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14. ABSTRACT

During the reporting period we have made substantial progress toward our main purpose of characterizing the *mechanisms and neuroprotective potential of purines – adenosine, caffeine, and urate -- linked to better outcomes in Parkinson's disease* (PD), and toward all three of the original Specific Ams (SAs). Major accomplishments included a definitive demonstration that caffeine's neuroprotective effect in a toxin model of PD requires the adenosine A_{2A} receptor, and conversely that a transgenic alpha-synuclein model of PD also requires the A_{2A} receptor (**SA 1**). We also discovered that a mutation in the gene encoding the urate-metabolizing enzyme urate oxidase (*UOx*) raised brain levels of urate and protected mice in another toxin model of PD, supporting the neuroprotective potential of targeting urate elevation in PD (**SA 2**). Lastly, we explored the mechanism by which urate may confer protection in PD by identifying an unexpected astrocyte-dependent, Nrf2 antioxidant pathway-mediated basis for neuroprotection by urate (**SA 3**). The project has had an unusually rapid translational impact and has supported the development of new, clinical trials of purines (caffeine and inosine) as potential disease modifying therapy for PD, a major unmet need of neurotherapeutics research. The results also provide biological insight into prior epidemiological links between purines (caffeine and urate) and PD, and into the broad principle that genetic and environmental factors – both toxins and protectants – interact to determine PD risk and to identify novel approaches to preventing PD or slowing its progression.

15. SUBJECT TERMS urate, tri-oxy-purine, synuclein, neuroprotection, neurotoxin, Parkinson's disease

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<u>**Appendices**</u> (App's) Key publications (2012-2016) *acknowledging W81XWH--1-0150*)

Appendix A: Kachroo A, Schwarzschild MA. (2012) Adenosine A_{2A} receptor gene disruption protects in an α-synuclein model of Parkinson's disease. *Annals Neurol*. 71:278-82.

Appendix B: Schwarzschild MA. (2012) Caffeine in Parkinson disease: better for cruise control than snooze patrol? *Neurology*. 79:616-8. [editorial/review]

Appendix C: Burdett TC, Desjardins CA, Logan R, McFarland NR, Chen X, Schwarzschild MA. (2013) Efficient determination of purine metabolites in brain tissue and serum by high-performance liquid chromatography with electrochemical and UV detection. *Biomed Chromatogr*. 27(1):122-9.

Appendix D: Chen X, Wu G, Schwarzschild MA. (2012) Urate in Parkinson's disease: more than a biomarker? Curr *Neurol Neurosci Rep*. 12(4):367-75. [review]

Appendix E: Cipriani S, Desjardins CA, Burdett TC, Xu Y, Xu K, Schwarzschild MA. (2012) Urate and its transgenic depletion modulate neuronal vulnerability in a cellular model of Parkinson's disease. *PLoS One*. 7:e37331

Appendix F: Cipriani S, Desjardins CA, Burdett TC, Xu Y, Xu K, Schwarzschild MA. (2012) Protection of dopaminergic cells by urate requires its accumulation in astrocytes. *J Neurochem*. 123:172-81.

Appendix G: Chen X, Burdett TC, Desjardins CA, Logan R, Cipriani S, Xu Y, Schwarzschild MA. (2013) Disrupted and transgenic urate oxidase alter urate and dopaminergic neurodegeneration. *Proc Natl Acad Sci USA*. 110(1):300-5.

Appendix H: Salamone JD, Collins-Praino LE, Pardo M, Podurgiel SJ, Baqi Y, Müller CE, Schwarzschild MA, Correa M. (2012) Conditional neural knockout of the adenosine A_{2A} receptor and pharmacological A_{2A} antagonism reduce pilocarpine-induced tremulous jaw movements: Studies with a mouse model of

parkinsonian tremor. *Eur Neuropsychopharmacol.* 23:972-7.

Appendix I: Kachroo A, Schwarzschild MA. Allopurinol reduces levels of urate and dopamine but not dopaminergic neurons in a dual pesticide model of Parkinson's disease. (2014) *Brain Res.* 14;1563:103-9.

Appendix J: Cipriani S, Bakshi R, Schwarzschild MA. (2014) Protection by inosine in a cellular model of Parkinson's disease. *Neuroscience.* Aug 22, 2014;274:242-9.

Appendix K: McFarland NR, Dimant H, Kibuuka L, Ebrahimi-Fakhari D, Desjardins CA, Danzer KM, Danzer M, Fan Z, Schwarzschild MA, Hirst W, McLean PJ. (2014) Chronic treatment with novel small molecule Hsp90 inhibitors rescues striatal dopamine levels but not α-synuclein-induced neuronal cell loss. *PLoS One.* 20;9(1):e86048.

Appendix L: Hung AY, Schwarzschild MA. Treatment of Parkinson's disease: what's in the non-dopaminergic pipeline? *Neurotherapeutics.* 2014 Jan;11(1):34-46. [review]

Appendix M: McFarland NR, Burdett T, Desjardins CA, Frosch MP, Schwarzschild MA. (2013) Postmortem brain levels of urate and precursors in Parkinson's disease and related disorders. *Neurodegener Dis.* 12(4):189-98.

Appendix N: Simon KC, Eberly S, Gao X, Oakes D, Tanner CM, Shoulson I, Fahn S, Schwarzschild MA, Ascherio A; Parkinson Study Group. (2014) Mendelian randomization of serum urate and parkinson disease progression. *Ann Neurol.* Dec 2014;76(6):862-8.

Appendix O: Bakshi R, Zhang H, Logan R, Joshi I, Xu Y, Chen X, Schwarzschild MA. (2015) Neuroprotective effects of urate are mediated by augmenting astrocytic glutathione synthesis and release. *Neurobiol Dis.* 82:574-9.

Appendix P: Jackson EK, Boison D, Schwarzschild MA, Kochanek PM. (2016) Purines: forgotten mediators in traumatic brain injury. *J Neurochem.* 137(2):142-53. [review]

Appendix Q: Bhattacharyya S, Bakshi R, Logan R, Ascherio A, Macklin EA, Schwarzschild MA. (2016) Oral Inosine Persistently Elevates Plasma antioxidant capacity in Parkinson's disease. *Mov Disord.* Mar;31(3):417-21.

Appendix R: Xu K, Di Luca DG, Orrú M, Xu Y, Chen JF, Schwarzschild MA. (2016) Neuroprotection by caffeine in the MPTP model of parkinson's disease and its dependence on adenosine A_{2A} receptors. *Neuroscience.* 13;322:129-37.

Appendix S: McClurg LG, Kachroo A, Schwarzschild MA. (2011) Does chronic 2,4-dichlorophenoxyacetic acid (2,4-D) exposure in mice produce a model of Parkinson's disease? *Society for Neuroscience Annual Meeting* (Washington, DC); abstract # 244.13.

Appendix T: Schwarzschild MA, Fitzgerald K, Bakshi R, Macklin EA, Scherzer C, Ascherio A. (2015) Association of α-synuclein gene expression with Parkinson's disease is attenuated with higher serum urate in the PPMI cohort. *XXI World Congress on Parkinson's Disease and Related Disorders* (Milan); abstract #P2.125.

Introduction (unchanged from proposal SOW)

The overarching aim of the proposed work is to characterize the **mechanisms and neuroprotective potential of purines linked to better outcomes in Parkinson's disease** *(PD)*. We will pursue 3 Specific Aims (SAs) outlined in Section 3 below, and schematized in Figure 1 in the context of purine metabolism and dopaminergic neuron death. **SA1** seeks to determine the effects of the adenosine A_{2A} receptor antagonist caffeine as well as of neuronal A_{2A} receptor knockout (KO) in unilateral toxin models of PD. The potential role of excitotoxic glutatmate release will be investigated. **SA2** will assess the effects of the antioxidant urate (a.k.a. uric acid) on neurotoxicity *in vivo* using complementary pharmacologic and genetic approaches. Inosine, a therapeutically relevant urate precusor, will be tested along with genetic manipulations of urate metabolism, including global KO or conditional KO (cKO) of the *urate oxidase* (*UOx*) or *xanthine oxidoreductase* (*XOR*) genes. **SA3** will explore oxidative and α-synuclein mechanisms of urate protection in a neuronal cell culture models of PD. We propose to systematically pursue the following work on each SA.

