phosphorylation mutants result in dopaminergic neurotoxicity similar to wt. Further study is needed to clarify the effects of these S129 mutants and α-synuclein phosphorylation in mammalian systems.

Key Words

Adeno-associated virus, dopamine, Lewy bodies, striatum, substantia nigra, tyrosine hydroxylase.

Introduction

Parkinson disease (PD) is the most common neurodegenerative movement disorder and affects about 1% of the population over the age 65. The pathological hallmarks of PD are progressive loss of nigrostriatal dopamine neurons, swollen dystrophic neurites, and characteristic, filamentous intracellular inclusions, called Lewy bodies. These inclusions are not unique to PD, but are also found in related disorders, such as dementia with Lewy bodies and multiple system atrophy (1-3). A principle component of Lewy bodies is α-synuclein; a soluble, 140-amino acid protein that is abundantly expressed in brain and enriched in synaptic terminals (4). Although its normal function remains unclear, several point mutations in the α-synuclein gene (*PARK1/SNCA*) have been identified and linked to families with autosomal dominant parkinsonism (5-7). Gene duplication and triplication have also been linked to familial parkinsonism, and demonstrate a gene-dosage effect which correlates with earlier disease onset and symptom severity (8-10). The fact that Lewy bodies are also found in these familial forms of parkinsonism (11), further suggests a role for α-synuclein in PD pathogenesis.

Abnormal folding, aggregation, and deposition of α-synuclein may be key steps in the pathogenesis of sporadic and familial PD (12). *In vitro* α-synuclein self-aggregates and fibrillizes, forming aggregates suggestive of those seen in Lewy bodies and Lewy neurites (13, 14). Identified missense mutations in α-synuclein also result in accelerated fibril formation (15, 16) and oligomerization (17). Moreover, dose increases (duplication, triplication) of the α-synuclein gene promote its aggregation and deposition in insoluble fractions, consistent with inclusion formation (18). Animal models of human α-synuclein overexpression likewise demonstrate increased aggregates, neurotoxicity, and recapitulate features of PD (19-21).

Epigenetic factors, such as phosphorylation, also influence α-synuclein fibrillization and aggregate formation. For example, the C-terminal portion of α-synuclein contains several phosphorylation sites (22, 23) and some evidence suggests that Ser-129 phosphorylation accelerates α-synuclein fibrillization and enhances neurotoxicity. S129 is extensively phosphorylated in brains from patients with both sporadic and familial PD (24, 25), as well as DLB (26). Although a small proportion (< 5%) of α-synuclein is normally phosphorylated, nearly 90% of α-synuclein in Lewy bodies is phosphorylated at S129. Furthermore, *in vitro* S129

phosphorylation enhances fibril formation (24). In transgenic flies expressing human α-synuclein with mutation of S129 to either alanine (S129A) to prevent phosphorylation or aspartate (S129D) to mimic phosphorylation, blocking S129 phosphorylation suppresses dopamine neuronal loss, whereas S129D enhances α-synuclein neurotoxicity (27). In addition, overexpression of the *Drosophila* G-protein-coupled receptor kinase 2 (GRK2), which selectively phosphorylates α-synuclein at S129, results in enhanced neurotoxicity. Blockade of S129 phosphorylation (S129A) also increases inclusion formation and correlates with decreased α-synuclein neurotoxicity, suggesting that aggregates may be neuroprotective and sequester potentially toxic α-synuclein species. However, a recent study in rats found the opposite and demonstrated that the S129A mutant was more toxic than wild-type human α-synuclein and associated with decreased intracellular aggregates, whereas the phosphomimic S129D appeared to be neuroprotective (28). Thus, whether or not phosphorylation at S129 plays a critical role in mediating α-synuclein-induced dopaminergic neurotoxicity remains uncertain.

In this study we examine the effect of α-synuclein S129 phosphorylation state on dopaminergic neurotoxicity in a rat model of PD. Previous studies have established that viral-vector based overexpression (using either recombinant adeno-associated virus, rAAV, or lentivirus) of human α-synuclein in the rat nigrostriatal system results in progressive dopaminergic cell loss, synuclein-rich aggregates, and dystrophic neurites (29, 30). New rAAV serotypes are increasingly being used and show increased transduction efficiency and delivery of transgene in the nervous system over the commonly used rAAV2/2 (31-33). Here, we present new data using rAAV2/8 for delivery of α-synuclein S129 phosphorylation mutants and show no significant differences in nigrostriatal dopaminergic toxicity or morphology of α-synuclein aggregates among wt and S129A/D mutants.

Materials and Methods

Virus production. Construction of rAAV vectors used to express human wild-type α-synuclein is as previously described (34). Overlap extension PCR was used to generate α-synuclein S129 mutants (S129A and S129D) using the mammalian pcDNA-Synuclein (human wt, genbank L08850) vector as template and previously designed primers (available by request). S129 mutant and wt α-synuclein were then cloned into AAV-CBA-WPRE vector using the subclone pIRES2-

EGFP (Clontech, Mountain View, CA). We also constructed an empty vector, AAV-CBA-WPRE, with no transgene (kindly provided by Dr. Miguel Sena-Esteves, Massachusetts General Hospital, Boston, MA). Recombinant AAV2/8 virus was generated via tripartite transfection of the *cis*-transgene, packaging (*rep* and *cap*) genes, and helper plasmid into HEK 293A cells (Harvard Gene Therapy Initiative, Harvard Medical School). Viral particles were purified by iodixanol density gradient, isolated, and titered by dot blot hybridization. Final titers for virus were for α-synuclein wt $1.4x10^{13}$ gc/mL, S129D $1.4x10^{13}$ gc/mL, S129A $7.7x10^{12}$ gc/mL, and empty vector $1.16x10^{14}$ gc/mL.

Stereotaxic viral injections. All animal protocols and procedures were approved by the MGH Subcommittee on Research Animal Care. The nigrostriatal system is essentially unilateral, thus each animal received bilateral stereotaxic injections of rAAV into the substantia nigra (SN). In most cases virus with empty vector was injected contralateral to virus encoding human wt or mutant S129A/D α-synuclein. Sprague Dawley rats (300-350 g) were anesthetized, placed in a Kopf stereotax, and bilateral small skull holes were drilled to expose the dura over the injection sites. Coordinates for SN injections from bregma were AP -5.2, ML ±2.0, and DV -7.4 from the dural surface and targeted the central SN pars compacta. For each virus, $1.4x10^{10}$ gc in 2 µL were injected at 0.2 µL/min using a microinjection pump (Stoelting Co., Wood Dale, IL) with 10 µL Hamilton syringe and 33-gauge needle. After injection the syringe remained in situ for 5 minutes before withdrawal.

Tissue preparation and immunohistochemistry. At 2 or 6 weeks post-injection, rats were deeply anesthetized and transcardially perfused with cold 0.1M phosphate buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in PBS. Brains from a subset of 6-week animals were removed without fixation, and the striatum and midbrain quickly dissected on ice, and snap-frozen in isopentane for use in dopamine content measurement and immunoblotting. Perfused brains were postfixed for 24 hours, then cryoprotected in 30% sucrose/PBS, and serially sectioned at 40 µm on a sliding microtome into 12 wells (forebrain and midbrain separately blocked). Sections were collected and stored in cryoprotectant, 30% sucrose and 30% ethylene glycol in PBS, until processed and analyzed. Briefly, free-floating sections were rinsed with PBS, then treated 3-5 min. with 10% methanol and 3% H_2O_2 to inhibit endogenous peroxidases,

permeablized with 0.3% Triton X-100 in PBS, and blocked in 5% normal goat serum. Coronal sections through the striatum and nigra were immunostained with primary antibodies to TH (1:10,000 dilution; Millipore, Billerica, MA) or α-synuclein LB509 (1:1000 dilution; Zymed Laboratories, Inc., San Francisco, CA) overnight at 4 °C. After washing, immunostaining was visualized with either fluorescent secondary (1:200 dilution; Alexa Fluor 488, Molecular Probes, Eugene, OR; or Cy3, Jackson ImmunoResearch, West Grove, PA) or biotinylated secondary, followed by avidin-biotin (Vectastain Elite Kit), and 3,3'-diaminobenzidine reaction. Immunostained sections were washed, mounted on Superfrost slides, and coverslipped (GVA mount, Zymed Laboratories, Inc., San Francisco, CA; or Permount, Sigma Chemicals). In some cases, adjacent coronal sections were also Nissl stained with 0.05% cresyl violet per standard protocols.

Microscopy and Stereology. Immunostained sections were viewed using an Olympus BX51 microscope with brightfield and epifluorescence attachment. Photomicrographs were taken with an Olympus DP70 digital camera and adjusted only for suitable contrast and brightness. To maintain detail, wide-field images were photomontaged from high-power photos using Adobe Photoshop CS3. Double-label immunofluorescence images of cells were obtained using a Zeiss LSM510 confocal microscope system. Images were obtained with multi-tracking to minimize spectral overlap.

Nigrostriatal cell loss was assessed by counting TH-immunoreactive cells in the SN pars compacta, including the adjacent pars lateralis and caudal dense cell group ventral to the medial lemniscus and retrorubral area, using unbiased stereology according to the optical fractionator principle (35). At least 8 sections (each 240 μm apart) though the SN for each case were analyzed and counted using the Olympus CAST Stereology System. Sampling intensity was sufficient for a coefficient of error for the counting protocol of less than 0.1.

Striatal TH-terminal expression; Semi-quantitative analysis of TH-expression in nigrostriatal terminals at 6 weeks post-injection virus was performed by measurement of optical density of immunofluorescence intensity scans using a Scan Array Express (Perkin Elmer, Boston, MA). All sections for analysis were handled, washed, and immunostained at the same time, using

common reagents to minimize interanimal variability. Striatal sections approximately 1 mm rostral to the decussation of the anterior commissure were immunostained with antibody to TH and fluorescent secondary-Cy3(Molecular Probes), and scanned using 543-laser excitation. Using ImageJ (NIH), mean gray value of fluorescence intensity within the dorsolateral striatum was obtained for each section and normalized to background, corpus callosum.

Immunoblotting: Striatal and midbrain tissue were separately suspended in 300 µL lysis buffer (50 mM Tris-HCL, pH 7.4; 175 mM NaCl; 5M EDTA, pH 8.0; and protease inhibitor (Roche)) and homogenized on ice for 10-15 s. Each sample was centrifuged for 15 minutes at 4 °C, filtered, and then 1% Triton X-100 added to the lysate. After 1 hour incubation on ice, lysates were centrifuged for 60 min at 4 °C to collect the triton-X insoluble fraction. Triton-soluble lysate was separated and the insoluble pellet resuspended in 2% SDS-containing lysis buffer (Triton-insoluble fraction), then sonicated for 10 s. Protein concentration for each lysate was determined by BCA assay. Five micrograms of each sample was separated on a 10-20% Tris-glycine pre-cast gel (Invitrogen), transferred to PVDF, and immunoblotted for TH (mouse TH-2 antibody, Sigma) or α-synuclein (mouse Syn1, BD Transduction Laboratories, or Syn (LB509), Zymed, antibody). All blots were immunostained for GAPDH (rabbit antibody, Abcam) as loading control. In some cases samples for each condition were pooled. Immunoblotted α-synuclein, TH and GAPDH were detected with secondary antibody conjugated to HRP and reacted with ECL (GE Healthcare), per protocol. Films were scanned using a FluorChem system and analyzed with ImageJ software (NIH). TH and α-synuclein content for each sample was normalized to GAPDH.

Dopamine content: Striatal tissue was thawed, homogenized, and mixed in lysis buffer with dihydrobenzylamine added as internal control. Dopamine and 3,4-dihydroxyphenlyacetic acid (DOPAC) were measured by HPLC with electrochemical detection and normalized to protein content per sample (see 36).

Statistics: All data were expressed as group mean ± SEM. Stereological estimates of nigral TH cell survival, mean striatal terminal density, and dopamine measurements were analyzed using

one-way ANOVA with Bonferonni's multiple comparison post-hoc (Prism GraphPad 4.03, San Diego, CA) unless otherwise stated.

Results

We used the pseudotyped rAAV 2/8 for delivery of transgene, because recent rAAV serotype comparisons have shown increased transduction efficiency over that of rAAV2/2 (33, 36). Transgene expression for rAAV2/8 is also markedly enhanced over rAAV2/2 and rapidly reaches a steady-state by 2-4 weeks (37, 38). Rat substantia nigra (SN) were stereotaxically injected with equal volume and genome copies ($1.4x10^{10}$ gc) of rAAV vector encoding either human wt or S129 phosphorylation mutant (S129A for substitution to alanine, or S129D for aspartate) α-synuclein followed by an internal ribosomal entry site (IRES) and the reporter gene, EGFP (enhanced green-fluorescent protein) resulting in bicistronic expression. To control for possible viral toxicity and injection effects, the contralateral SN of several rats was injected with empty rAAV vector and used as an internal control. Based on preliminary experiments with rAAV2/8, its rapid transgene expression and spread, we selected 2 and 6 week time-points post-injection for analysis of wt and S129A/D α-synuclein toxicity in the nigrostriatal system.

The pattern of transgene expression for all rAAV was similar. For α-synuclein viruses, EGFP expression marked cells transduced with rAAV in the SN. All cells with EGFP co-expressed α-synuclein as assessed by double immunofluorescence. Also, rAAV2/8 was neurotrophic; we did not observe transduction of glial elements. At 2 and 6 weeks post-injection, EGFP expression was widespread for α-synuclein rAAV and included cells not only in the SN pars compacta (SNc), but also in the adjacent pars reticulata (SNr), ventral tegmental nucleus, and a portion of the ventral mesencephalic reticular formation (Figure 1). These findings are consistent with the reported enhanced transduction efficiency of rAAV2/8 compared to rAAV2/2 in the SN (33, 34, 37).

In addition to cellular expression of α-synuclein within the SN, immunostaining for human α-synuclein using the LB509 antibody (specific for amino acid residues 115-122) also demonstrated extensive transport of α-synuclein to nigrostriatal terminals (Figure 2). Cases without extensive nigrostriatal labeling suggested failed or misplaced injections, and were

excluded from the analysis. Synuclein-positive terminals were widespread and found throughout the dorsal striatum (caudatoputamen), extending rostrally to the striatal pole and several millimeters caudal to the anterior commissure. These findings are consistent with the topographic distribution of nigrostriatal projections (39) and confirm transduction of a large proportion of SNc neurons for each case. Double labeling of SN with antibodies to α-synuclein (or GFP) and tyrosine hydroxylase (TH) also demonstrated that rAAV injections transduced a majority of dopaminergic nigrostriatal neurons in the SNc and adjacent regions (data not shown).

Viral expression. To assess rAAV α-synuclein expression levels for wt, S129A and S129D mutants, we performed Western blot analyses on striatal and midbrain extracts from 3 animals per group obtained at 6 weeks post-injection and compared these to extracts from the contralateral hemisphere injected with empty vector control. Blots from samples taken from wt, S129A, and S129D α-synuclein cases probed with the human-specific LB509 synuclein antibody (Zymed/Invitrogen, Carlsbad, CA) demonstrated strong human α-synuclein expression ipsilateral to the injection as expected. We also probed blots with the Syn-1 antibody (BD Transduction, San Jose, CA), which recognizes both endogenous rat and human α-synuclein, to estimate the level of human α-synuclein overexpression for each rAAV compared to endogenous levels (Figure 3). Comparison of the α-synuclein injected side to the contralateral striatum or midbrain, empty rAAV vector control, revealed levels of α-synuclein overexpression with a mean ratio (ipsilateral/contralateral side) of approximately twice that of endogenous rat α-synuclein.