Figure 1

SA 1: **Mechanisms of protection by caffeine in toxin models of PD** *in vivo*

SA 2: **Neruoprotection by urate in a unilateral toxin model of PD** *in vivo.*

SA 3: **Mechanisms of protection by urate in toxin models of PD in neuronal cultures.**
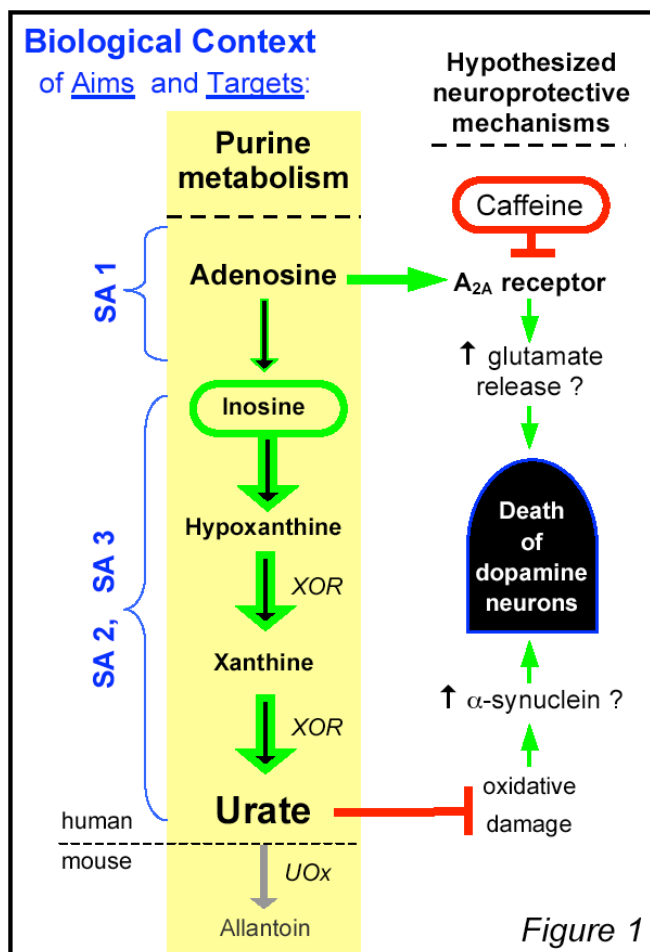
Body of the Report (briefly summarized, with details provided via key publications included as
appendices)

During the reporting period we have made substantial progress toward our main purpose of
characterizing the ***mechanisms and neuroprotective potential of purines – adenosine,
caffeine, and urate -- linked to better outcomes in Parkinson's disease*** (PD), and toward all
three of the original Specific Aims (SAs). Major accomplishments included a definitive
demonstration that caffeine's neuroprotective effect in a toxin model of PD requires the
adenosine A_{2A} receptor [App. R], and conversely that a transgenic alpha-synuclein model of PD
also requires the A_{2A} receptor [App. A] (**SA 1**). We also discovered that a mutation in the gene
encoding the urate-metabolizing enzyme urate oxidase (*UOx*) raised brain levels of urate and
protected mice in another toxin model of PD [App. G], supporting the neuroprotective potential
of targeting urate elevation in PD (**SA 2**). Lastly, we explored the mechanism by which urate
may confer protection in PD by identifying an unexpected astrocyte-dependent, Nrf2 antioxidant
pathway-mediated basis for neuroprotection by urate [Apps. E, F and O] (**SA 3**). The project has
had an unusually rapid translational impact and has supported the development of new, clinical
trials of purines (caffeine and inosine) as potential disease modifying therapy for PD, a major
unmet need of neurotherapeutics research. The results also provide biological insight into prior
epidemiological links between purines (caffeine and urate) and PD, and into the broad principle
that genetic and environmental factors – both toxins and protectants – interact to determine PD
risk and to identify novel approaches to preventing PD or slowing its progression.

Project personnel paid during the project:
- Michael A. Schwarzschild, MD PhD (PI)
- Xiqun Chen, MD PhD   (Co-investigator)
- Anil Kachroo, PhD   (Co-investigator)
- Rachit Bakshi, PhD (Postdoctoral fellow)
- Sarah Cipriani, PhD   (Postdoctoral Fellow)
- Marco Orru, PhD (Postdoctoral fellow)
- Yuehang Xu (Laboratory Technologist)
- Robert Logan (Laboratory Technician)
- Michael Maguire (Laboratory Technician)

Key Research Accomplishments

The project's greatest accomplishment is establishing a biological foundation for the
neuroprotective potential of purines known to be inverse risk factors for PD. As detailed
below in the context of each Specific Aim (SA), key research accomplishments have
contributed to this foundation while providing mechanistic, epidemiological and
therapeutic insights into this neurodegenerative disease.

**SA 1: Mechanisms of protection by adenosine A_{2A} antagonist caffeine in models of PD
*in vivo***

➢ Demonstrated that mutant alpha-synuclein-induced neurodegeneration in mice requires adenosine A_{2A} receptors, providing evidence of a gene-environment interactions influencing the putative protective effects of adenosine A_{2A} antagonists like caffeine. (App. A.)

➢ Direct (gene knockout) evidence that caffeine's neuroprotective effect in a toxin model of PD requires the adenosine A_{2A} receptor, substantiating a neurobiological mechanism by which caffeine caffeine could confer protection against PD pathophysiology. (App. R)

➢ Preliminary evidence of a herbicide 2,4-D (2,4-dichlorophenoxyacetic acid)-based mouse model of Parkinson's disease (See App. S), though technical and time limitations precluded confirmation or characterization of 2,4-D effect.

SA 2: Neruoprotection by urate in a unilateral toxin model of PD *in vivo*.

➢ Systematic genetic (knockout and transgenic *urate oxidase*) evidence for a critical role for endogenous urate as a neuroprotectant in a standard toxin model of Parkinson's disease. (App. G)

➢ Evidence that *urate oxidase* mutations during human evolution may have conferred a mechanism for neuroprotection. (App. G)

➢ Pharmacological evidence that urate reduction may exacerbate neurotoxicity in a pesticide model of PD. (App. I)

➢ Developing efficient reliable analytical methods for measurement of purines (like urate) and catechols (like dopamine) simultaneously using HPLC coupled to electrochemical and ultraviolet detectors. (App. C)

SA 3: Mechanisms of protection by urate in toxin models of PD in neuronal cultures*.*

➢ Robust demonstration of the neuroprotectant and antioxidant properties of urate in cellular models of Parkinson's disease. (Apps. E, F, O)

➢ Discovery of an astrocyte-dependent mechanism of neuroprotection by urate in cellular models of PD. (Apps. E, F, O)

➢ First evidence that urate transporters may be targeted as a novel neuroprotective strategy for Parkinson's disease. (See App. F.)

➢ Publication demonstrating that urate induces astrocytes to release a neuroprotective factor via the Nrf2 antioxidant response pathway. (Apps. F, O)

➢ Evidence that inosine (a purine precursor of urate) currently in clinical development may produce urate-independent protective effects on dopaminergic cells. (App. J)

<u>Reportable Outcomes</u>

• <u>Journal Publications/Manuscripts</u> (acknowledging W81XWH--1-0150)

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- Bakshi R, Zhang H, Logan R, Joshi I, Xu Y, Chen X, Schwarzschild MA. (2015) Neuroprotective effects of urate are mediated by augmenting astrocytic glutathione synthesis and release. *Neurobiol Dis.* 82:574-9. (See App. O)

- Bhattacharyya S, Bakshi R, Logan R, Ascherio A, Macklin EA, Schwarzschild MA. (2016) Oral Inosine Persistently Elevates Plasma antioxidant capacity in Parkinson's disease. *Mov Disord.* Mar;31(3):417-21. (See App. Q)

- Burdett TC, Desjardins CA, Logan R, McFarland NR, Chen X, Schwarzschild MA. (2013) Efficient determination of purine metabolites in brain tissue and serum by high-performance liquid chromatography with electrochemical and UV detection. *Biomed Chromatogr.* 27(1):122-9. (See App. C)

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- Chen X, Wu G, Schwarzschild MA. (2012) Urate in Parkinson's disease: more than a biomarker? Curr *Neurol Neurosci Rep.* 12(4):367-75. [review] (See App. D)

- Cipriani S, Bakshi R, Schwarzschild MA. (2014) Protection by inosine in a cellular model of Parkinson's disease. *Neuroscience.* Aug 22, 2014;274:242-9. (See App. J)

- Cipriani S, Desjardins CA, Burdett TC, Xu Y, Xu K, Schwarzschild MA. (2012) Urate and its transgenic depletion modulate neuronal vulnerability in a cellular model of Parkinson's disease. *PLoS One.* 7:e37331. (See App. E)

- Cipriani S, Desjardins CA, Burdett TC, Xu Y, Xu K, Schwarzschild MA. (2012) Protection of dopaminergic cells by urate requires its accumulation in astrocytes. *J Neurochem.* 123:172-81. (See App. F)

- Hung AY, Schwarzschild MA. Treatment of Parkinson's disease: what's in the non-dopaminergic pipeline? *Neurotherapeutics.* 2014 Jan;11(1):34-46. [review] (See App. L)

- Jackson EK, Boison D, Schwarzschild MA, Kochanek PM. (2016) Purines: forgotten mediators in traumatic brain injury. *J Neurochem.* 137(2):142-53. [review] (See App. P)

- Kachroo A, Schwarzschild MA. (2012) Adenosine A_{2A} receptor gene disruption protects in an α-synuclein model of Parkinson's disease. *Annals Neurol.* 71:278-82. (See App. A)

- Kachroo A, Schwarzschild MA. Allopurinol reduces levels of urate and dopamine but not dopaminergic neurons in a dual pesticide model of Parkinson's disease. (2014) *Brain Res.* 14;1563:103-9. (See App. I)

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- Matos M, Augusto E, Santos-Rodrigues AD, Schwarzschild MA, Chen JF, Cunha RA, Agostinho P. (2012) Adenosine A_{2A} receptors modulate glutamate uptake in cultured astrocytes and gliosomes. *Glia*. 2012 May;60(5):702-16.

- McFarland NR, Burdett T, Desjardins CA, Frosch MP, Schwarzschild MA. (2013) Postmortem brain levels of urate and precursors in Parkinson's disease and related disorders. *Neurodegener Dis*. 12(4):189-98. (See App. M)

- McFarland NR, Dimant H, Kibuuka L, Ebrahimi-Fakhari D, Desjardins CA, Danzer KM, Danzer M, Fan Z, Schwarzschild MA, Hirst W, McLean PJ. (2014) Chronic treatment with novel small molecule Hsp90 inhibitors rescues striatal dopamine levels but not α-synuclein-induced neuronal cell loss. *PLoS One*. 20;9(1):e86048. (See App. K)

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- Schwarzschild MA. (2012) Caffeine in Parkinson disease: better for cruise control than snooze patrol? *Neurology*. 79:616-8. [editorial/review] (See App. B)

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- <u>Abstracts and Presentations</u> – numerous meeting abstracts and lectures on research supported by this award # *W81XWH--1-0150* have been presented regionally, nationally and internationally over the course of the project, as detailed in prior annual progress reports. Included in the final report are two meeting abstracts illustrating preliminary research that was not further pursued further due to technical/time limitations (App. S), and that present preliminary data obtained the end of the project period (App. T).

- <u>Grants secured</u> (*relying on W81XWH--1-0150 project progress*)

2013-2015   *"*Role of urate in protecting mitochondrial function in the brain"
             NIH/NINDS, R21 NS084710 (Schwarzschild dual PI with David Simon of
             BIDMC)
             The goal of the project is to explore the potential of urate to maintain
             mitochondrial integrity during aging, particularly of the CNS.

2014-2016   "Purine biomarkers of LRRK2 PD"
             Michael J. Fox Foundation, LRRK2 Consortium (Schwarzschild, PI)
             The goal of the project is to determine whether purines are predictors of
             age of onset in LRRK2+ Parkinson's disease.

2014-2016   "Pre-clinical foundation of urate elevating therapy for ALS"
             Target ALS / Columbia University (Schwarzschild, Consortioum PI)
             This proposal is aimed at testing the molecular mechanisms of urate-
             mediated neuroprotection in pre-clinical models of ALS.

2014-2018   "Identification of Premotor Parkinson's Disease"
             DOD, W81XWH-14-1-0131 (A. Ascherio, PI)
             Epidemiology of prodromal features of PD in large prospective cohorts.

2015-2016   "Why are Melanoma & PD Linked?: Role of MC1R"
             Michael J. Fox Foundation, Target Validation program (X Chen, PI)
             Pilot pharmacological validation of MC1R as a therapeutic target in a
             toxin model of PD.

2015-2016   "Clinical Pharmacology supporting inosine phase 3 advance"
             Michael J. Fox Foundation (Schwarzschild, PI)
             The goal of the project is to conduct FDA-recommended clinical
             pharmacology studies to permit initiation of a phase 3 trial of inosine in
             Parkinson's disease.

2015-2017   "Neuroprotection by MC1R as the basis for the melanoma-PD link"
             NIH/NINDS, R21 NS090246 (X. Chen, PI)
             An investigation of the role of MC1R in survival and degeneration of
             nigrostriatal dopaminergic neurons using genetic (non-pharmacological)
             probes of MC1R.

2015-2020   "Phase 3 trial of inosine for Parkinson's disease CCC"
             NIH/NINDS, U01 NS090259 (Schwarzschild, PI)
             The goal of the project is to determine whether urate-elevating inosine
             treatment slows progression of Parkinson's disease.

Conclusions

The project has successfully pursued its original aims toward characterizing the **mechanisms and neuroprotective potential of purines – adenosine, caffeine, and urate -- linked to better outcomes in PD**. It has demonstrated that caffeine's protective effect in a toxin model of PD requires the adenosine A_{2A} receptor [App. R], and conversely that a transgenic alpha-synuclein model of PD also requires the A_{2A} receptor [App. A] (SA 1). It has shown that a mutation in the gene encoding the urate-metabolizing enzyme urate oxidase (*UOx*) raised brain levels of urate and protected mice in another toxin model of PD [App. G], supporting the neuroprotective potential of targeting urate elevation in PD (SA 2). Lastly, the project explored the mechanism by which urate may confer protection in PD and identified an unexpected astrocyte-dependent, Nrf2 antioxidant pathway-mediated basis for neuroprotection by urate [Apps. E, F and O] (SA 3).

Our characterization of the roles of these purines in mouse models of PD neurodegeneration through this preclinical project has already demonstrated considerable potential to inform and accelerate clinical trial development of neuroprotective candidates for the disease. Human studies are under way investigating adenosine-targeted strategies in patients with PD. Caffeine itself (http://clinicaltrials.gov/show/NCT01738178) as well as more specific antagonism of the adenosine A_{2A} receptor (http://clinicaltrials.gov/show/NCT01968031; https://clinicaltrials.gov/show/NCT02453386) have entered clinical development in PD and now have a clearer path toward investigating a potential therapeutic indication for disease modification. Similarly our own clinical development of inosine as a urate precursor targeted as a candidate neuroprotective strategy has reported results of phase 2 testing (http://clinicaltrials.gov/ct2/show/NCT00833690; The Parkinson Study Group SURE-PD Investigators, 2014) and led to a major NIH-funded, randomized, blinded, phase 3 efficacy testing of urate-elevating inosine treatment for disease-modification, which is on target to begin enrollment in June 2016 at 60 PD trial centers across the US (https://clinicaltrials.gov/show/NCT02642393). The 5-year project is based in part on the results of the current preclinical DoD/NETPR project and is expected to rigorously test of the hypothesis that treatment with oral inosine dosed to nearly double serum urate to 7-8 mg/dL for two years slows clinical progression in early PD.

In addition to its high translational impact, the results of our purines experiments in preclinical models of PD have substantial epidemiological and military significance. The mechanistic insights pursued under this project reflect a prototypic interaction between putative environmental protectants (e.g., caffeine, urate) and toxins. As reflected in a recent presentation of progress under this DoD award by the PI at the National Neurotrauma Society (July 2015 and resulting publication; App. P) and his preliminary research proposals, the advances made under this award may be ripe for lateral translation to the field of traumatic brain injury (TBI), a major clinical challenge of military service and civilian life, beyond its impact on Parkinson's disease.

References – please see literature cited in the appendices and the Reportable Outcomes above.

10

—————————— BRIEF COMMUNICATION ——————————

Adenosine A$_{2A}$ Receptor Gene Disruption Protects in an α-Synuclein Model of Parkinson's Disease

Anil Kachroo, MD, PhD, and
Michael A. Schwarzschild, MD, PhD

To investigate the putative interaction between chronic exposure to adenosine receptor antagonist caffeine and genetic influences on Parkinson's disease (PD), we determined whether deletion of the adenosine A$_{2A}$ receptor in knockout (KO) mice protects against dopaminergic neuron degeneration induced by a mutant human α-synuclein (hm^2-αSYN) transgene containing both A53T and A30P. The A$_{2A}$ KO completely prevented loss of dopamine and dopaminergic neurons caused by the mutant α-synuclein transgene without altering levels of its expression. The adenosine A$_{2A}$ receptor appears required for neurotoxicity in a mutant α-synuclein model of PD. Together with prior studies the present findings indirectly support the neuroprotective potential of caffeine and more specific A$_{2A}$ antagonists.