Comparison of nigrostiatal toxicity. Unbiased stereological estimates of TH-positive cells in the SN were performed at 2 and 6 weeks post-injection of rAAV expressing wt or mutant, S129A or S129D, α-synuclein and compared to empty vector injections. In preliminary studies, at 2 weeks subtle TH cell loss was observed in both wt and mutant S129A and S129D groups (n = 2-3 for each group). These findings were somewhat surprising given the early time point, but dopaminergic neurotoxicity for S129A has been reported as early as 4 weeks previously (28). Qualitative comparison TH-cell loss with cresyl violet stained sections confirmed proportional nigral cell loss and gliosis. Given the modest extent of cell loss at 2 weeks, we examined a larger cohort (6-10 per group) at 6 weeks to further compare toxicity of wt and S129 mutant α-synucleins.

At 6 weeks post-injection, there was evident TH cell loss in the SNc for both wt and S129 mutant α-synuclein groups (Figure 4). Cell loss, as opposed to TH-phenotype loss, was confirmed by analysis of Nissl stained sections and qualitative comparison with nigral TH cell loss. Stereological estimates of remaining TH cells in the SN demonstrated significant cell loss compared to empty vector control for wt (20.5 ± 6.8%, $p < 0.05$), S129A (29.9 ± 2.4%, $p < 0.001$), and S129D (27.0 ± 6.7%, $p < 0.01$) mutants ($F[3,24] = 9.96$, $p = 0.0002$) (Figure 5A). The mean TH-cell loss was 25.8% and ranged from 7-45% for all cases. Post-hoc analysis revealed no significant differences in TH cell counts between wt, S129A, or S129D. Qualitative comparison of nigral TH staining at 6 weeks also did not differ.

Nigrostriatal TH and dopamine content. Immunostaining of striatal sections for TH did not show nigrostriatal terminal loss at 2 weeks. By contrast, at 6 weeks TH-immunostaining revealed loss of terminal density in the dorsal striatum for both S129A and S129D cases compared to wt (Figure 2). Semi-quantitative analysis, using scan array measurement of immunofluorescence optical density, also revealed a small decrease in dorsal striatal TH+ terminal density for wt and mutant S129A and S129D cases relative to control, but these finding did not achieve statistical significance (Figure 5B). Relative side-to-side comparison in cases with internal empty vector control showed a similar trend of decreased dorsal striatal TH+ terminal density for S129A and S129D mutant cases, but also did not achieve significance.

To further investigate potential changes in striatal dopaminergic innervation, we also quantified striatal dopamine content at 6 weeks post-injection via HPLC fractionation and electrochemical detection. Dopamine was extracted from striatal tissue ipsilateral to the SN injection of either wt or mutant (S129A or S129D) α-synuclein and compared to that extracted from the contralateral control striatum (SN injected with empty rAAV vector). In wt cases striatal dopamine content and levels of its metabolite, DOPAC, did not differ (Figure 5C). In contrast, relative dopamine content for S129A did significantly decrease, mean ratio (ipsilateral/contralateral striatum) 0.66 ± 0.04 ($p = 0.006$, one sample t-test for hypothesized ratio of 1). Dopamine (DA) levels also decreased for S129D (mean ratio 0.59 ± 0.15), but did not achieve statistical significance ($p = 0.055$). Similarly, striatal DOPAC content for both S129A and S129D demonstrated a trend

toward decrease, but were not significant. These results are consistent with the loss of 25% of TH+ neurons in the SN.

Synuclein toxicity, cellular aggregates, and terminal morphology. Examination of sections stained for human α-synuclein (LB509) at 2 and 6 weeks using brightfield and confocal microscopy revealed progressive changes in nigral cell morphology. Synuclein-positive cells in wt and mutant S129A and S129D cases were densely immunostained throughout the nucleus and cytoplasm (Figure 6). At 6 weeks a subset of cells contained synuclein-positive aggregates. Some larger cell aggregates co-stained for α-synuclein and ubiquitin. There were no qualitative differences in aggregate size or distribution between wt and S129A or S129A cases. Transduced cells frequently had dystrophic process as previously described (29). Examination of nigrostriatal terminals immunostained for human α-synuclein also showed dystrophic changes and neurites, but no qualitative differences were noted between wt, S129A or S129D.

Discussion

We examined the role of α-synuclein phosphorylation at Ser-129 by comparing the effects of overexpression of human wt and mutant α-synuclein S129A (alanine blocks phosphorylation) and S129D (mimics constitutive phosphorylation) in the rat nigrostriatal system. A targeted viral vector-based approach with rAAV2/8 was used to overexpress α-synuclein in the SNc. Neither Ser-129 α-synuclein mutant appeared to significantly alter dopaminergic nigrostriatal toxicity in the rat PD model. At 2 weeks, early neurodegenerative changes and TH cell loss were apparent, and by 6 weeks overexpression of wt and S129A and S129D mutants all resulted in significant (20-30%) nigral dopaminergic cell loss (confirmed on Nissl-stained sections) compared to empty vector control. However, there were no significant differences in dopaminergic nigrostriatal cell toxicity between wt, S129A, or S129D α-synuclein at 6 weeks. Furthermore, the size and distribution of α-synuclein aggregates did not differ among wt and either S129 mutant. Consistent with these findings, we did not detect major changes in striatal TH-terminal density or expression, although striatal dopamine content was decreased for S129A and S129D. The normal striatal dopamine levels for wt α-synuclein are not surprising because over 6 weeks non-geriatric rodents show the ability to recover dopamine content after nigrostriatal lesion, such as that caused by MPTP (40). The lack of recovery of dopamine content for both S129 mutants thus

may suggest a decrease in functional neurochemical compensation or plasticity. Morphologically, though, α-synuclein positive nigrostriatal terminals for S129 cases did not differ significantly from those of wt. However, S129 mutants may alter dopamine release and synaptic function as these are putatively regulated by α-synuclein (41).

These findings contrast with those from previous studies in similar Drosophila and rat PD models that also compared wt α-synuclein and S129A and S129D phosphorylation mutants (27, 28). In the fly, S129D was more toxic to dopamine cells and S129A was not only protective, but also associated with an increase in α-synuclein aggregates. Recent data in rats found the opposite result and demonstrated increased toxicity for S129A and possible neuroprotective effects for S129D (28). Toxicity, though, was similarly associated with a decrease in large, insoluble α-synuclein aggregates, whereas neuroprotection correlated with the presence of cytoplasmic inclusions. By contrast, we observed no differences in toxicity or aggregate formation for wt α-synuclein or the phosphorylation mutants S129A and S129D in the rat.

Differences in study design may in part explain these differing results among studies. We used rAAV2/8 to deliver and overexpress α-synuclein in the rat SN, whereas Gorbatyuk et al. (28) employed a different serotype, rAAV2/5. Both of these rAAV's show enhanced transduction efficiency (increase in cell number and volume transduced) over that of rAAV2/2 in the rodent nigrostriatal system (33, 38, 42). However, rAAV2/8 results in higher transgene expression than other rAAV serotypes, including 2/2 and 2/5 (37). One concern is that elevated levels of α-synuclein expression from high-titer AAV2/8 injections may mask differences in toxicity between wt and the S129 mutants. We thus injected a moderate dose of each virus ($1.4x10^{10}$ gc), resulting in only 20-30% nigral TH cell loss at 6 weeks. Using a similar dose of AAV2/5, Gorbatyuk et al. reported 40-60% cell loss for both wt and S129A, albeit at 8 weeks post-injection. Moreover, Western blot estimates of human α-synuclein expression compared to endogenous rat α-synuclein were also lower (only 2-fold) than that previously reported. These finding suggest that the amount of virus injected and, indirectly, levels of α-synuclein expression in our study did not "saturate" the system. The fact that we saw toxicity for S129D also argues that the viral dose was not too low, as previously it appeared less toxic that wt (28).

Another difference between studies is the length of viral incubation. The time-course for expression of the transgene is similar for both rAAV 2/5 and 2/8, although perhaps a bit more rapid for rAAV2/8 (38). Two and 6-week time points were thus chosen for analysis, and may have been too early to detect differences in toxicity among wt and S129 mutants. However, even at 4 weeks toxicity was noted for S129A by Gorbatyuk et al. (28) and was evident by 2 weeks in this study. Moreover, by 6 weeks wt and both S129 mutants showed significant dopaminergic cell toxicity.

Recent *in vitro* data on S129 phosphorylation and phosphomimics, S129D/E, may also help to explain differences in results from rat and fly PD models. S129 phosphorylation increases α-synuclein conformational flexibility and appears to inhibit fibril formation. Although designed as phosphomimics, S129D/E do not exactly reproduce the effects of native phosphorylation (43). S129 phosphomimics only demonstrate local changes in conformational dynamics and *in vitro* form fibrils similar to wt, but not as rapidly as that of S129A. Consistent with these findings we found similar toxicity for wt and S129D α-synuclein. Rapid fibrillization due to S129A presumably leads to increased α-synuclein aggregate formation that is thought to sequester toxic species. This hypothesis is supported by reduced S129A toxicity and increased α-synuclein inclusions seen in the fly (27). However, contradictory results for S129A are seen in the rat (28), possibly suggesting that while fibrils form, the S129A mutant in a mammalian system may also enhance formation of toxic α-synuclein oligomers. Together, these findings emphasize the importance of phosphorylation at S129 as a regulator of α-synuclein function, but do not fully clarify its effects on toxicity.

Increasing evidence suggests that large α-synuclein aggregates may be neuroprotective and that S129 phosphorylation plays a role in aggregate formation (44). A high proportion of α-synuclein in Lewy bodies is phosphorylated at S129 (24, 25). Furthermore, co-expression of human α-synuclein and protein kinases, such as G-protein-receptor kinase 5 (GRK5)—one of many kinases that phosphorylate at S129 (27, 45)—is associated with α-synuclein aggregate formation in cell culture (46). However, recent data indicate that S129 phosphorylation can also inhibit fibril formation and α-synuclein oligomerization, but with longer incubation times, fibrillary aggregates such as those found in Lewy bodies eventually form (43). Lingering questions remain

though, including what influences α-synuclein phosphorylation, when it occurs (before, during or after fibrillization and aggregate formation), and how it may mediate neurotoxicity.

Several kinases phosphorylate α-synuclein at S129, including casein kinases 1 and 2, and GRK2 and 5 (22, 45, 47), but specific phosphatases have yet to be identified. Regulation of α-synuclein phosphorylation state *in vivo* thus remains unclear, in particular due to the multiple cellular compartments in which α-synuclein is found. Phosphorylation of α-synuclein by GRKs is enhanced by phospholipids (45). Liposomal binding of α-synuclein and GRK appears to increase its kinase activity (48). α-Synuclein phosphorylation by casein kinase 2 is similarly enhanced by liposomes. There is also evidence for Ca^{2+}/calmodulin interaction with GPK5 and activation of α-synuclein phosphorylation (49). Further study remains, though, to identify other specific kinases and protein and membrane interactions involved in α-synuclein phosphorylation.

The function of α-synuclein remains largely unknown and further complicates our understanding of its role in PD pathophysiology. Putative functions include regulation of synaptic dopamine transport (41), vesicle formation and trafficking (51), mitochondrial function (41, 50), and nuclear transcription (51). How α-synuclein phosphorylation affects these functions is unclear, but disruption in any one of these could lead to dysfunction and potential neurotoxicity. Findings presented here, though, suggest that phosphorylation state at S129 and its effects *in vivo* are more complex than previously thought, and alone, may not play as significant a role in mediating dopaminergic toxicity. A better understanding of α-synuclein phosphorylation and aggregate formation is clearly needed and will help target novel therapies.

References

1. Baba M, Nakajo S, Tu PH, Tomita T, Nakaya K, Lee VM, Trojanowski JQ, Iwatsubo T. Aggregation of alpha-synuclein in Lewy bodies of sporadic Parkinson's disease and dementia with Lewy bodies. Am J Pathol 1998:152;879.
2. Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M. Alpha-synuclein in Lewy bodies. Nature 1997:388;839.
3. Tu PH, Galvin JE, Baba M, Giasson B, Tomita T, Leight S, Nakajo S, Iwatsubo T, Trojanowski JQ, Lee VM. Glial cytoplasmic inclusions in white matter oligodendrocytes of multiple system atrophy brains contain insoluble alpha-synuclein. Ann Neurol 1998:44;415.
4. Iwai A, Masliah E, Yoshimoto M, Ge N, Flanagan L, de Silva HA, Kittel A, Saitoh T. The precursor protein of non-A beta component of Alzheimer's disease amyloid is a presynaptic protein of the central nervous system. Neuron 1995:14;467.
5. Kruger R, Kuhn W, Muller T, Woitalla D, Graeber M, Kosel S, Przuntek H, Epplen JT, Schols L, Riess O. Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. Nat Genet 1998:18;106.
6. Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, Nussbaum RL. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science 1997:276;2045.
7. Zarranz JJ, Alegre J, Gomez-Esteban JC, Lezcano E, Ros R, Ampuero I, Vidal L, Hoenicka J, Rodriguez O, Atares B, Llorens V, Gomez Tortosa E, del Ser T, Munoz DG, de Yebenes JG. The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. Ann Neurol 2004:55;164.
8. Chartier-Harlin MC, Kachergus J, Roumier C, Mouroux V, Douay X, Lincoln S, Levecque C, Larvor L, Andrieux J, Hulihan M, Waucquier N, Defebvre L, Amouyel P, Farrer M, Destee A. Alpha-synuclein locus duplication as a cause of familial Parkinson's disease. Lancet 2004:364;1167.
9. Singleton AB, Farrer M, Johnson J, Singleton A, Hague S, Kachergus J, Hulihan M, Peuralinna T, Dutra A, Nussbaum R, Lincoln S, Crawley A, Hanson M, Maraganore D,

Adler C, Cookson MR, Muenter M, Baptista M, Miller D, Blancato J, Hardy J, Gwinn-Hardy K. alpha-Synuclein locus triplication causes Parkinson's disease. Science 2003:302;841.

10. Wood-Kaczmar A, Gandhi S, Wood NW. Understanding the molecular causes of Parkinson's disease. Trends Mol Med 2006:12;521.
11. Kotzbauer PT, Giasson BI, Kravitz AV, Golbe LI, Mark MH, Trojanowski JQ, Lee VM. Fibrillization of alpha-synuclein and tau in familial Parkinson's disease caused by the A53T alpha-synuclein mutation. Exp Neurol 2004:187;279.
12. Feany MB. New genetic insights into Parkinson's disease. NEnglJMed 2004:351;1937.
13. Conway KA, Harper JD, Lansbury PT, Jr. Fibrils formed in vitro from alpha-synuclein and two mutant forms linked to Parkinson's disease are typical amyloid. Biochemistry 2000:39;2552.
14. Hashimoto M, Hsu LJ, Sisk A, Xia Y, Takeda A, Sundsmo M, Masliah E. Human recombinant NACP/alpha-synuclein is aggregated and fibrillated in vitro: relevance for Lewy body disease. Brain Res 1998:799;301.
15. Conway KA, Harper JD, Lansbury PT. Accelerated in vitro fibril formation by a mutant alpha-synuclein linked to early-onset Parkinson disease. NatMed 1998:4;1318.
16. Greenbaum EA, Graves CL, Mishizen-Eberz AJ, Lupoli MA, Lynch DR, Englander SW, Axelsen PH, Giasson BI. The E46K mutation in alpha-synuclein increases amyloid fibril formation. JBiolChem 2005:280;7800.
17. Conway KA, Lee SJ, Rochet JC, Ding TT, Harper JD, Williamson RE, Lansbury PT, Jr. Accelerated oligomerization by Parkinson's disease linked alpha-synuclein mutants. Ann NY Acad Sci 2000:920;42.
18. Miller DW, Hague SM, Clarimon J, Baptista M, Gwinn-Hardy K, Cookson MR, Singleton AB. Alpha-synuclein in blood and brain from familial Parkinson disease with SNCA locus triplication. Neurology 2004:62;1835.
19. Feany MB, Bender WW. A Drosophila model of Parkinson's disease. Nature 2000:404;394.
20. Chesselet MF. In vivo alpha-synuclein overexpression in rodents: a useful model of Parkinson's disease? Exp Neurol 2008:209;22-7.
21. Springer W, Kahle PJ. Mechanisms and models of alpha-synuclein-related neurodegeneration. Curr Neurol Neurosci Rep 2006:6;432-6.

22. Okochi M, Walter J, Koyama A, Nakajo S, Baba M, Iwatsubo T, Meijer L, Kahle PJ, Haass C. Constitutive phosphorylation of the Parkinson's disease associated alpha-synuclein. J Biol Chem 2000:275;390.
23. Ellis CE, Schwartzberg PL, Grider TL, Fink DW, Nussbaum RL. alpha-synuclein is phosphorylated by members of the Src family of protein-tyrosine kinases. J Biol Chem 2001:276;3879.
24. Fujiwara H, Hasegawa M, Dohmae N, Kawashima A, Masliah E, Goldberg MS, Shen J, Takio K, Iwatsubo T. alpha-Synuclein is phosphorylated in synucleinopathy lesions. Nat Cell Biol 2002:4;160.
25. Anderson JP, Walker DE, Goldstein JM, de Laat R, Banducci K, Caccavello RJ, Barbour R, Huang J, Kling K, Lee M, Diep L, Keim PS, Shen X, Chataway T, Schlossmacher MG, Seubert P, Schenk D, Sinha S, Gai WP, Chilcote TJ. Phosphorylation of Ser-129 is the dominant pathological modification of alpha-synuclein in familial and sporadic Lewy body disease. J Biol Chem 2006:281;29739-52.
26. Saito Y, Kawashima A, Ruberu NN, Fujiwara H, Koyama S, Sawabe M, Arai T, Nagura H, Yamanouchi H, Hasegawa M, Iwatsubo T, Murayama S. Accumulation of phosphorylated alpha-synuclein in aging human brain. J Neuropathol Exp Neurol 2003:62;644.
27. Chen L, Feany MB. Alpha-synuclein phosphorylation controls neurotoxicity and inclusion formation in a Drosophila model of Parkinson disease. NatNeurosci 2005:8;657.
28. Gorbatyuk OS, Li S, Sullivan LF, Chen W, Kondrikova G, Manfredsson FP, Mandel RJ, Muzyczka N. The phosphorylation state of Ser-129 in human alpha-synuclein determines neurodegeneration in a rat model of Parkinson disease. Proc Natl Acad Sci U S A 2008:105;763-8.
29. Kirik D, Rosenblad C, Burger C, Lundberg C, Johansen TE, Muzyczka N, Mandel RJ, Bjorklund A. Parkinson-like neurodegeneration induced by targeted overexpression of alpha-synuclein in the nigrostriatal system. J Neurosci 2002:22;2780.
30. Lo Bianco C, Ridet JL, Schneider BL, Deglon N, Aebischer P. alpha -Synucleinopathy and selective dopaminergic neuron loss in a rat lentiviral-based model of Parkinson's disease. Proc Natl Acad Sci USA 2002:99;10813.
31. Gao G, Vandenberghe LH, Wilson JM. New recombinant serotypes of AAV vectors. Curr Gene Ther 2005:5;285-97.

32. Howard DB, Powers K, Wang Y, Harvey BK. Tropism and toxicity of adeno-associated viral vector serotypes 1, 2, 5, 6, 7, 8, and 9 in rat neurons and glia in vitro. Virology 2007.
33. Taymans JM, Vandenberghe LH, Haute CV, Thiry I, Deroose CM, Mortelmans L, Wilson JM, Debyser Z, Baekelandt V. Comparative analysis of adeno-associated viral vector serotypes 1, 2, 5, 7, and 8 in mouse brain. Hum Gene Ther 2007:18;195-206.
34. St Martin JL, Klucken J, Outeiro TF, Nguyen P, Keller-McGandy C, Cantuti-Castelvetri I, Grammatopoulos TN, Standaert DG, Hyman BT, McLean PJ. Dopaminergic neuron loss and up-regulation of chaperone protein mRNA induced by targeted over-expression of alpha-synuclein in mouse substantia nigra. J Neurochem 2007.
35. West MJ, Slomianka L, Gundersen HJ. Unbiased stereological estimation of the total number of neurons in thesubdivisions of the rat hippocampus using the optical fractionator. Anat Rec 1991:231;482.
36. Broekman ML, Comer LA, Hyman BT, Sena-Esteves M. Adeno-associated virus vectors serotyped with AAV8 capsid are more efficient than AAV-1 or -2 serotypes for widespread gene delivery to the neonatal mouse brain. Neuroscience 2006:138;501-10.
37. Klein RL, Dayton RD, Leidenheimer NJ, Jansen K, Golde TE, Zweig RM. Efficient neuronal gene transfer with AAV8 leads to neurotoxic levels of tau or green fluorescent proteins. Mol Ther 2006:13;517-27.
38. Reimsnider S, Manfredsson FP, Muzyczka N, Mandel RJ. Time course of transgene expression after intrastriatal pseudotyped rAAV2/1, rAAV2/2, rAAV2/5, and rAAV2/8 transduction in the rat. Mol Ther 2007:15;1504-11.
39. Maurin Y, Banrezes B, Menetrey A, Mailly P, Deniau JM. Three-dimensional distribution of nigrostriatal neurons in the rat: relation to the topography of striatonigral projections. Neuroscience 1999:91;891-909.
40. Schmidt N, Ferger B. Neurochemical findings in the MPTP model of Parkinson's disease. J Neural Transm 2001:108;1263-82.
41. Wersinger C, Prou D, Vernier P, Sidhu A. Modulation of dopamine transporter function by alpha-synuclein is altered by impairment of cell adhesion and by induction of oxidative stress. FASEB J 2003:17;2151-3.
42. Burger C, Gorbatyuk OS, Velardo MJ, Peden CS, Williams P, Zolotukhin S, Reier PJ, Mandel RJ, Muzyczka N. Recombinant AAV viral vectors pseudotyped with viral capsids

from serotypes 1, 2, and 5 display differential efficiency and cell tropism after delivery to different regions of the central nervous system. Mol Ther 2004:10;302-17.

43. Paleologou KE, Schmid AW, Rospigliosi CC, Kim HY, Lamberto GR, Fredenburg RA, Lansbury PT, Jr., Fernandez CO, Eliezer D, Zweckstetter M, Lashuel HA. Phosphorylation at Ser-129 but not the phosphomimics S129E/D inhibits the fibrillation of alpha-synuclein. J Biol Chem 2008:283;16895-905.
44. Cookson MR. The biochemistry of Parkinson's disease. Annu Rev Biochem 2005:74;29-52.
45. Pronin AN, Morris AJ, Surguchov A, Benovic JL. Synucleins are a novel class of substrates for G protein-coupled receptor kinases. J Biol Chem 2000:275;26515.
46. Arawaka S, Wada M, Goto S, Karube H, Sakamoto M, Ren CH, Koyama S, Nagasawa H, Kimura H, Kawanami T, Kurita K, Tajima K, Daimon M, Baba M, Kido T, Saino S, Goto K, Asao H, Kitanaka C, Takashita E, Hongo S, Nakamura T, Kayama T, Suzuki Y, Kobayashi K, Katagiri T, Kurokawa K, Kurimura M, Toyoshima I, Niizato K, Tsuchiya K, Iwatsubo T, Muramatsu M, Matsumine H, Kato T. The role of G-protein-coupled receptor kinase 5 in pathogenesis of sporadic Parkinson's disease. J Neurosci 2006:26;9227-38.
47. Wakamatsu M, Ishii A, Ukai Y, Sakagami J, Iwata S, Ono M, Matsumoto K, Nakamura A, Tada N, Kobayashi K, Iwatsubo T, Yoshimoto M. Accumulation of phosphorylated alpha-synuclein in dopaminergic neurons of transgenic mice that express human alpha-synuclein. J Neurosci Res 2007:85;1819-25.
48. Pronin AN, Carman CV, Benovic JL. Structure-function analysis of G protein-coupled receptor kinase-5. Role of the carboxyl terminus in kinase regulation. J Biol Chem 1998:273;31510-8.
49. Chuang TT, Paolucci L, De Blasi A. Inhibition of G protein-coupled receptor kinase subtypes by Ca2+/calmodulin. J Biol Chem 1996:271;28691-6.
50. Parihar MS, Parihar A, Fujita M, Hashimoto M, Ghafourifar P. Mitochondrial association of alpha-synuclein causes oxidative stress. Cell Mol Life Sci 2008:65;1272-84.
51. Kontopoulos E, Parvin JD, Feany MB. Alpha-synuclein acts in the nucleus to inhibit histone acetylation and promote neurotoxicity. Hum Mol Genet 2006:15;3012.

Figure Legends

Figure 1. Fluorescence immunostaining of SN injected with rAAV2/8 expressing wt α-synuclein and (ires-)EGFP . Tyrosine hydroxylase (TH) staining shows the distribution of dopaminergic SN neurons at representative rostral (**A-C**) and caudal (**D-F**) levels. GFP+ transduced cells are found widely throughout the SN and almost exclusively co-localize with TH+ neurons. Bar = 250 μm.

Figure 2. Striatal photos show the distribution of nigrostriatal terminals immunostained for TH and human α-synuclein (LB509) at 6 weeks. Columns 1 and 2 compare striatal TH terminal labeling after empty vector control (**A, D, G**) and α-synuclein (**B, E, H**) injection in the same animal. There is evident striatal TH terminal loss in the dorsal striatum ipsilateral to α-synuclein injection that appears more prominent for both S129 mutants than for wt. However, the distribution of α-synuclein-positive (LB509 antibody) striatal terminals (**C, F, I**) was similar among wt and mutant cases. Insets reveal high-power detail of α-synuclein+ nigrostriatal terminals. Bars indicate respective magnification (μm).

Figure 3. Measurement of α-synuclein and TH expression in midbrain tissue 6 weeks post viral injection. Western blots show samples from midbrain ipsilateral to α-synuclein (wt, S129A , and S129D) injection and are compared to protein extracts from the contralateral midbrain injected with empty vector (–) control in the same animal. Five micrograms of protein from the left and right midbrain samples were Western blotted with primary antibody to α-synuclein and TH, followed by secondary HRP-conjugated antibody and ECL detection. Bands were quantified and normalized to GAPDH loading control. The ratio of α-synuclein and TH for the α-synuclein (ipsilateral) versus empty vector (contralateral) side was calculated for each animal. Blots show α-synuclein overexpression for most animals, but variable expression was evident as shown for wt. Decrease in TH expression is likewise shown for wt and S129 mutants.

Figure 4. Photomicrographs of representative SN sections from cases at 6 weeks demonstrate neurodegenerative changes and TH cell loss. **A-D** case 98, wt α-synuclein. **E-H** case 99, S129A. **I-L** case 100, S129D. **A, E, I** show TH immunostaining in the contralateral SN after empty

vector control injection. **B, F, J** illustrate TH cell loss in the SN for wt and S129 mutant cases. **C, G, K** show the distribution of human α-synuclein (LB509) expression (gray-black immunostaining) within the SN. **D, H, L** are adjacent sections stained with cresyl violet which demonstrate SN cell loss and gliosis at rAAV injection sites. Bar = 250 μm.

Figure 5. Graphs of nigral TH cell loss, striatal TH terminal density, and dopamine content. **A**, Stereological estimates of TH cell loss in the SN at 6 weeks are expressed relative to empty vector control. Expression of both wt and S129 mutant α-synuclein resulted in significant TH cell loss (mean 25.8%) compared to control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; $F[3,24] = 9.96$, $p = 0.0002$). However, TH cells did not differ among wt and S129A and S129D groups. **B**, Optical density scan array analysis of striatal TH terminal density showed a 16-25% decrease for wt and S129 mutants, but no significant difference among groups ($F[3,27] = 2.514$, $p = 0.080$). **C**, Relative striatal dopamine (compared to contralateral striatum injected with empty vector control), but not DOPAC content was reduced for S129 mutants only.

Figure 6. High-power photomicrographs of human α-synuclein (LB509) immunostaining of nigral neurons in wt (**A-C**), S129A (**D-F**), and S129D (**G, H**) cases. Arrows mark large intracytoplasmic α-synuclein+ aggregates. Most aggregates were either perinuclear or in the periphery (**A** and **G**). **B** shows typical nuclear ghosting, consistent with neurodegenerative change. Dystrophic neurites in **C** have characteristic α-synuclein+ inclusions (arrowheads). Bar = 5 μm.