Adenosine A$_{2A}$ receptor antagonists are emerging as promising candidates for nondopaminergic therapy for Parkinson's disease (PD) in part due to symptomatic effects on motor deficits in preclinical models, and selective expression of the A$_{2A}$ receptor within the basal ganglia. Consumption of caffeine a nonspecific A$_{2A}$ receptor antagonist has been consistently linked to reduced risk of developing PD.[1] Caffeine protects against dopaminergic nigrostriatal toxicity in a number of PD models.[2–5] Similar protective effects are consistently observed with specific A$_{2A}$ antagonists[6] and in mice lacking the A$_{2A}$ receptor due to global[2] or neuronal knockout (KO)[7] of its gene. Recently, polymorphisms in the human A$_{2A}$ receptor gene (ADORA2A) have been linked to a reduced risk of PD.[8] To explore the effect of chronically disrupting adenosine A$_{2A}$ receptor signaling in a progressive genetic model of neurodegeneration in PD, we crossed A$_{2A}$ KO mice with 1 of the few transgenic α-synuclein lines that feature progressive loss of dopamine (DA) and dopaminergic neurons characteristic of the disease.[9,10] Assessments of the integrity of the dopaminergic nigrostriatal system of their offspring in late life indicated an essential role of adenosine A$_{2A}$ receptors in the neurodegenerative effect of mutant α-synuclein in a mouse model of PD.

Subjects and Methods

Animals

Heterozygous A$_{2A}$ (+/−) KO mice in a C57Bl/6 background (back-crossed 8 generations; N8) were mated with heterozygous A$_{2A}$ (+/−) KO mice that were also transgenic for wild-type (WT) hw-αSYN or the doubly mutant hm^2-αSYN form of the human α-synuclein gene under the control of a 9kb rat tyrosine hydroxylase (TH) promoter.[9] The latter mice were generated by crossing N8 homozygous A$_{2A}$ (−/−) KO mice to transgenic hw-αSYN and hm^2-αSYN mice, which had been backcrossed with C57Bl/6J mice 3 to 4 times after receipt from E.K. Richfield. Nontransgenic (NT) controls generated from these crosses were also used. The 6 genotypes used in this experiment included: A$_{2A}$WT [NT (n = 6M, 6F)); hw-αSYN (n = 4M, 6F); hm^2-αSYN (n = 4M, 5F)]; A$_{2A}$KO [NT (n = 7M, 6F); hw-αSYN (n = 4M, 4F); hm^2-αSYN (n = 6M, 5F)]. Behavioral (see Supplemental Text and Figs. S1–S4) and neurochemical assessments were conducted on both sexes, with anatomical measures performed only on male samples.

Tissue Processing and Analysis

Mice were sacrificed by cervical dislocation at 20 to 24 months of age. The brain was removed and rostral and caudal portions separated by an axial cut made across the whole brain at the tail end of the striatum. Both striata were removed and frozen at −80°C until use. The remaining caudal brain portion was immediately fixed, placed in cryoprotectant, and stored at −80°C until use. The striatum was assayed for DA and 3,4-dihydroxyphenylacetic acid (DOPAC) by standard reverse phase high-performance liquid chromatography with electrochemical detection as routinely performed in our laboratory.[2] Fixed brains were cut on a Leica microtome into 30μm-thick sections and stored for immunolabeling studies in a cryoprotectant consisting of 30% sucrose and 30% ethylene glycol in 0.1M phosphate buffer. Sections were chromogenically stained for TH immunoreactivity (IR) followed by counterstaining with Nissl.[9] Double-label fluorescence immunohistochemistry (IHC) for both TH and hα-SYN was performed on 4 brain sections each
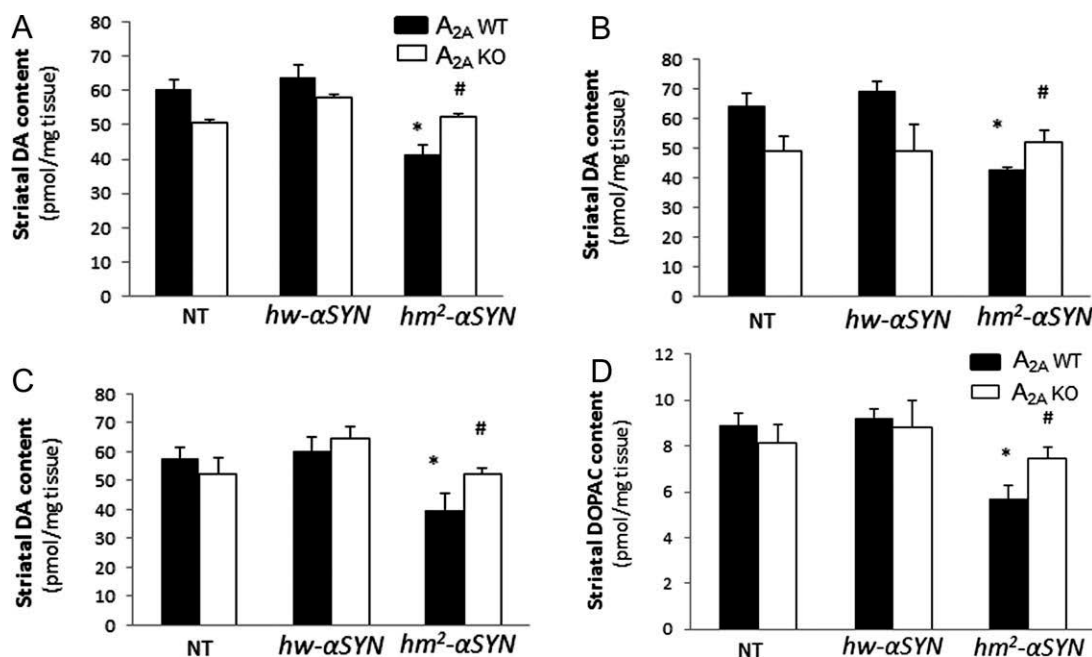
FIGURE 1: Mutant α-synuclein-induced striatal dopamine and DOPAC loss requires the A_{2A} receptor. (A) Striatal dopamine (DA) content was measured at 20 to 24 months of age in nontransgenic (NT) mice and those transgenic for the wild-type (*hw*-αSYN) and the double mutant (*hm²*-αSYN) human synuclein gene. See Subjects and Methods section for numbers of mice/group. *$p < 0.001$ vs NT and *hw*-αSYN; #$p < 0.01$ vs A_{2A}WT [*hm²*-αSYN]; individual 1-way ANOVAs with transgene as the between factor and subsequent post hoc analysis to determine differences between transgenic groups within an A_{2A} genotype; and unpaired *t* test for within transgene comparison between A_{2A} genotypes. (B) Striatal DA level for male mice. *$p < 0.01$ vs NT and *hw*-αSYN; #$p < 0.01$ vs A_{2A}WT [*hm²*-αSYN]; individual 1-way ANOVAs with transgene as the between factor and subsequent post hoc analysis to determine differences between transgenic groups within an A_{2A} genotype; and unpaired *t* test for within transgene comparison between A_{2A} genotypes. (C) Striatal DA level for female mice. *$p < 0.05$ vs NT and *hw*-αSYN; #$p < 0.05$ vs A_{2A}WT [*hm²*-αSYN]; individual 1-way ANOVAs with transgene as the between factor and subsequent post hoc analysis to determine differences between transgenic groups within an A_{2A} genotype; and unpaired *t* test for within transgene comparison between A_{2A} genotypes. (D) Striatal DOPAC content for male and female mice.*$p < 0.001$ vs NT and *hw*-αSYN; #$p < 0.05$ vs A_{2A}WT [*hm²*-αSYN]; individual 1 way ANOVAs with transgene as the between factor and subsequent post hoc analysis to determine differences between transgenic groups within an A_{2A} genotype; and unpaired *t* test for within transgene comparison between A_{2A} genotypes.

from mice in the 2 *hm²*-αSYN groups and data was analyzed using an optical density (OD) measure. To determine α-synuclein expression, the OD of the α-synuclein and TH immunoreactivities was measured in 100 randomly sampled TH+ neurons within the substantia nigra pars compacta (SNpc) using Fluoview software to determine the ratio of *h*-αSYN:TH+ ODs. Quantitative OD values for each neuron were generated at ×40 magnification for both TH and α-synuclein expression using green and red filters, respectively. Stereological assessment of neuronal loss in midbrain sections performed as previously described[5] was limited to the SNpc. All counts were performed by a single investigator blinded as to the groups.