Figure 1

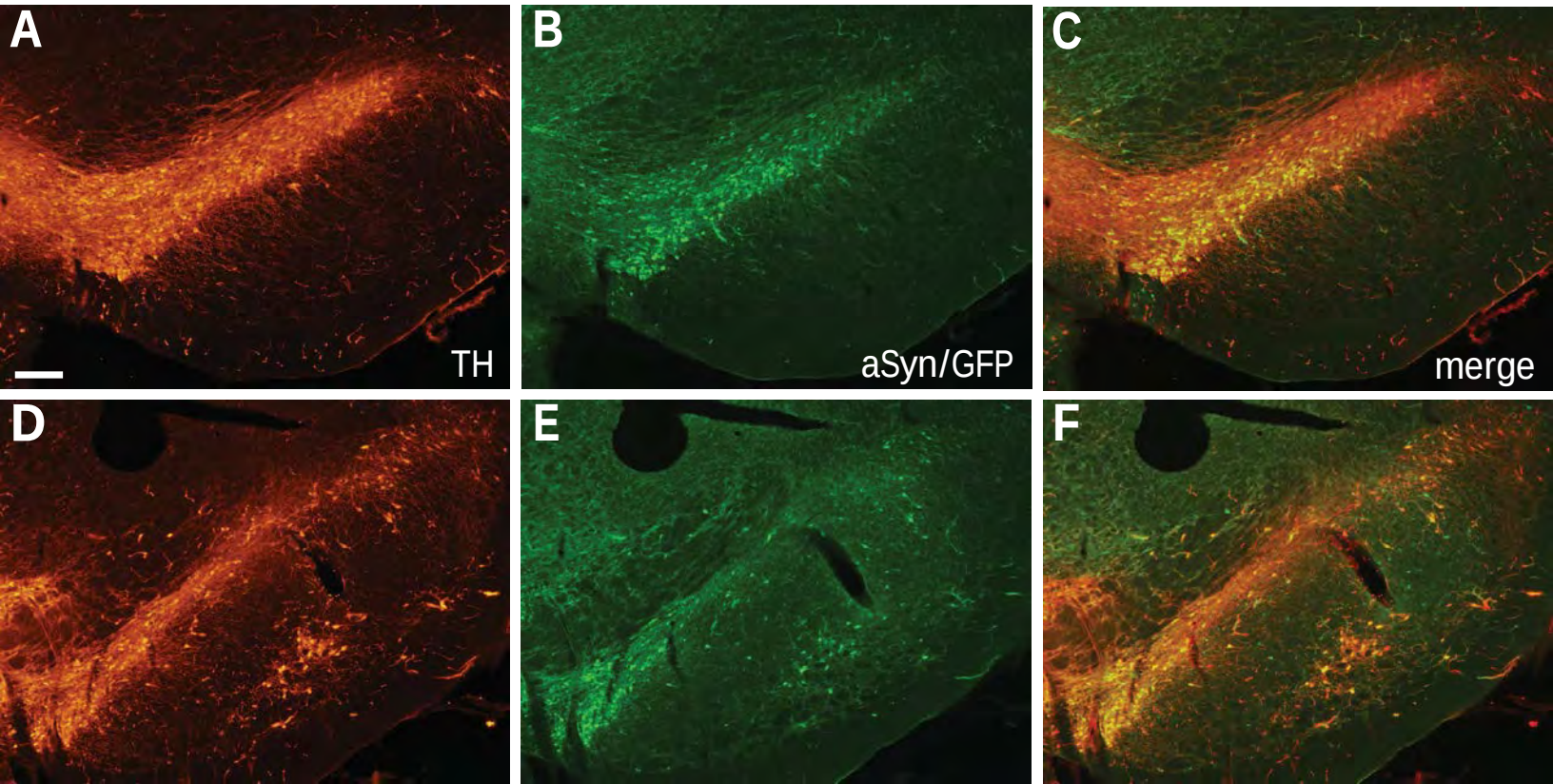


Figure 2

Appendix C

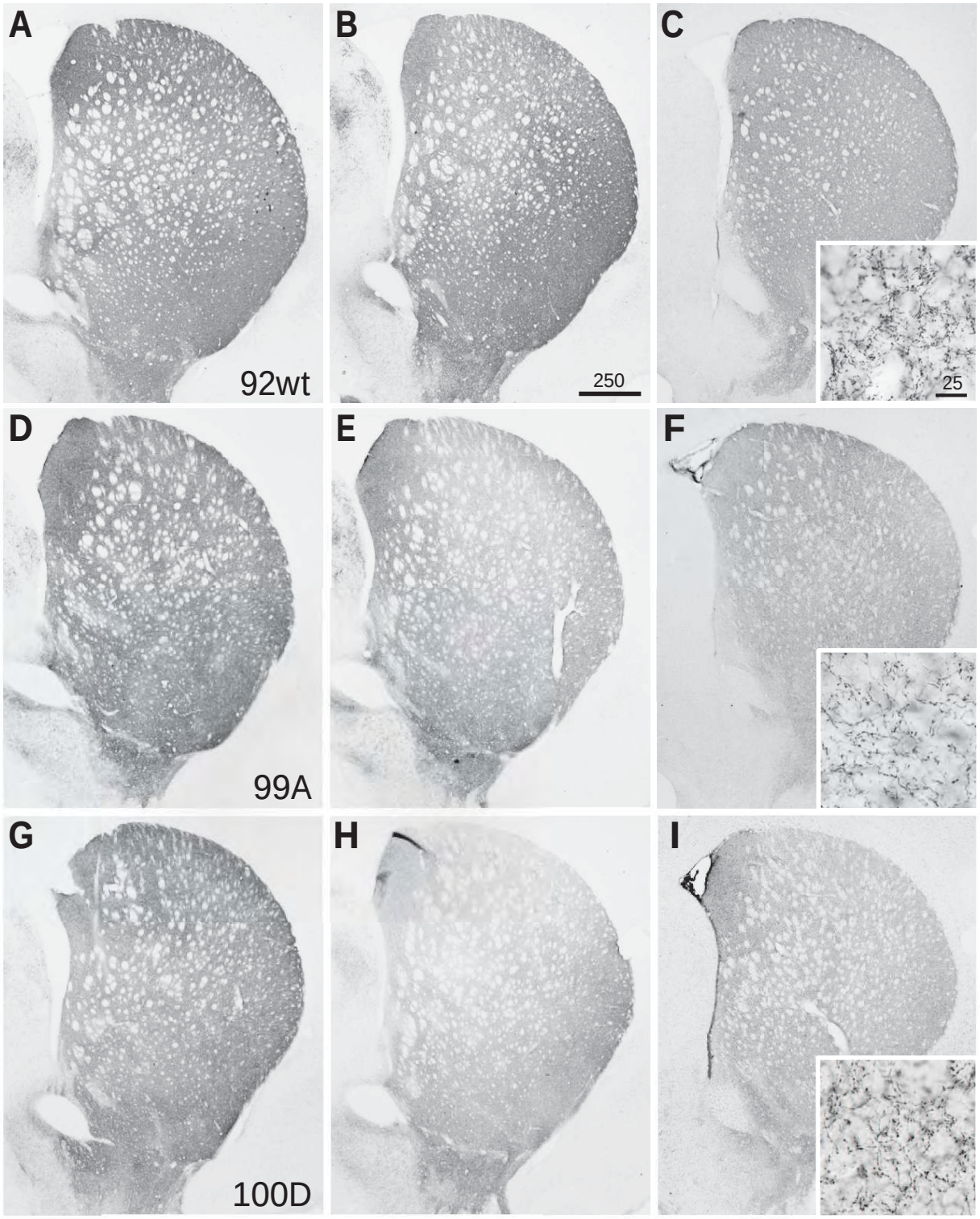


Figure 3

Appendix C

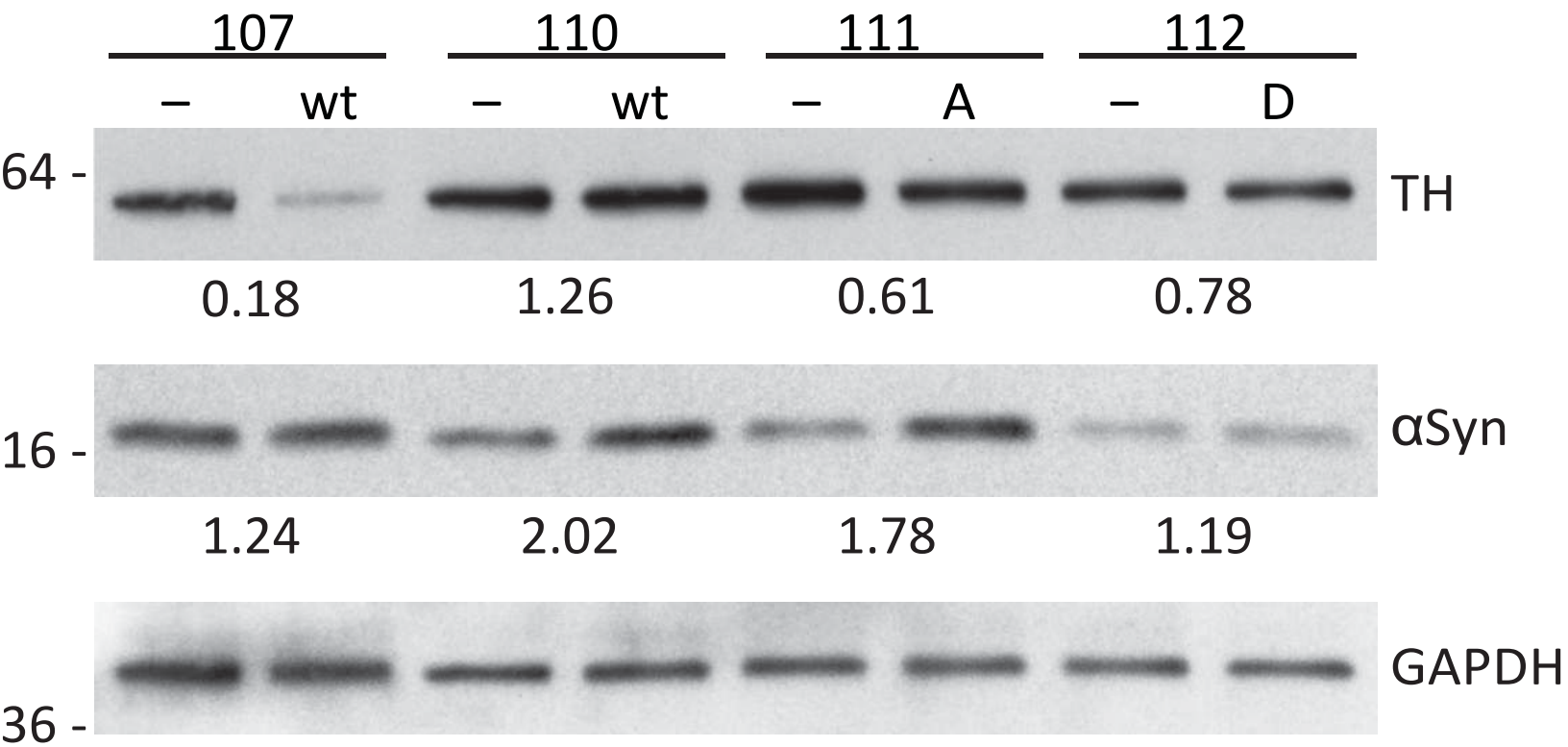
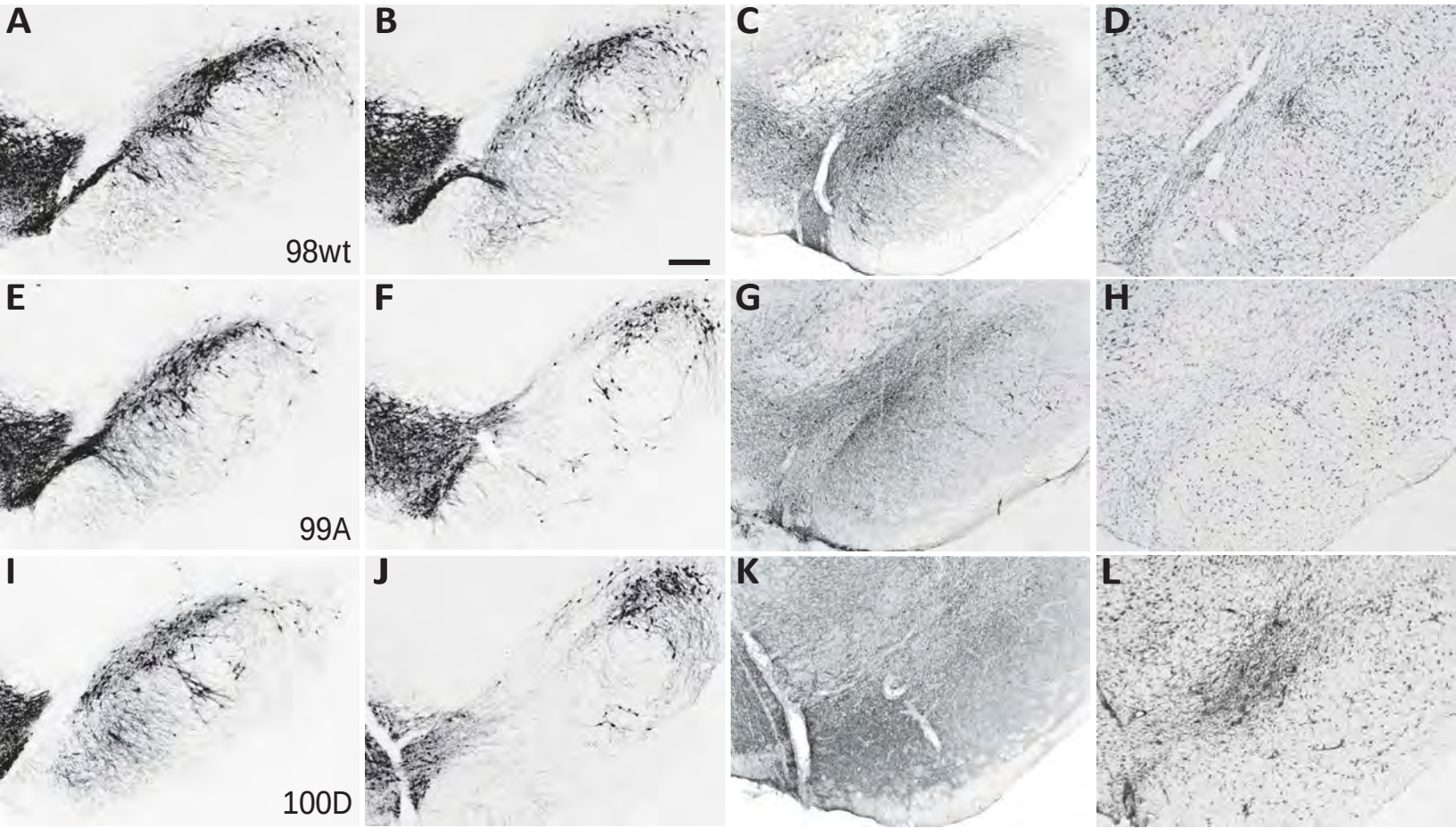
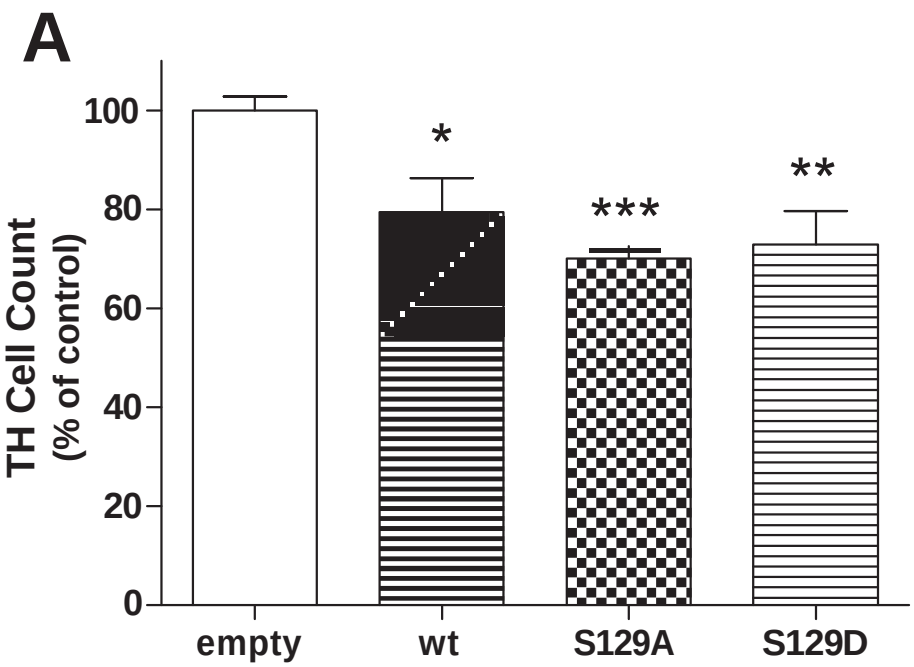
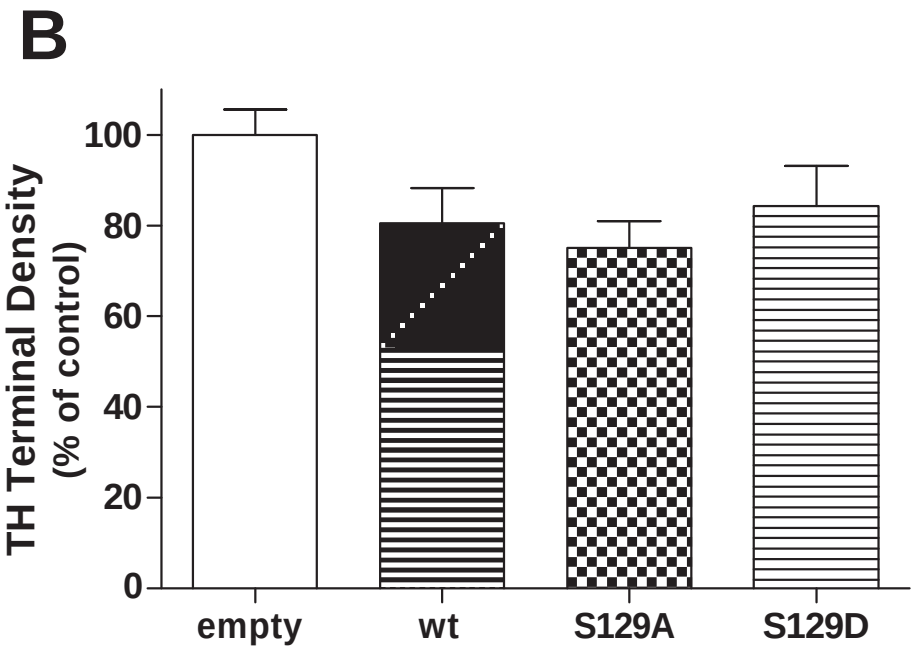


Figure 4

Appendix C




A
TH Cell Count
(% of control)
100
80
60
40
20
0
*
***
**
empty
wt
S129A
S129D


B
TH Terminal Density
(% of control)
100
80
60
40
20
0
empty
wt
S129A
S129D

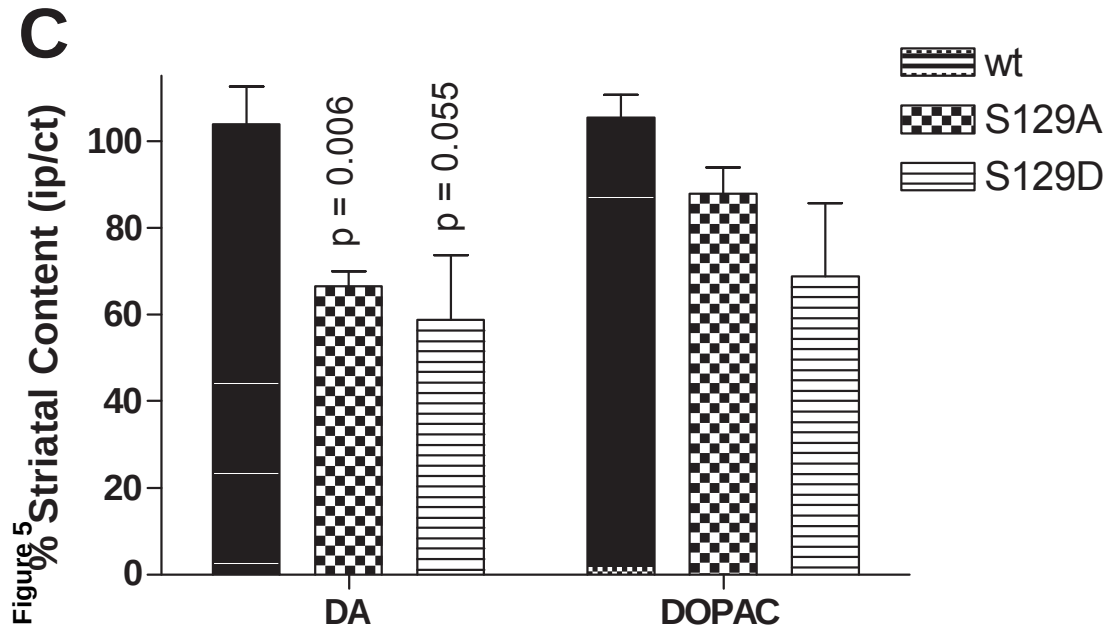
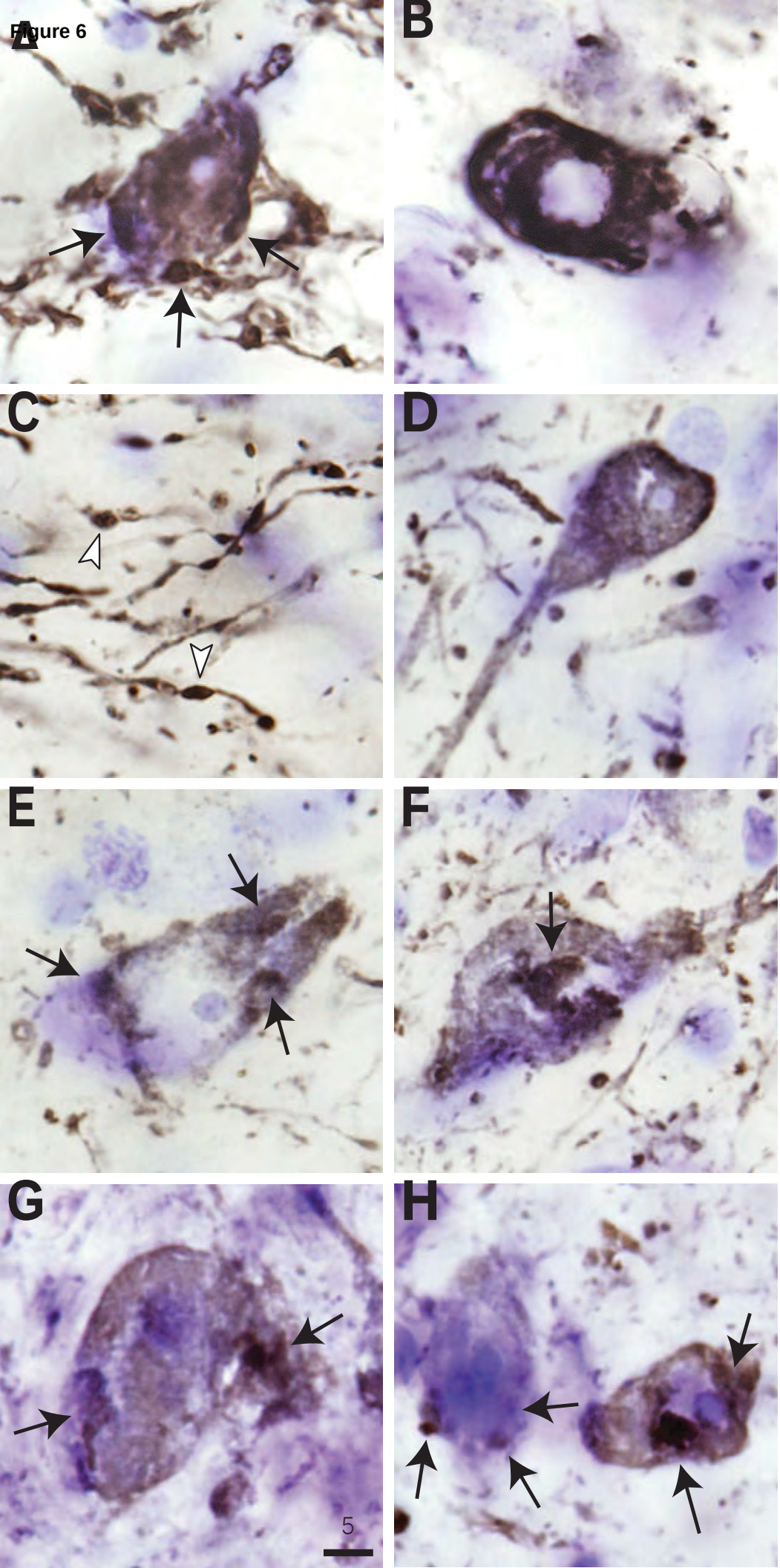

C
% Striatal Content (ip/ct)
100
80
60
40
20
0
p = 0.006
p = 0.055
wt
S129A
S129D
DA
DOPAC

Figure 5

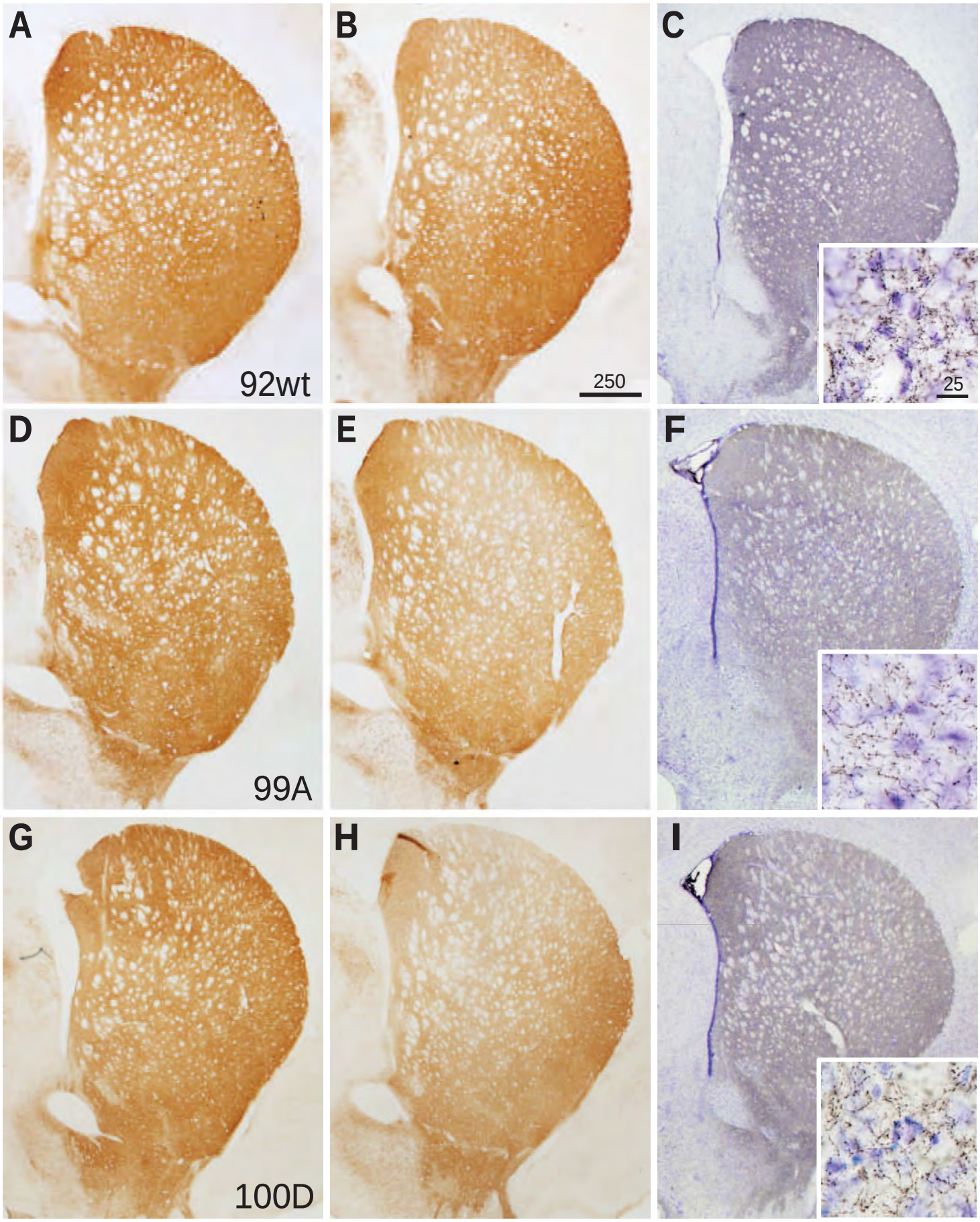
Figure 6

Appendix C



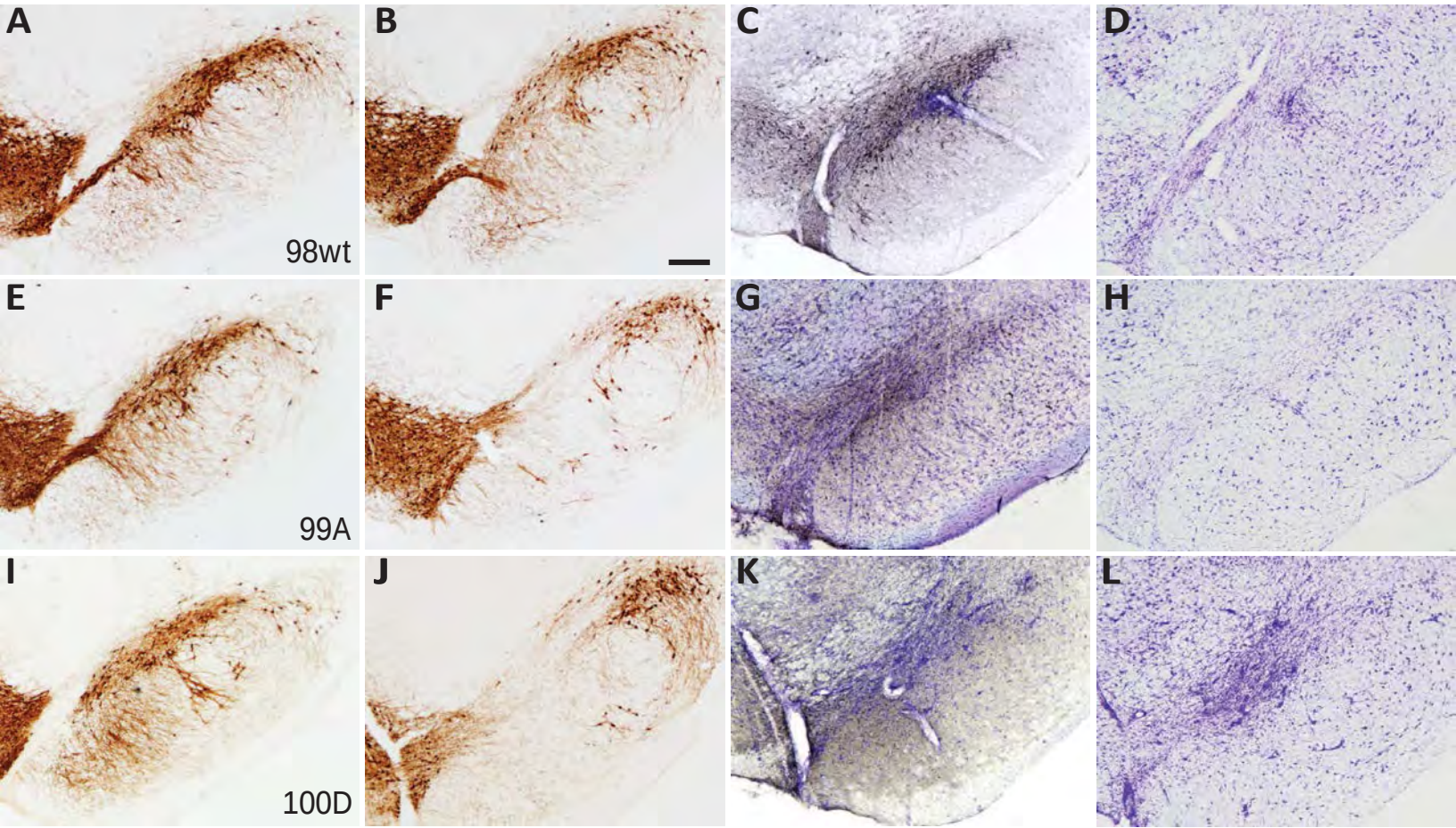
Color Figure 2

Appendix C



Color Figure 4

Appendix C



Appendix D

Print this Page

Presentation Abstract

Program#/Poster#: 142.5/F14

Title: Adenosine A_{2A} receptor gene deletion confers protection of nigral neurons in an α-synuclein model of Parkinson's disease

Location: South Hall A

Presentation Time: Sunday, Oct 18, 2009, 8:00 AM - 9:00 AM

Authors: ***A. KACHROO**[1], E. K. RICHFIELD[2], M. A. SCHWARZSCHILD[1]; [1]Mass Gen. Hosp/Harvard Med. Sch., Charlestown, MA; [2]Envrn. and Occup. Hlth. Sci. Institute, Univ. of Med. and Dent. of New Jersey, Piscataway, NJ

Abstract: Parkinson's disease (PD), a progressive neurodegenerative disorder, results from the interaction of genetic and environmental factors in association with aging. From a genetic standpoint human α-synuclein (*h-αSYN*) has been implicated in the PD phenotype. Mutations in the (h-αSYN) gene (e.g., A53T and A30P) may cause autosomal dominant familial PD. Transgenic mice expressing a *h-αSYN* gene with these two mutations (*hm^2-αSYN* mice) develop age-related reductions in dopaminergic nigral neurons, striatal dopamine (DA) and locomotor activity. Pharmacological blockade of the adenosine A_{2A} receptor using caffeine (non-specific A_{2A} antagonist) and specific antagonists, as well as genetic elimination of the A_{2A} receptor are all neuroprotective in mouse models of PD. The present study sought to determine whether deletion of the A_{2A} receptor (*A_{2A}*[-/-] mice) would afford neuroprotection in *hm^2-αSYN* mice. Heterozygous *A_{2A}*[+/-] mice were mated with *A_{2A}*[+/-] mice that were also transgenic for

the wild-type (*hw-αSYN*) or doubly mutated (*hm^2-αSYN)* form of the human synuclein gene under control of a 9-kb rat tyrosine hydroxylase (TH) promoter - all in the C57B/6J background. Offspring were sacrificed at 20-24 months of age. Striatal DA and metabolites were measured by HPLC-ECD. Stereological counts were performed in the substantia nigra pars compacta. Alternate brain sections collected were also stained using double immunohistochemistry for both TH-positive neurons and synuclein to assess the levels of *h-αSYN* in nigral dopaminergic neurons from brain sections of A_{2A}[+/+] and A_{2A}[-/-] hm^2-αSYN male mice. In the presence of a functional A_{2A} receptor, stereological counts were significantly lower (by approx 43%) in hm^2-αSYN mice compared to either hw-αSYN or non-transgenic (NT) mice. By contrast, in A_{2A}[-/-] mice the hm^2-αSYN transgene did not reduce neuronal cell counts compared to either control. Striatal DA data showed a profile similar to that observed with the stereological counts. No differences were observed in the expression levels of *hm^2-αSYN* immunoreactivity between the two A_{2A} genotypes, indicating that a reduction in *hm^2-αSYN* expression does not account for the reduced toxicity observed in the A_{2A}[-/-] hm^2-αSYN mice. Thus the A_{2A} receptor appears to be required for neurotoxicity in this α-synuclein mouse model of PD, supporting the neuroprotective potential of caffeine and more specific A_{2A} antagonists in the chronic dopaminergic neuron degeneration that characterizes PD.