Statistical Analysis

Data values reported for DA, DOPAC content, and stereological cell counts are expressed as mean ± standard error of the mean (SEM). Within-group and between-group comparisons were performed using *t* test and 1-way analysis of variance (ANOVA) followed by Bonferroni post hoc analysis, respectively.

Results

Mutant α-Synuclein-Induced Striatal DA Loss Requires the A_{2A} Receptor

In line with the previous finding of an age-dependent loss of striatal DA in *hm²*-αSYN mice,[9] the striatal DA content of aged *hm²*-αSYN mice was reduced by approximately 35% compared to transgenic *hw*-αSYN and NT controls (Fig 1A). By contrast, mutant α-synuclein appeared to have no effect on striatal DA level in mice lacking the A_{2A} receptor. Similarly, the level of DA metabolite DOPAC was reduced in striatum of *hm²*-αSYN mice in the presence of adenosine A_{2A} receptors but not in their A_{2A} KO littermates (see Fig 1D). Separating the DA data out by sex showed a similar profile for male and female mice (see Fig 1B and C, respectively). Despite the DA deficiency observed in *hm²*-αSYN mice no associated behavioral deficit was detected (see Supplementary Materials), possibly reflecting compensatory mechanisms.
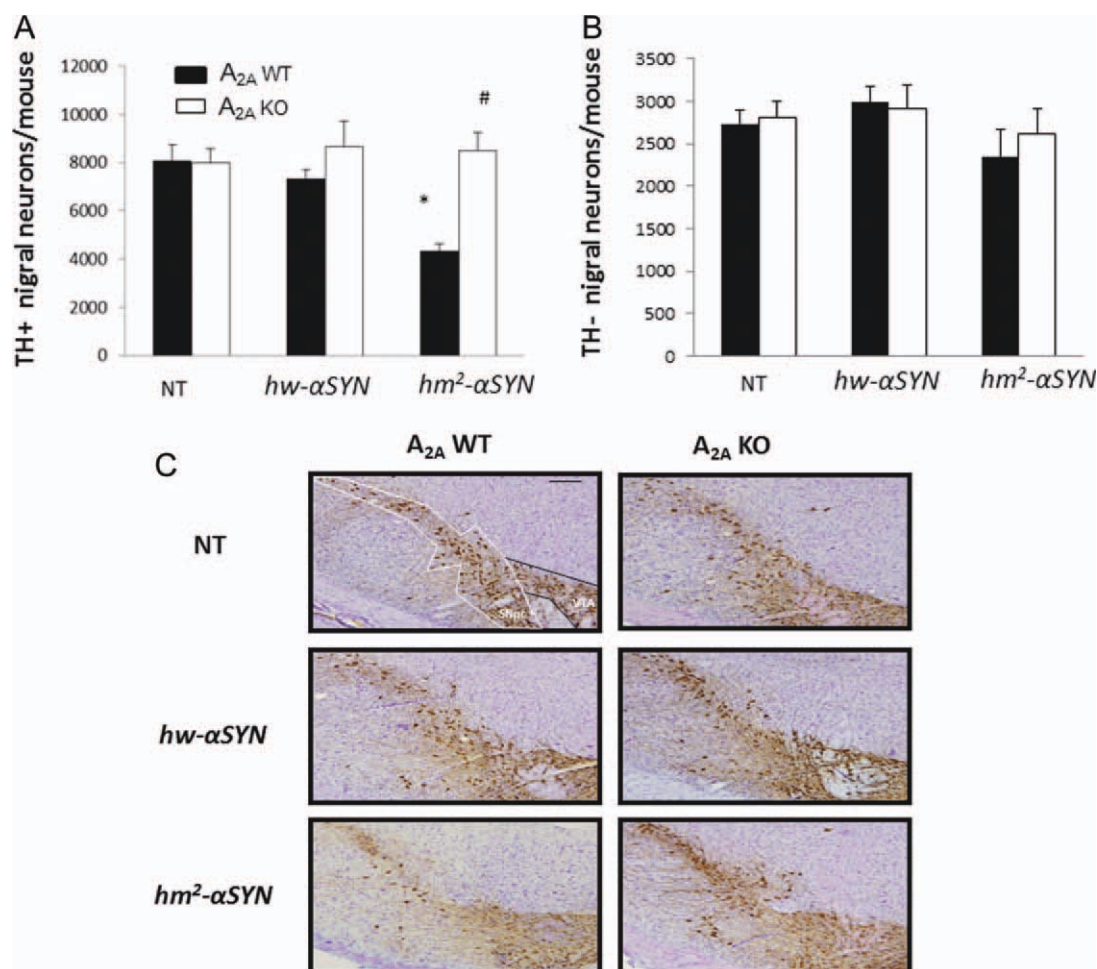
FIGURE 2: Dopaminergic neuron degeneration induced by transgenic mutant human α-synuclein is prevented in mice lacking the adenosine A_{2A} receptor. (A) Stereological cell counts of TH-immunoreactive (TH+) neurons from male mouse brains. See Subjects and Methods section for numbers of mice/group. *$p < 0.01$ vs NT and *hw-αSYN*; #$p < 0.01$ vs A_{2A}WT [*hm²-αSYN*]; individual 1-way ANOVAs with transgene as the between factor and subsequent post hoc analysis to determine differences between transgenic groups within an A_{2A} genotype; and unpaired *t* test for within transgene comparison between A_{2A} genotypes. (B) TH− nigral (Nissl) neurons were assessed in brain sections from male mice. $p > 0.05$; 1-way ANOVA with post hoc analysis and *t* test. (C) Representative photomicrographs showing chromogenically stained TH+ and TH− neurons of the SNpc. Bar = 60μm. [Color figure can be viewed in the online issue, which is available at www.annalsofneurology.org.]

Dopaminergic Neuron Degeneration Induced by Transgenic Mutant Human α-Synuclein Is Prevented in Mice Lacking the Adenosine A_{2A} Receptor

Given the similar profiles in neurochemical changes between the sexes as well as lesser variability of nigral neuron number among male mice, only male mice were used to assess α-synuclein-A_{2A} interaction at the level of neuronal cell counts. Consistent with the characteristic age-dependent loss of dopaminergic nigral neurons in *hm²-αSYN* mice,[9] the mutant α-synuclein mice (at an average age of 22 months) possessed 40% fewer TH+ nigral neurons than its WT *h-αSYN* and NT controls. By contrast, in the absence of A_{2A} receptors this attenuation was completely prevented (Fig 2A, C). Differences of TH+ nigral

neurons between groups could not be attributed to altered TH expression since there were no differences in TH− nigral neuronal counts between groups (see Fig 2B, C).

Absence of Mutant α-Synuclein-Induced Neurodegeneration in A_{2A}KO Mice is Not Due to Reduced Transgene Expression

We explored whether altered *h-αSYN* expression might have contributed to the lack of a mutant α-synuclein effect on striatal DA or TH+ nigral neuronal cell counts in A_{2A} KO mice. The expression of *h-αSYN* protein product in dopaminergic nigral neurons was compared in *hm²-αSYN* male mice with or without A_{2A} receptors, using double-label IHC to normalize human α-synuclein-IR to TH-IR in the cell bodies of the SNpc. TH and h-αSYN immunoreactivities co-localized
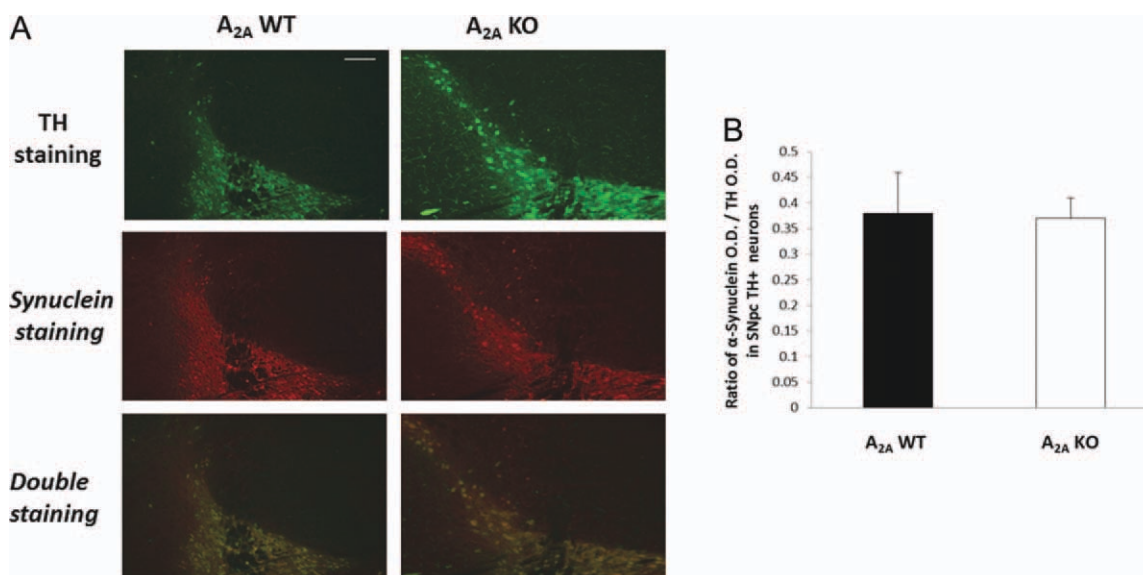
FIGURE 3: Absence of mutant α-synuclein-induced neurodegeneration in A_{2A}KO mice is not due to reduced expression of mutant α-synuclein. Brain sections from mice transgenic for the double mutant (*hm²-αSYN*) human synuclein gene were used. Double label fluorescence IHC for expression of TH and α-synuclein was performed. (A) Fluorescent images (×10) generated from double label staining for TH *(green)*, α-synuclein *(red)*, and merged *(yellow)* are shown. (B) Ratio of α-synuclein OD/TH+ OD in SNpc TH+ neurons; $p > 0.05$; Student *t* test. Bar = 60μm. [Color figure can be viewed in the online issue, which is available at www.annalsofneurology.org.]