Disclosures: **A. Kachroo**, None; **E.K. Richfield**, None; **M.A. Schwarzschild**, None.

Keyword(s): synuclein

adenosine

parkinsons

Support: NIEHS R01010804

DOD W81XWH-04-1-0881

AFAR/Beeson program

[Authors]. [Abstract Title]. Program No. XXX.XX. 2009 Neuroscience Meeting Planner. Chicago, IL: Society for Neuroscience, 2009. Online.

Deletion of adenosine A_1 or A_{2A} receptors or treatment with caffeine reduces levodopa-induced dyskinesia in a model of Parkinson's disease

Danqing Xiao[1*], Jared Cassin[1], Brian Healy[2], Jian-Fan Chen[3], Bertil B. Fredholm[4], Michael A. Schwarzschild[1*]

[1.] Department of Neurology, Massaschusetts Institute of Neurological Disorder, Charlestown, MA

[2]. Biostatistics Center, Massachusetts General Hospital, Boston, MA

[3.] Department of Neurology, Boston University School of medicine, Boston, MA

[4]. Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden.

Number of pages: 23

Number of figures: 3

Number of tables: 2

Number of words in the text: 2956

Running title: Adenosine receptors in L-DOPA-induced dyskinesia

*Correspondence to:

Drs. Michael A. Schwarzschild and Danqing Xiao

Department of Neurology

Massaschusetts Institute of Neurological Disorder

Building 114, Sixteenth Street

Charlestown, MA 02129, USA.

Abstract

Adenosine A_{2A} receptor antagonism provides a promising approach to developing nondopaminergic therapy for Parkinson's disease (PD). Initial clinical trials of A_{2A} antagonists have targeted PD patients with L-3,4-dihydroxyphenylalanine (L-DOPA)-induced dyskinesia (LID) in an effort to improve parkinsonian symptoms. The role of adenosine in the development of LID is little known, especially regarding its actions via A_1 receptors. We examined the effects of genetic deletion and pharmacological blockade of A_1 and/or A_{2A} receptors on the development of LID, on the induction of molecular markers of LID including striatal preproenkephalin (PPE), and on the integrity of dopaminergic nigrostriatal neurons in hemiparkinsonian mice. Following a unilateral 6-hydroxydopamine (6-OHDA) lesion A_1, A_{2A} and double A_1-A_{2A} knockout (KO) and wild-type (WT) littermate mice, or caffeine- (and saline-) pretreated mice, were treated daily for 18-21 days with a low dose of L-DOPA. Total abnormal involuntary movement (AIM, a measure of LID) was significantly attenuated in all KOs and in caffeine-pretreated mice ($p<0.05$). An elevation of PPE mRNA ipsilateral to the lesion in WT mice was reduced in all KO mice. In addition, neuronal integrity assessed by striatal dopamine content was similar in all KO and caffeine-pretreated mice following 6-OHDA lesioning. Our findings raise the possibility that A_1 as well as A_{2A} or combined antagonists may also confer a disease-modifying benefit of reduced *risk* of disabling LID. Furthermore, the report may strengthen the rationale for conducting trials of adenosine antagonists as initial rather than late adjunctive therapy for PD.

Introduction

Blockade of adenosine A_{2A} receptors is being attempted as a non-dopaminergic alternative treatment of Parkinson's disease (PD). In particular, the usefulness of A_{2A} receptor antagonism to treat PD complicated by L-DOPA-induced dyskinesia (LID) has been studied in both animal models and clinical trials (Chen, 2003; Morelli et al., 2007). The rationale behind the use of A_{2A} antagonists for symptomatic benefit with reduced risk of adverse effects in PD and LID depends inter alia on the discrete CNS distribution and colocalization of the A_{2A} receptor with D_2 receptors in the 'indirect' pathway of the basal ganglia motor circuitry (Ferre et al., 1997; Kase, 2001; Fredholm et al., 2003). Experimentally it has been shown that elimination or blockade of A_{2A} receptors expressed by forebrain neurons attenuate LID and related behaviors in hemiparkinsonian rodents or parkinsonian non-human primates (Bibbiani et al., 2003; Xiao et al., 2006).

However, adenosine also activates the adenosine A_1 receptor, which – in contrast to discretely expressed A_{2A} receptor – is widely distributed throughout the CNS including the hippocampus and cortex as well as on the striatal neurons of the 'direct' pathway of the basal ganglia (Fastbom et al., 1987; Johansson et al., 1997; Tohyama, 1998). It has been proposed that blocking A_1 receptors on striatonigral neurons of the direct pathway may facilitate motor activity by disinhibiting the motor stimulant actions of colocalized dopamine D_1 receptors on these neurons, whereas blocking A_{2A} receptors on striatopallidal neurons of the indirect pathway may produce a parallel behavioral activation by inhibiting the motor suppressant actions of colocalized D_2 receptors on these neurons (Ferre et al., 1997). However, A_1 receptors are as abundant in

striatopallidal and striatonigral neurons (Ferre et al., 1996) and a selective action is not easily conceived.

Thus adenosine may modulate LID through opposing or cooperative actions on two of its receptors in the CNS. To explore the roles of these receptors alone and in combination in a mouse model of LID in PD, we characterized single A_1 and A_{2A} KO, as well as double A_1-A_{2A} receptor KO phenotypes in hemiparkinsonian mice that are all littermates of double heterozygote crosses in a congenic C57Bl/6 background. In addition to this genetic approach to addressing adenosine receptor involvement in LID, we investigated the effect of pharmacological blockade of adenosine receptors using the widely consumed non-specific adenosine antagonist caffeine. The investigation of caffeine was also prompted by preliminary clinical data that raised the possibility of a link between higher levels of caffeine consumption among PD patients and a reduced risk of subsequent dyskinesia development (Schwarzschild et al., 2003). We chose both a moderate dose of caffeine, which elicits hyperlocomotion (15 mg/kg), and a low dose of caffeine (3 mg/kg), which is capable of modifying neuroplasticity (as in that of conditioning preference) with little accompanying motor stimulant effect (Fredholm et al., 1999).

Materials and methods

Generation of adenosine receptor KO mice

Wild-type (WT) control ($A_1^{+/+}$, $A_{2A}^{+/+}$), A_1 KO ($A_1^{-/-}$, $A_{2A}^{+/+}$), A_{2A} KO ($A_1^{+/+}$, $A_{2A}^{-/-}$) and A_1-A_{2A} double KO ($A_1^{-/-}$, $A_{2A}^{-/-}$) mice were generated by double heterozygous mating ($A_1^{+/-}$, $A_{2A}^{+/-}$ x $A_1^{+/-}$, $A_{2A}^{+/-}$) and genotyped by PCR analysis of tail DNA as described previously (Bastia et al., 2005; Kachroo et al., 2005; Xiao et al., 2006). The double heterozygotes were the offspring of crosses between homozygous A_1 KO and homozygous A_{2A} KO mice from lines that we rendered congenic for the C57Bl/6 strain background (BanburyConference, 1997).

6-OHDA lesion of striatum

All mice were maintained in home cages with a 12 h light/dark cycle. All experiments were performed in accordance with the Massachusetts General Hospital and National Institutes of Health guidelines on the ethical use of animals. The dopaminergic nigrostriatal pathway on the left side of each mouse was lesioned by a stereotactic intrastriatal infusion of 10 ug 6-OHDA (hydrobromide) as described previously (Fredduzzi et al., 2002; Xiao et al., 2006). The mice were fed with both veterinary (Nutri-Cal) and human infant (Enfamil) nutritional supplements immediately after surgery for several days, followed by soft food (Nutra-Gel Diet) to help ensure survival. Twelve days following surgery each mouse underwent a cylinder test (Cenci and Lundblad, 2007), in which asymmetry of forepaw placement on the inner wall of a plexiglass cylinder was used as an indirect assessment of lesion extent. Mice with <85% of dopamine loss (~15% of the lesioned mice) in the lesioned striatum (compared to the contralateral, non-lesioned

striatum) were excluded from all data analysis (except for that of dopamine content). The mice included in the final analyses of the KO experiment comprised 29 male and 9 female mice (3-14 months old with average age 7.5 months) and were balanced for age, weight and gender across genotypes (n=8-12 in each genotype group).

Pharmacological treatment and behavioral testing

Two weeks after 6-OHDA lesioning, mice were treated daily with L-DOPA (freshly prepared, 2 mg/kg ip) for 18-21 days. In the adenosine receptor KO experiment, benserazide (2 mg/kg ip in saline) was administered 20 min prior to each dose of L-DOPA. In the adenosine antagonist experiment, 20 mice were divided into three groups, saline (n=7), caffeine (3 mg/kg ip, n=6) and caffeine (15 mg/kg, ip, n=7) groups (balanced for performance on the cylinder test and home cage residence), in which each daily L-DOPA (mixed with benserazide 2 mg/kg in saline) was preceded by saline or caffeine by 10 min. Behaviors (AIMs and rotations) were recorded every 2-3 days as described previously (Xiao et al., 2006).

Molecular and neurochemical assessments

Three days after the last L-DOPA injection for both KO and caffeine experiments, whole brains were dissected out. The rostral half of the striata was used for mRNA expression quantification of PPE and preprodynorphin (PPD) by *in situ* hybridization, as described previously (Benn et al., 2004; Xiao et al., 2006). The probe sequence (5' to 3') for PPE mRNA was as follows: ATC TGC ATC CTT CTT CAT GAA ACC GCC ATA CCT CTT GGC AAT GAT CTC. The probe sequence (5' to 3') for PPD mRNA was as follows: ATG GGG GCT TCC TGC GGC GCA TTC GCC CCA AGC TGA AGT GGG

ACA. Quantitative densitometric analysis of the mRNA signals was performed using Image J 1.40g software (National Institutes of Health, USA). Optical density (OD) of the transcripts was quantified for the striatum and expressed as the ratio on the 6-OHDA-lesioned side to that on intact (contralateral) side.

The remaining frozen brains containing the caudal half of the striata were sectioned to a thickness of 400 µm at -17 ^{o} C, and the striata were micropunched (Stoelting, 1.25 mm diam). The cores from the left and right striata were analyzed by HPLC with electrochemical detection for dopamine (DA) and DOPAC content (Chen et al., 2001; Xu et al., 2006).

Statistical analysis

All data are expressed as group average ± SEM. The difference between treatments (WT versus KO; caffeine versus saline) during the plateau phase of LID (from day 11 on for each analysis) was evaluated using a repeated measures model with a compound symmetry covariance structure. No significant time by treatment interaction was observed in any model, so only the main effect of group is reported. If a global difference between groups was observed, post-tests were performed and Holm's correction was used to account for multiple comparisons (Holm, 1979). A Wilcoxon signed rank test was used to compare each the plateau measurements to the day 1 measurements for each genotype. The analysis was generated using SAS software, Version 9.1 of the SAS System for Windows (SAS Institute Inc., Cary, NC, USA.) and GraphPad. Student's *t*-test was used for the remaining statistical analyses of molecular

correlates and dopamine loss (Tables). A value of $p < 0.05$ was considered to be significant.

Results

Attenuation of behavioral sensitization in adenosine receptor knockout mice

Following daily L-DOPA treatment, the hemiparkinsonian mice developed behavioral sensitization, as recorded by contralateral rotations, and dyskinesia, quantified by abnormal involuntary movement (AIMs) scale (Figure 1). Acutely (on day 1), responses to L-DOPA were indistinguishable between adenosine receptor genotypes. Chronically, rotational sensitization on the plateau phase (day 11-21) in A_1 KO, A_{2A} KO or A_1-A_{2A} double KO mice was attenuated over the 21 days ($p=0.12$, for each comparison, Holm's corrected p-values) in comparison to WT (Figure 1A). Total AIMs in A_1 KO, A_{2A} KO or A_1-A_{2A} double KO mice were attenuated significantly ($p=0.005$, $p=0.017$, $p=0.030$, respectively, Holm's corrected p-values) in comparison to WT (Figure 1B). The attenuation appeared incomplete in all KO genotypes, with responses in KO mice during the plateau phase still significantly increased compared to their initial (day 1) response ($p<0.05$, Figure 1). Importantly, the effects of A_1 and A_{2A} deletion on total AIMs were non-additive. In fact, for total AIMs, the A_1-A_{2A} double KO mice had higher levels than the A_1 KO mice at all time points.

Total AIMs were further divided into rotational and non-rotational AIMs subscales. Rotational AIMs comprised most (~90%) of total AIMs, and they were attenuated in all genotypes ($p=0.005$, $p=0.018$, $p=0.018$, respectively, Holm's corrected p-values), whereas non-rotational AIMs showed no difference that reached statistical significance compared to WT littermates.

Effect of Caffeine on L-DOPA-induced behavioral sensitization

The mixed adenosine A_1-A_{2A} antagonist caffeine, at the low dose of 3mg/kg, significantly attenuated AIMs that developed in response to repeated L-DOPA administration ($p=0.02$, Figure 2B), and tended to reduce sensitized rotational responses ($p=0.13$, Figure 2A). The initial motor stimulating effect may confound the attenuation effect of caffeine on chronic contralateral rotations. Low dose of caffeine reduced rotational components of AIMs ($p=0.03$; data not shown), but not non-rotational AIMS ($p=0.22$). The discrepancy between rotation and AIMs recording is most likely due to the differences between these two measurements (Carta et al., 2006). An increase in contralateral rotations could be due to the anti-parkinsonian effect of caffeine rather than an increase in dyskinesia (Lane et al., 2006; Marin et al., 2006). Not surprisingly and consistent with our previous finding (Yu et al., 2006), the higher dose of caffeine (15 mg/kg) did not significantly attenuate either L-DOPA-induced rotations or AIMs ($p>0.05$, data not shown), possibly due to its general motor stimulant actions.

Modulation of striatal gene expression and residual dopamine

In WT mice striatal PPE mRNA levels were significantly elevated on the lesioned side after chronic L-DOPA treatment (Figure 3), as demonstrated previously and consistent with the activation of PPE-expressing striatopallidal neurons with the development of LID in parkinsonian rodents and primates [e.g. review (Xu et al., 2005)]. This elevation of PPE was absent in all adenosine receptor KOs, which showed significantly lower striatal PPE mRNA ipsilateral to 6-OHDA lesioning after chronic L-DOPA treatment, compared to their WT littermates (Figure 3). The PPD on the lesioned

side tended to be reduced in comparison to the non-lesioned side (Student *t*-test, p=0.07) in A_{2A} KO mice, consistent with our previous finding (Fredduzzi et al., 2002), in A_1 KO (p=0.09) and A_1-A_{2A} KO (p=0.09). However, the intact side of adenosine receptor KO mice showed no difference in PPE (p>0.05 for all KOs mice) and PPD (p>0.05 for all KOs mice, student *t*-test) in comparison with WT control, suggesting that L-DOPA treatment had no differential effect on gene expression in unlesioned striata across genotypes .