(Fig 3A) as previously reported.[9] The data showed no appreciable difference for the ratio of h-αSYN-IR:TH-IR optical densities in TH+ cells, between mice lacking or expressing the A_{2A} receptor (see Fig 3B).

Discussion

The present findings confirm the neurodegenerative phenotype in aging double mutant α-synuclein transgenic mice[9] and identify a requisite facilitative role of the adenosine A_{2A} receptor in this toxicity. Significant losses of striatal DA and nigral dopaminergic neurons were demonstrated in *hm²-αSYN* mice, compared to both their transgenic (*hw-αSYN*) and nontransgenic controls, and were attenuated or prevented in mice lacking the adenosine A_{2A} receptor. Reversal of mutant α-synuclein toxicity by A_{2A} receptor depletion highlights the interplay between toxic and protective influences on dopaminergic neuron viability, raising the possibility that adenosine A_{2A} receptor antagonists, including caffeine, produce their well-documented neuroprotective effects in PD models by preventing synuclein-induced toxicity.

Although the A_{2A} KO phenotype has consistently recapitulated the neuroprotective effects of A_{2A} antagonists in multiple neurotoxin models of PD,[11,12] caution is warranted in extrapolating from the present genetic evidence for an adenosine A_{2A} receptor/α-synuclein link in mice. Despite advantages of absolute specificity and complete inactivation, knockout approaches to receptor function have their own limitations and do not always predict antagonist actions.[13] Accordingly, it remains to be determined whether chronic pharmacological blockade of A_{2A} receptors prevents α-synuclein pathology.

We considered whether attenuated *hm²-αSYN* toxicity observed in A_{2A} KO mice could be attributed to a simple technical artifact of reduced transgene expression in the knockout. However, analysis of the ratio of human α-synuclein and TH immunoreactivities in dopaminergic neurons of the SNpc in *hm²-αSYN* mice showed indistinguishable values between A_{2A} KO and WT littermates, suggesting that neuroprotection afforded in *hm²-αSYN* mice by elimination of the A_{2A} receptor is not through attenuation of h-αSYN expression.

It remains unclear how genetic deletion or pharmacological blockade of the A_{2A} receptor attenuates the death of dopaminergic neurons in models of PD, although multiple mechanisms have been advanced, including the attenuation of excitotoxic and inflammatory effects of A_{2A} receptor activity.[12] Similar uncertainty exists over the mechanisms by which human *α-SYN* mutations or overexpression can produce neurodegeneration in PD and its models. However, consistent with evidence that α-synuclein toxicity may be mediated by proteasomal (ubiquitin system) dysfunction,[14] the ubiquitin proteasomal system (UPS) is impaired in aged transgenic mutant *hm²-αSYN* mice like those studied here, compared to their transgenic WT *hw-αSYN* and nontransgenic controls.[15] Whether the prevention of cell loss observed in A_{2A} KO mice is due to attenuation of UPS dysfunction or downstream mediator of

α-synuclein toxicity remains to be clarified. Another plausible explanation involves a limitation of genetic deletion studies, such that the absence of the A_{2A} receptor throughout development may have resulted in an adult KO phenotype that in its own right might have influenced α-*SYN* toxicity. Although morphological and neurochemical assessments of the constitutive A_{2A} KO mice have not supported a developmental phenotype.[16] This question could be definitively addressed in future studies with the use of a conditional brain-specific A_{2A} KO-transgenic synuclein model.

With multiple specific adenosine A_{2A} antagonists as well as caffeine currently progressing through phase II and III clinical trials for the symptomatic treatment of PD,[17] this class of agent is well positioned for clinical testing of its neuroprotective potential. The present findings strengthen the rationale for disease modification trials of A_{2A} receptor antagonism. They complement epidemiological data on caffeine links to a reduced risk of PD, and substantially broaden the preclinical evidence for A_{2A} receptor-dependent neurodegeneration from acute toxin (eg, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [MPTP] and 6-hydroxydopamine [6-OHDA]) models[12] to an established chronic progressive (mutant human α*SYN*) model of PD. The results also strengthen the contemporary view that PD etiopathogenesis reflects an interplay between genetic (eg, mutant α-synuclein) and environmental (eg, adenosine A_{2A} receptor disruption) influences, and highlight the therapeutic potential of modifying the latter.

Potential Conflicts of Interest

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Caffeine in Parkinson disease : Better for cruise control than snooze patrol?

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EDITORIAL

Caffeine in Parkinson disease
Better for cruise control than snooze patrol?

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Caffeine, the world's most widely used psychomotor stimulant, potentiates the antiparkinsonian effects of levodopa in preclinical models, as noted nearly 40 years ago.[1] The findings prompted early placebo-controlled crossover studies of caffeine as an adjunct to levodopa or a dopamine agonist in Parkinson disease (PD).[2,3] No motor effect of caffeine was demonstrated other than exacerbation of dyskinesia. However, these small studies assessed caffeine at high doses (~1,100 mg/day, the equivalent of ~8 cups of brewed coffee/day), at which most subjects reported restlessness and insomnia. By contrast, another small study reported that caffeine at a much lower dose of 100 mg/day helped improve freezing of gait, though tolerance to caffeine seemed to limit benefit.[4]

In this issue of *Neurology®*, Postuma et al.[5] report the results of a randomized controlled trial of caffeine as a treatment of excessive daytime sleepiness in PD. Although efficacy for improving wakefulness assessed under the primary outcome did not reach statistical significance (yielding Class I evidence against such an indication in PD), a secondary outcome analysis provided evidence in support of an antiparkinsonian motor effect of caffeine. Sixty-one subjects with PD with documented daytime sleepiness and moderate motor symptoms, treated with ~600 mg per day of levodopa on average, were randomized 1:1 to placebo vs 100 mg caffeine twice a day for 3 weeks before advancing to 200 mg twice daily for 3 more weeks. After 6 weeks, those in the caffeine group showed improvement relative to controls on a standard clinical scale of parkinsonian dysfunction (close to 5 points on the total Unified Parkinson's Disease Rating Scale [UPDRS]), including its objective motor component and subscores for bradykinesia and rigidity, with similar findings at 3 weeks on the lower dose.

Several limitations of the study, as discussed by the authors, include the exploratory nature of the motor findings given the primary hypothesis of a nonmotor benefit; the possibility of incomplete blinding; and the brevity of treatment, leaving open the question of tolerance to caffeine. Nevertheless, these findings are noteworthy, the first to suggest antiparkinsonian effects of caffeine in a randomized clinical trial.