To address the possibility of any change of the prepeptide genes due to the gene KO itself, we also studied the baseline changes, if any, of PPE and PPD in the colonies of A_1, A_{2A} and A_1-A_{2A} double KO mice. We found there was no difference of these peptide gene expression among the different genotypes (p>0.05, one-way ANOVA and Student *t*-test, supplementary figure), consistent with previous finding (Chen et al., 1999).

Consistent with adenosine A_1-A_{2A} receptor KO, low dose of caffeine (3 mg/kg) pretreatment tended to reduce the expression of PPE on the 6-OHDA lesioned side (n=6, p=0.08, student *t*-test), corresponding to caffeine's attenuating effect on LID.

Genotype or pharmacological blockade did not affect the dopamine levels in the 6-OHDA-lesioned and contralateral (non-lesioned) striata (Table 1 and Table 2, respectively).

Discussion

Genetic elimination of adenosine receptors – either A_1 or A_{2A} or both – attenuated the development of AIMs and associated sensitized rotational responses to L-DOPA in a 6-OHDA lesion model of PD. Though we had previously demonstrated the A_{2A} receptor-dependence of these phenomena (Xiao et al., 2006), our demonstration of a similar dependence on A_1 receptors prompted a complementary pharmacological test of the mixed A_1-A_{2A} caffeine in this model of LID. At a low dose (at the threshold of its motor stimulatory effect) caffeine also reduced AIMs. We also found that that induction of PPE, a marker of striatal (nigropallidal) neuron activity, was attenuated in the adenosine receptor KO striata. Finally, striatal dopamine levels were unaffected in KO or caffeine-treated mice, suggesting that A_1 and A_{2A} receptors facilitate the maladaptive neuroplasticity of this LID model rather than the nigrostriatal lesion that is required for its full manifestation.

The role of A_1 and A_{2A} receptors in L-DOPA-induced dyskinesia

Earlier studies have shown that blockade of A_{2A} receptors can improve symptoms of PD and decrease neurodegeneration (Chen, 2003; Chen et al., 2007). Here we show that LID can be reduced by A_{2A} receptor blockade, consistent with our previous finding (Fredduzzi et al., 2002; Xiao et al., 2006). Despite our own evidence that A_{2A} receptor depletion can confer neuroprotection in an MPTP model of PD, in others including the unilateral 6-OHDA-leison model used here, we've observed no effects of A_{2A} depletion on the neurochemical (dopamine content) or neuroanatomical (dopamine transporter ligand binding) indicators of dopaminergic neuron injury [(Fredduzzi et al., 2002; Xiao et

al., 2006); and Tables 1 and 2 in this paper]. Furthermore, caffeine was administered two weeks after the 6-OHDA lesioning in the present experiment, rendering unlikely the possibility that adenosine receptors contribute to LID indirectly through facilitation of neurodegeneration. Thus the reductions in LID reported here suggest a role for adenosine receptors in the neuroplasticity underlying LID.

The effects of blocking A_1 receptor in LID are less well understood, as there is clear evidence for multiple sites of presynaptic, as well as postsynaptic expression and actions of A_1 receptors in the striatum. For example, presynaptic striatal A_1 receptors inhibiting glutamate (Ambrosio et al., 1996; Ciruela et al., 2006), dopamine or acetylcholine release in the striatum and elsewhere might contribute to this complexity (Fredholm and Dunwiddie, 1988; Jin et al., 1993).

PPE expression in the 6-OHDA-lesioned striata was elevated relative to the intact side after L-DOPA-treatment as expected, likely reflecting overactivation of the striatopallidal pathway in LID [reviewed in (Xu et al., 2005)]. The increased PPE expression was attenuated in adenosine receptor KO compared to WT mice, consistent with previous findings that blockade of adenosine receptor reverses the elevated gene expression of PPE in lesioned striatum (Aoyama et al., 2002). Together these studies suggest that the so-called indirect pathway (in which A_{2A} receptor-expressing striatopallidal neurons reside) plays a crucial role in the modulation of LID by adenosine receptors.

A_1 and A_{2A} receptors interaction in L-DOPA-induced dyskinesia

Although the attenuation of LID was partial in both the A_1 and A_{2A} KO mice, there was no clear additivity or synergy of their attenuating effects on LID. In seeming contrast, the combination of A_1 and A_{2A} antagonists by (Karcz-Kubicha et al., 2003) produced an additivity of their individual motor-activating effects. Adenosine receptor mechanisms and hence their interactions in acute motor activation likely differ from those in LID. Similarly, less than additive effects of D_1 and D_2 dopamine receptor antagonists have been reported on complex adaptive behaviors, whereas acute effects of these drugs in combination are typically at least additive on motor activity (Schneider et al., 1991). In LID A_1 and A_{2A} receptors may have sufficiently redundant effects in parallel to preclude an additional effect of blocking one receptor after the other has been inactivated. Alternatively, A_1 and A_{2A} receptor roles in LID may occur sequentially – either when colocalized to a single cell or when interacting in differenct cells at a network level [(e.g. review by (Lopes et al., 2002; Xu et al., 2005)]. The lack of clear additivity observed here may also reflect the chronicity of complete, lifelong inactivation of A_1 and/or A_{2A} receptors in constitutive KO mice, compared to the acuity of interaction with most pharmacological treatments.

Furthermore, the similar attenuations of LID in adenosine receptor KO and after adenosine antagonist (caffeine) application in adult mice support a contention that the observed KO phenotype reflects actions of A_1 and A_{2A} receptors in the adult brain. Though the complete elimination of a receptor subclass as occurs in the homozygous mutant state can offer an incisive initial assessment of its biological role, it may not be the best predictor of pharmacotherapy. Effects of a receptor antagonist like caffeine are

mediated by blockade of only a subset of its receptors, such that a subtler or even distinct phenotype of a heterozygous compared to homozygous KO mouse may be most instructive. Indeed we have recently demonstrated that some behavioral characteristics of doubly (A_1 and A_{2A}) heterozygous mice can match some motor effects of repeated caffeine exposure (Yang et al., 2009).

Translational significance

The results of this study suggest that both A_1 as well as A_{2A} receptor antagonists could be useful therapeutically to lower the risk of LID in PD patients. The results also raise the possibility that blocking both A_1 and A_{2A} receptors simultaneously, as occurs with caffeine use, could provide a prophylaxis against LID development similar to that of a specific A_1 or A_{2A} antagonist alone. Currently caffeine (Postuma, 2009) and multiple specific A_{2A} receptor antagonists (Xu et al., 2005; Biogen Idec. Cambridge, 2008; Black, 2008; Schering-Plough, 2009) are undergoing randomized clinical trials for possible symptomatic benefits in later stages of the disease after dopaminergic treatment has been initiated. Our findings lend further support to the emerging rationale for investigating the early adjunctive use of adenosine antagonists in PD as potential disease-modifying therapy (Ravina et al., 2003; Schwarzschild et al., 2006).

Acknowledgement

This work was supported by N IH grants NS054978 and NS060991, DoD grant W81XWH-04-1-0881, and the Michael J. Fox Foundation. W e thank Tom C. Bur dett, Andrew McAleavey, and Yuehang Xu for excellent technical assistance.

Figure legends

Figure 1. Attenuation of contralateral rotations and abnormal involuntary movement (AIM) in A_1, A_{2A} and A_1-A_{2A} double KO mice compared to WT. Mice were treated daily with L-DOPA (2 mg/kg, i.p.) in combination with benserazide (2 mg/kg, i.p.) for 3 weeks. **A**, Contralateral turns in mice treated with L-DOPA. **B**, Total AIMs in mice treated with L-DOPA. Significant differences in the total AIMs in the plateau phase were observed between the WT (n=12) and the A_1KO (n=9), A_{2A}KO (n=8) or A_1-A_{2A} KO (n=8) groups ($p<0.05$ for each comparison, Holm's correction). The attenuation of AIMs during the plateau phase significantly increased compared to day 1 response, except day 14 and day 17 for A_1 KO.

Figure 2. Attenuation of abnormal involuntary movements (AIMs) by a low dose of caffeine. Mice were treated daily for 18 days with L-DOPA (2 mg/kg, i.p. in combination with benserazide 2 mg/kg, i.p.) 10 mins after i.p. adminstration of saline or caffeine (3mg/kg). **A**, Contralateral turns in mice pretreated with saline or caffeine ($p=0.13$, comparing caffeine to saline in the plateau phase). **B**, Total AIMs in mice pretreated with saline or caffeine ($p<0.05$, comparing caffeine to saline in the plateau phase).

Figure 3. Reduction of PPE mRNA in lesioned striata of adenosine receptor knockout mice following chronic L-DOPA treatment. PPE mRNA levels (**A**) in the 6-OHDA-lesioned striata were quantified as optical density (OD) at the mid-striatum level and expressed as a ratio to OD of the contralateral (unlesioned) striatum (**B**). Chronic treatment with L-DOPA reduced the 6-OHDA-induced increase in striatal PPE levels in

A_1 KO (n=4), A_{2A} KO (n=5) and A_1-A_{2A} KO (n=4) mice (*$p<0.05$; Student's *t*-test compared with the WT group, n=5).

Table 1. Neurochemical measure of nigrostriatal innervation in wild-type and adenosine receptor KOs mice chronically treated with L-DOPA after a unilateral 6-OHDA lesion.

Genotype	DA (pm/mg tissue)	DOPAC (pm/mg tissue)
WT (n=12)		
Ipsilateral (lesioned)	4.2 ± 1.4*	0.0 ± 0.0*
Contralateral (intact)	143.6 ± 7.5	11.3 ± 1.7
A_{2A} KO (n=9)		
Ipsilateral (lesioned)	4.3 ± 3.0*	0.7 ± 0.5*
Contralateral (intact)	129 ± 10	15.9 ± 7.4
A_1 KO (n=8)		
Ipsilateral (lesioned)	6.9 ± 3.0*	0.4 ± 0.4*
Contralateral (intact)	120 ± 14	9.7 ± 0.9
A_1-A_{2A} KO (n=7)		
Ipsilateral (lesioned)	10.4 ± 8.2*	1.2 ± 0.5*
Contralateral (intact)	118 ± 18	9.6 ± 0.9

* $p < 0.05$ *versus* respective contralateral (intact) striatum. The dopamine level in the intact (right) side between wild-type and three other genotypes was not significant different ($p>0.05$).

Table 2. Neurochemical measure of nigrostriatal innervation in saline and caffeine (3mg/kg) pretreated mice daily treated with L-DOPA after a unilateral 6-OHDA lesion

Treatment	DA(pm/mg tissue)	DOPAC(pm/mg tissue)
Saline (n=6-7)		
Ipsilateral (lesioned)	0.4 ± 0.2*	1.0 ± 0.8*
Contralateral (intact)	90.8 ± 14.5	21.8 ± 14.9
Caffeine (n=6)		
Ipsilateral (lesioned)	4.6 ± 2.6*	0.6 ± 0.4*
Contralateral (intact)	103.1 ± 17.6	23.4 ± 15.1

* $p < 0.05$ *versus* respective contralateral striatum. The dopamine level in the intact (contralateral) side between saline and caffeine groups was not significantly different ($p>0.05$).

References:

Ambrosio AF, Malva JO, Carvalho AP, Carvalho CM (1996) Modulation of Ca2+ channels by activation of adenosine A1 receptors in rat striatal glutamatergic nerve terminals. Neurosci Lett 220:163-166.

Aoyama S, Koga K, Mori A, Miyaji H, Sekine S, Kase H, Uchimura T, Kobayashi H, Kuwana Y (2002) Distribution of adenosine A(2A) receptor antagonist KW-6002 and its effect on gene expression in the rat brain. Brain Res 953:119-125.

BanburyConference (1997) Mutant mice and neuroscience: recommendations concerning genetic background. Banbury Conference on genetic background in mice. Neuron 19:755-759.

Bastia E, Xu YH, Scibelli AC, Day YJ, Linden J, Chen JF, Schwarzschild MA (2005) A crucial role for forebrain adenosine A(2A) receptors in amphetamine sensitization. Neuropsychopharmacology 30:891-900.

Benn CL, Farrell LA, Cha JH (2004) Neurotransmitter receptor analysis in transgenic mouse models. Methods Mol Biol 277:231-260.

Bibbiani F, Oh JD, Petzer JP, Castagnoli N, Jr., Chen JF, Schwarzschild MA, Chase TN (2003) A2A antagonist prevents dopamine agonist-induced motor complications in animal models of Parkinson's disease. Exp Neurol 184:285-294.

Biogen Idec. Cambridage MU (2008) Dose-finding safety study of BIIB014 in combination with levodopa in moderate to late stage Parkinson's disease. http://clinicaltrials.gov/ct2/show/NCT00438607.

Black KJ (2008) Study to evaluate SYN115 in Parkinson's disease. http://clinicaltrials.gov/ct2/show/NCT00605553.

Carta AR, Pinna A, Morelli M (2006) How reliable is the behavioural evaluation of dyskinesia in animal models of Parkinson's disease? Behav Pharmacol 17:393-402.

Cenci MA, Lundblad M (2007) Ratings of L-DOPA-induced dyskinesia in the unilateral 6-OHDA lesion model of Parkinson's disease in rats and mice. Curr Protoc Neurosci Chapter 9:Unit 9 25.

Chen JF (2003) The adenosine A(2A) receptor as an attractive target for Parkinson's disease treatment. Drug News Perspect 16:597-604.

Chen JF, Huang Z, Ma J, Zhu J, Moratalla R, Standaert D, Moskowitz MA, Fink JS, Schwarzschild MA (1999) A(2A) adenosine receptor deficiency attenuates brain injury induced by transient focal ischemia in mice. Journal of Neuroscience 19:9192-9200.

Chen JF, Sonsalla PK, Pedata F, Melani A, Domenici MR, Popoli P, Geiger J, Lopes LV, de Mendonca A (2007) Adenosine A2A receptors and brain injury: broad spectrum of neuroprotection, multifaceted actions and "fine tuning" modulation. Prog Neurobiol 83:310-331.

Chen JF, Xu K, Petzer JP, Staal R, Xu YH, Beilstein M, Sonsalla PK, Castagnoli K, Castagnoli N, Jr., Schwarzschild MA (2001) Neuroprotection by caffeine and A(2A) adenosine receptor inactivation in a model of Parkinson's disease. J Neurosci 21:RC143.

Ciruela F, Casado V, Rodrigues RJ, Lujan R, Burgueno J, Canals M, Borycz J, Rebola N, Goldberg SR, Mallol J, Cortes A, Canela EI, Lopez-Gimenez JF, Milligan G, Lluis C, Cunha RA, Ferre S, Franco R (2006) Presynaptic control of striatal

glutamatergic neurotransmission by adenosine A1-A2A receptor heteromers. J Neurosci 26:2080-2087.

Fastbom J, Pazos A, Probst A, Palacios JM (1987) Adenosine A1 receptors in the human brain: a quantitative autoradiographic study. Neuroscience 22:827-839.

Ferre S, Fredholm BB, Morelli M, Popoli P, Fuxe K (1997) Adenosine-dopamine receptor-receptor interactions as an integrative mechanism in the basal ganglia. Trends in Neurosciences 20:482-487.

Ferre S, O'Connor WT, Svenningsson P, Bjorklund L, Lindberg J, Tinner B, Stromberg I, Goldstein M, Ogren SO, Ungerstedt U, Fredholm BB, Fuxe K (1996) Dopamine D1 receptor-mediated facilitation of GABAergic neurotransmission in the rat strioentopenduncular pathway and its modulation by adenosine A1 receptor-mediated mechanisms. Eur J Neurosci 8:1545-1553.

Fredduzzi S, Moratalla R, Monopoli A, Cuellar B, Xu K, Ongini E, Impagnatiello F, Schwarzschild MA, Chen JF (2002) Persistent behavioral sensitization to chronic L-DOPA requires A2A adenosine receptors. Journal of Neuroscience 22:1054-1062.

Fredholm BB, Dunwiddie TV (1988) How does adenosine inhibit transmitter release? Trends Pharmacol Sci 9:130-134.

Fredholm BB, Cunha RA, Svenningsson P (2003) Pharmacology of adenosine A2A receptors and therapeutic applications. Current Topics in Medicinal Chemistry 3:413-426.

Fredholm BB, Battig K, Holmen J, Nehlig A, Zvartau EE (1999) Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacological Reviews 51:83-133.

Holm S (1979) A simple sequentially rejective multiple test procedure. Scandinavian Journal of Statistics 6:65-70.

Jin S, Johansson B, Fredholm BB (1993) Effects of adenosine A1 and A2 receptor activation on electrically evoked dopamine and acetylcholine release from rat striatal slices. Journal of Pharmacology & Experimental Therapeutics 267:801-808.

Johansson B, Georgiev V, Lindstrom K, Fredholm BB (1997) A1 and A2A adenosine receptors and A1 mRNA in mouse brain: effect of long-term caffeine treatment. Brain Research 762:153-164.

Kachroo A, Orlando LR, Grandy DK, Chen JF, Young AB, Schwarzschild MA (2005) Interactions between metabotropic glutamate 5 and adenosine A2A receptors in normal and parkinsonian mice. J Neurosci 25:10414-10419.

Karcz-Kubicha M, Antoniou K, Terasmaa A, Quarta D, Solinas M, Justinova Z, Pezzola A, Reggio R, Muller CE, Fuxe K, Goldberg SR, Popoli P, Ferre S (2003) Involvement of adenosine A1 and A2A receptors in the motor effects of caffeine after its acute and chronic administration. Neuropsychopharmacology 28:1281-1291.

Kase H (2001) New aspects of physiological and pathophysiological functions of adenosine A2A receptor in basal ganglia. Bioscience, Biotechnology & Biochemistry 65:1447-1457.

Lane EL, Cheetham SC, Jenner P (2006) Does contraversive circling in the 6-OHDA-lesioned rat indicate an ability to induce motor complications as well as therapeutic effects in Parkinson's disease? Exp Neurol 197:284-290.

Lopes LV, Cunha RA, Kull B, Fredholm BB, Ribeiro JA (2002) Adenosine A(2A) receptor facilitation of hippocampal synaptic transmission is dependent on tonic A(1) receptor inhibition. Neuroscience 112:319-329.

Marin C, Rodriguez-Oroz MC, Obeso JA (2006) Motor complications in Parkinson's disease and the clinical significance of rotational behavior in the rat: have we wasted our time? Exp Neurol 197:269-274.

MorelliM, Di Paolo T, Wardas J, Calon F, Xiao D, Schwarzschild MA (2007) Role of adenosine A2A receptors in parkinsonian motor impairment and l-DOPA-induced motor complications. Prog Neurobiol 83:293-309.

Postuma R (2009) Caffeine for excessive daytime somnolence in Parkinson's disease. http://clinicaltrials.gov/ct2/show/NCT00459420.

Ravina BM, Fagan SC, Hart RG, Hovinga CA, Murphy DD, Dawson TM, Marler JR (2003) Neuroprotective agents for clinical trials in Parkinson's disease: a systematic assessment. Neurology 60:1234-1240.

Schering-Plough (2009) Acute effects of SCH 420814 on dyskinesia and Parkinsonism in Levodopa treated patients (study P05550AM1). http://clinicaltrials.gov/ct2/show/NCT00845000.

Schneider LH, Watson CA, Gibbs J, Smith GP (1991) Infra-additivity of combined treatments with selective D1 and D2 receptor antagonists for inhibiting sucrose reinforcement. Brain Res 550:122-124.

Schwarzschild MA, Agnati L, Fuxe K, Chen JF, Morelli M (2006) Targeting adenosine A2A receptors in Parkinson's disease. Trends Neurosci 29:647-654.

Schwarzschild MA, Chen JF, Tennis M, Messing S, Kamp C, Ascherio A, Holloway RG, Marek K, Tanner CM, McDermott M, Lang AE, the Parkinson Study Group (2003) Relating caffeine consumption to Parkinson's disease progression and dyskinesias development. Mov Disord 18:1682-1683.

Tohyama M, Takatsuji, K. (1998) Atlas of neuroactive substances and their receptors in the rat. New York: Oxford University Press.

Xiao D, Bastia E, Xu YH, Benn CL, Cha JH, Peterson TS, Chen JF, Schwarzschild MA (2006) Forebrain adenosine A2A receptors contribute to L-3,4-dihydroxyphenylalanine-induced dyskinesia in hemiparkinsonian mice. J Neurosci 26:13548-13555.

Xu K, Bastia E, Schwarzschild M (2005) Therapeutic potential of adenosine A(2A) receptor antagonists in Parkinson's disease. Pharmacol Ther 105:267-310.

Xu K, Xu Y, Brown-Jermyn D, Chen JF, Ascherio A, Dluzen DE, Schwarzschild MA (2006) Estrogen prevents neuroprotection by caffeine in the mouse 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease. J Neurosci 26:535-541.

Yu L, Schwarzschild MA, Chen JF (2006) Cross-sensitization between caffeine- and L-dopa-induced behaviors in hemiparkinsonian mice. Neurosci Lett 393:31-35.

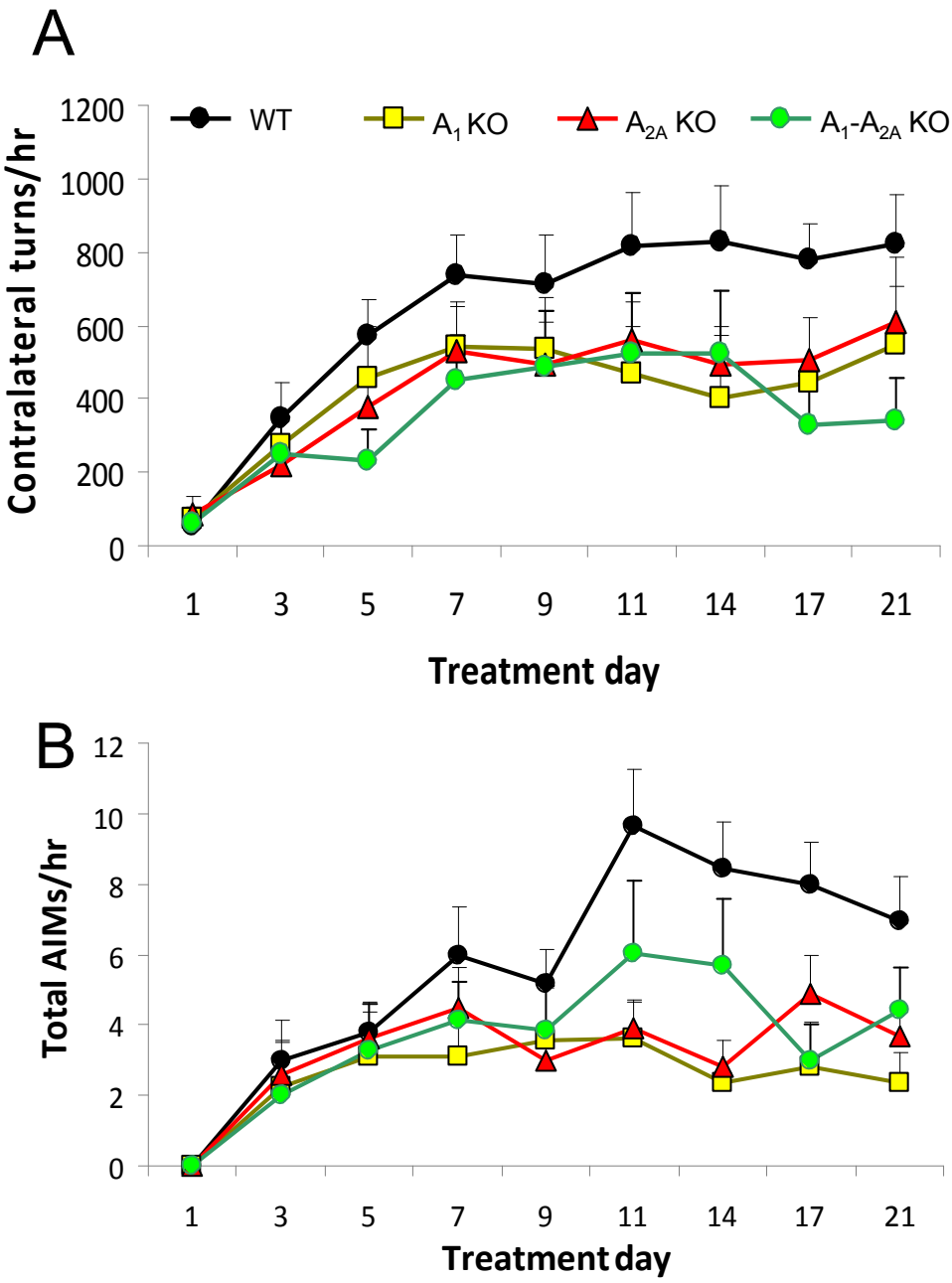


Figure 1
Xiao et al, 2009

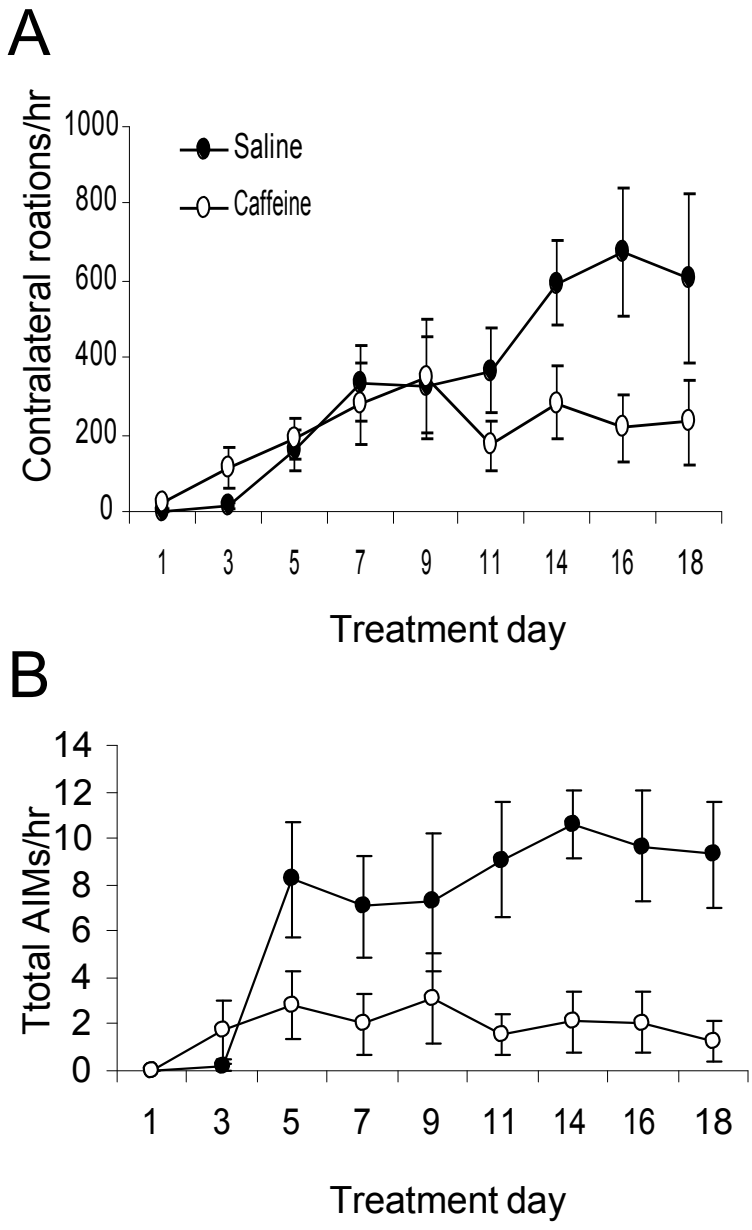
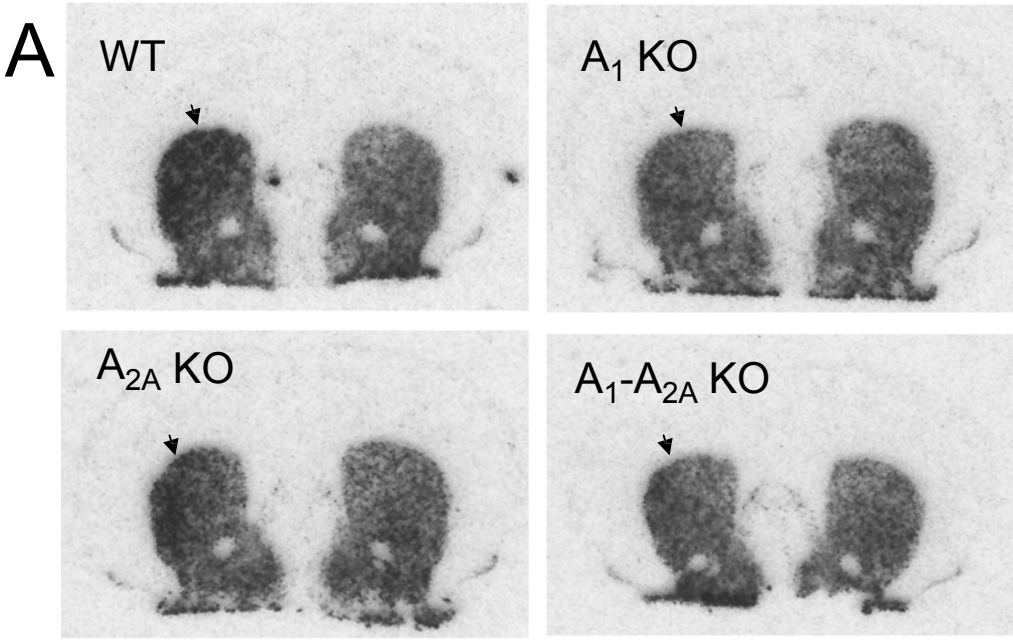


Figure 2
Xiao et al, 2009



"➘"6-OHDA lesioned side

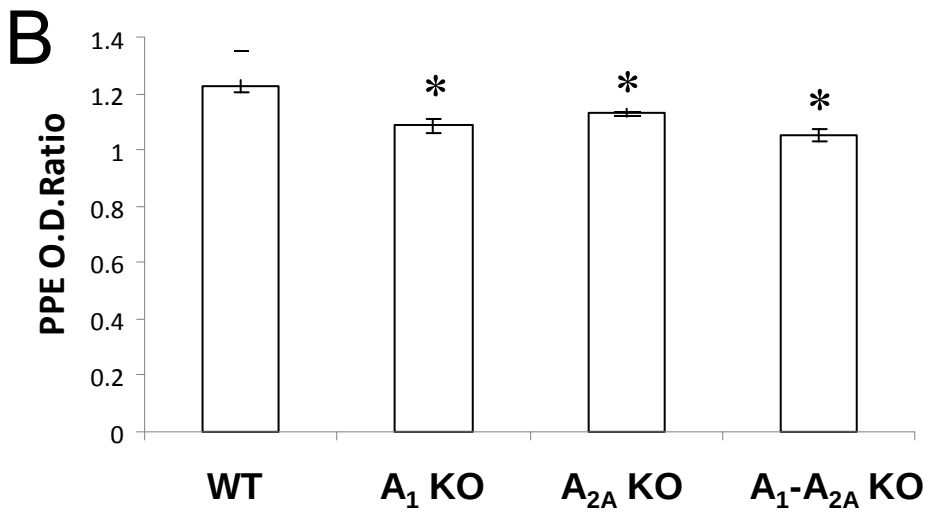


Figure 3
Xiao et al, 2009