This Class II evidence that motor function in PD can be improved by caffeine is bolstered by mechanistic and clinical advances identifying adenosine A_{2A} receptor antagonism as the molecular basis of caffeine's psychomotor stimulant properties, and as a promising antiparkinsonian strategy. The discovery by the early 1980s that caffeine likely acts through antagonism of adenosine receptors[6] coupled with caffeine's antiparkinsonian effects in animal models[1] accelerated research into the neurobiology and neurotherapeutic potential of adenosine receptor blockade. Enthusiasm for targeting adenosine A_{2A} receptors in particular as a candidate antiparkinsonian strategy grew after the colocalization of A_{2A} receptors with dopamine D_2 receptors in striatopallidal output neurons, where their opposing cellular influences account for antiparkinsonian actions of both A_{2A} antagonists and D_2 agonists.[6,7] Moreover, the relatively restricted expression of CNS A_{2A} receptors to and within the striatum[7] (figure, A) suggests a low liability for neuropsychiatric side effects of A_{2A} antagonists, in contrast to existing nondopaminergic antiparkinsonian agents targeting much more widespread CNS receptors. Neuroimaging and behavioral data confirmed that caffeine indeed blocks striatal A_{2A} receptors (figure, B),[8] which appear required for its motor stimulant properties (figure, C).[9]

Caffeine's candidacy as an antiparkinsonian agent is strengthened further by progress made with several more specific A_{2A} antagonists (including istradefylline, preladenant, and tozadenant). Positive results have prompted ongoing phase II and III clinical trials of their antiparkinsonian potential. Epidemiologic and laboratory evidence that caffeine and specific A_{2A} antagonists may offer additional benefits of slowing the underlying neurodegenerative process or
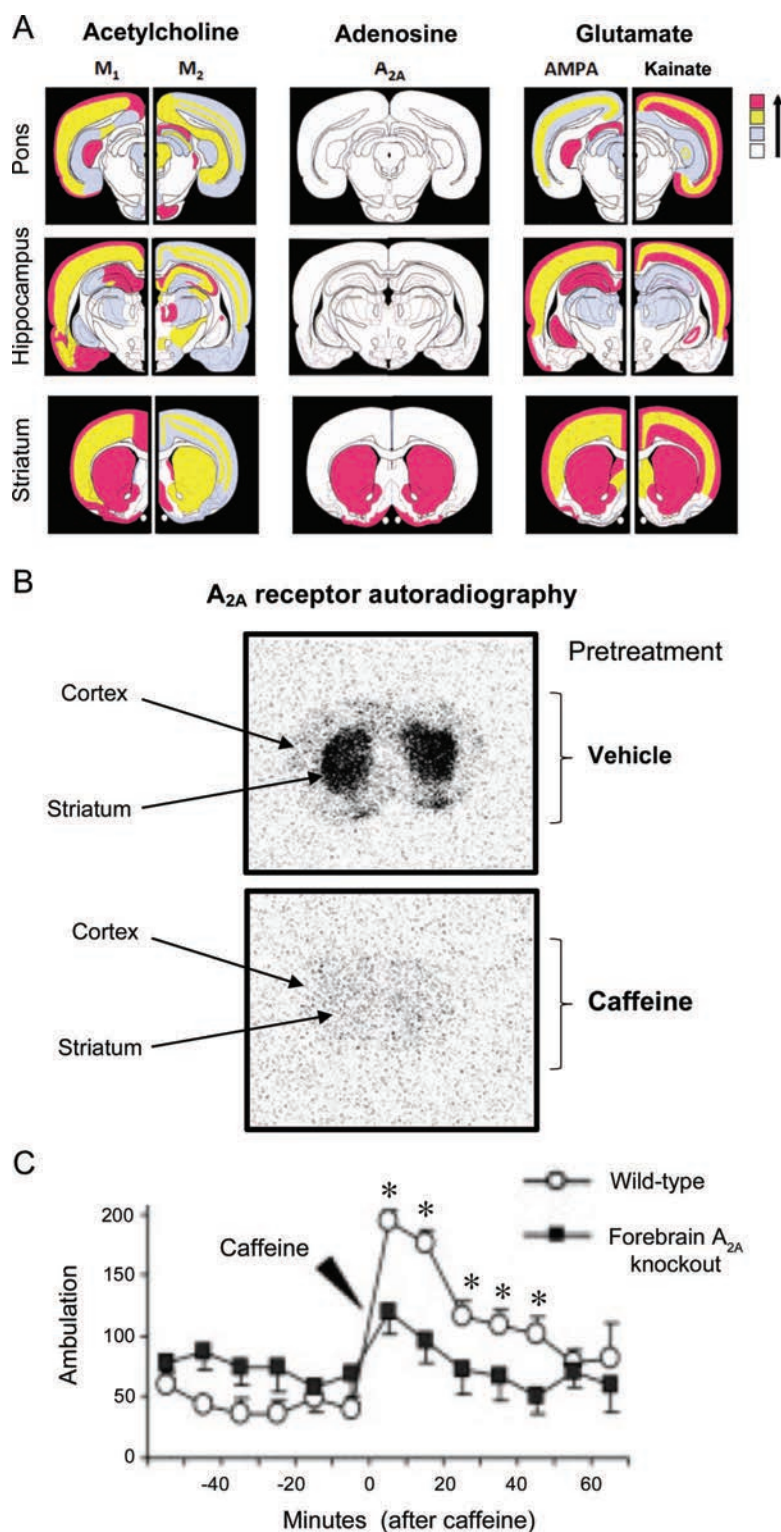
Figure Potential antiparkinsonian actions of caffeine through its blockade of adenosine A_{2A} receptors on striatal neurons

(A) Muscarinic acetylcholine receptors (left) and ionotropic glutamate receptors (right) are targeted by nondopaminergic antiparkinsonian drugs (e.g., trihexyphenidyl and amantadine, respectively). By contrast, the adenosine A_{2A} receptor (center) is discretely expressed in the CNS, primarily in the striatum.[7] (B) Caffeine blocks adenosine receptors including the A_{2A} receptor. A moderate dose of caffeine can markedly displace binding of endogenous adenosine or a radiolabeled A_{2A} receptor ligand.[8] (C) The psychomotor motor stimulant actions of caffeine require adenosine A_{2A} receptors, particularly those expressed on forebrain neurons as demonstrated by attenuated locomotor effects of caffeine in mice with a conditional knockout of neuronal A_{2A} receptors.[9] For details, see cited publications and references therein, from which panels are adapted with permission.

reducing the risk of dyskinesias,[7] while clinically untested, has helped justify a high level of investment in adenosine antagonism for PD.

Nevertheless, the findings of Postuma et al.[5] underscore the longstanding question of whether the greater selectivity for A_{2A} (over A_1 and other adenosine receptor subtypes) offered by adenosine antagonists in commercial development constitutes a clinically meaningful advantage over the relatively nonspecific adenosine antagonism of caffeine. Such benefits should be substantial to offset the unmatchable advantages of caffeine's long-term safety experience and cost. Moreover, as the authors note, their preliminary findings that caffeine improved total UPDRS score by 4–5 points, if substantiated, may be comparable to UPDRS improvements achieved to date with specific A_{2A} antagonists.

There are theoretical disadvantages of caffeine and its greater likelihood for "off-target" effects. For example, caffeine classically produces tolerance to its motor stimulant actions; by contrast, preclinical studies of a specific A_{2A} antagonist failed to demonstrate tolerance to motor stimulant and antiparkinsonian effects.[10] Ultimately, head-to-head comparisons may be required to distinguish the utility of A_{2A}-specific and mixed adenosine receptor antagonists for treating the motor symptoms or other features of PD. For the time being, the results of Postuma et al.[5] should encourage further investigation of a potential antiparkinsonian ("cruise control") benefit of caffeine without entirely discouraging pursuit of its putative alerting ("snooze patrol") action in PD. Although current data do not warrant a recommendation of caffeine as a therapeutic intervention in PD, they can reasonably be taken into consideration when discussing dietary caffeine use.

DISCLOSURE

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Efficient determination of purine metabolites in brain tissue and serum by high-performance liquid chromatography with electrochemical and UV detection

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ABSTRACT: The purine metabolic pathway has been implicated in neurodegeneration and neuroprotection. High-performance liquid chromatography (HPLC) is widely used to determine purines and metabolites. However, methods for analysis of multiple purines in a single analysis have not been standardized, especially in brain tissue. We report the development and validation of a reversed-phase HPLC method combining electrochemical and UV detection after a short gradient run to measure seven purine metabolites (adenosine, guanosine, inosine, guanine, hypoxanthine, xanthine and urate) from the entire purine metabolic pathway. The limit of detection (LoD) for each analyte was determined. The LoD using UV absorption was 0.001 mg/dL for hypoxanthine (Hyp), inosine (Ino), guanosine (Guo) and adenosine (Ado), and those using coulometric electrodes were 0.001 mg/dL for guanine (Gua), 0.0001 mg/dL for urate (UA) and 0.0005 mg/dL for xanthine (Xan). The intra- and inter-day coefficient of variance was generally <8%. Using this method, we determined basal levels of these metabolites in mouse brain and serum, as well as in post-mortem human brain. Peak identities were confirmed by enzyme degradation. Spike recovery was performed to assess accuracy. All recoveries fell within 80–120%. Our HPLC method provides a sensitive, rapid, reproducible and low-cost method for determining multiple purine metabolites in a single analysis in serum and brain specimens. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: HPLC; electrochemical detection; UV–vis detection; biological specimens; purines

Introduction

A growing body of evidence supports an important role of the purine metabolic pathway (Fig. 1) in various neurological disorders including brain injury, Parkinson's disease (PD) and other neurodegenerative diseases (Burnstock, 2008). Adenosine (Ado) is well known to modulate neuronal and synaptic function through its A1 and A2 receptors (Stone, 2005; Schwarzschild et al., 2006). Inosine (Ino) has been shown to be neuroprotective either directly (Irwin et al., 2006) or indirectly through metabolic conversion to downstream metabolites (Gomez and Sitkovsky, 2003). Similarly, guanine (Gua)-based guanosine (Guo) is implicated as a modulator of neural function (Schmidt et al., 2007). Hypoxanthine (Hyp) and xanthine (Xan) have been linked to glutamate-mediated excitotoxicity (Stover et al., 1997) and oxidative stress (Quinlan et al., 1997), and a recent study implicated a potential role of Xan as a biomarker of PD (LeWitt et al., 2011). Remarkably, a convergence of laboratory and epidemiological data has recently identified urate (UA), the enzymatic end product of purine degradation in humans, as a molecular predictor of both risk and progression of PD and as a candidate neuroprotectant for the treatment of PD (Ascherio et al., 2009; Cipriani et al., 2010). Therefore, extensive detection and quantification of the purine degradation pathway metabolites in brain may provide insight into their relevance to different physiological and pathological conditions.

High-performance liquid chromatography (HPLC) has been the prevalent method of measuring nucleotides, nucleosides and major purine bases in different biological samples (Bakay et al., 1978; Nissinen, 1980; Ryba, 1981; Zakaria and Brown, 1981; Iriyama et al., 1984; Wynants and Van Belle, 1985; Smolenski et al., 1990; Liu et al., 1995; Takahashi et al., 2010; Struck et al., 2011). Although many of those HPLC-based protocols are capable of separating and quantifying multiple purines, they often demand a large injection volume and long retention time, and have low throughput and relatively low sensitivity. The ability to measure much of the purine degradation pathway in a

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Abbreviations used: Ado, adenosine; DHBA, 3,4-dihydroxybenzylamine; Gua, guanine; Guo, guanosine; Hyp, hypoxanthine; Ino, inosine; MD, methyl-DOPA sesquihydrate; PD, Parkinson's disease; UA, urate; Xan, xanthine
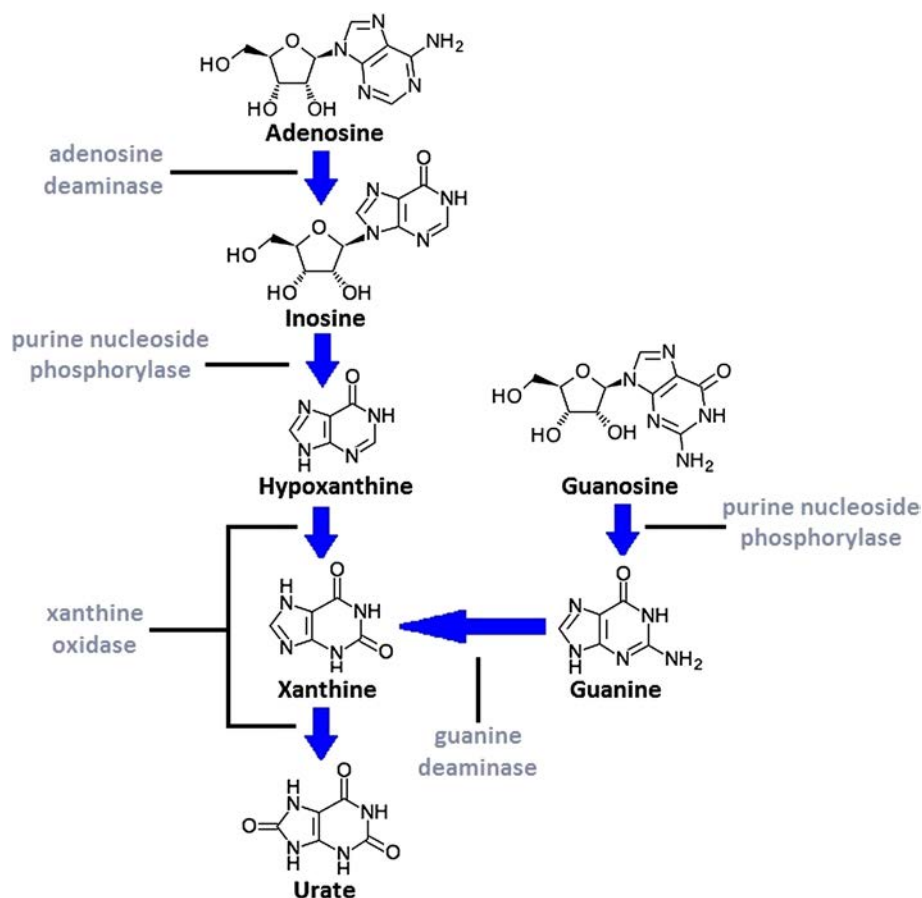
Figure 1. Purine degradation pathway. Adonosine is converted to inosine through the removal of the amine moiety by adenosine deaminase, and inosine is degraded to hypoxanthine through the removal of phospho-1-ribose by purine nucleoside phophorylase. Guanosine is converted to guanine via the action of purine nucleoside phosphorylase, and guanine is then degraded to xanthine through the action of guanine deaminase. In the presence of xanthine oxidase, hypoxanthine and xanthine are converted to urate. Urate constitutes the end product of purine catabolism in humans owing to lack of urate oxidase activity.

single analysis may prove to be a valuable tool in understanding its role in human diseases like PD, as well as in their animal models. A single-run analysis may lead to a better measurement of the purine pathway by eliminating the potential variation inherent in measuring analytes using separate analyses.

Towards this goal, we describe the development of a dual-pump gradient HPLC method using UV and electrochemical detection (ECD). This method achieves suitable separation and sensitivity in a short run time and is capable of measuring seven purine metabolites in tissue and serum with minimal sample preparation and high throughput.

Experimental protocol

Collection of mouse and human tissues

Mice 10 to 12 weeks old C57BL/6 (J) and weighing 29 ± 1.3 g were obtained from Jackson Laboratories (Bar Harbor, ME, USA). They were kept under standard conditions (temperature $21 \pm 2°C$, humidity 30–70%, 12 h light–dark cycle) and with water and standard pellet feed *ad libitum*. Mouse whole blood was collected via a lancet (Goldenrod Animal Lancet, Mineola, NY, USA) puncture of the submandibular vein. The mice were killed via cervical dislocation, and the brain was removed. The

striatum of each hemisphere were collected separately and all tissue samples were frozen on dry ice. Postmortem human brain samples were obtained from the MassGeneral Aging and Disability Resource Center/Harvard NeuroDiscovery Center neuropathology core B repository in accordance with institutional, state and federal regulations, as well as the wishes of the families of donors. Fresh frozen tissue (stored at $-80°C$) samples were collected from 10 male control brains, defined as those without evidence of neurodegenerative disease (such as Parkinson, Huntington or Alzheimer's disease) and with postmortem interval <24 h (mean 19.8 ± 6.0) and age limited to >50 years (mean 82.0 ± 11.2). Tissue samples (~100–200 mg) were dissected on dry ice from striatum. All tissue was kept frozen at $-80°C$ until processed for HPLC analysis.

Instrumentation

The reversed-phase HPLC system comprised two pumps, a model 584 and a model 582 isocratic pump, feeding a high-pressure gradient mixer. Samples were injected using a model 524 autosampler with a 100 μL sample loop and analysis was performed using a model 5600A CoulArray with a 528 UV–vis detector followed by two model 5011A coulometric cells. All equipment was obtained from ESA Biosciences (Chelmsford,

MA, USA). Analyte separation was achieved using a batch-tested Varian Microsorb-MV reversed-phase C_{18} column (150 × 4.6 mm i.d., 5 μm, 100 A; Varian Inc., Palo Alto, CA, USA).

Chemicals and reagents

Acetonitrile (HPLC-grade), potassium phosphate monobasic (HPLC-grade) and EDTA (electrophoresis grade, ≥99%) were supplied by Fisher Chemical (Pittsburgh, PA, USA). Ado, Guo, Gua, Hyp, Ino, UA, Xan and 3,4-dihydroxybenzylamine (DHBA) standards (≥99%) were supplied by Sigma Aldrich (St Louis, MO, USA). Sodium 1-pentanesulfonate (≥99%) and methyl-DOPA sesquihydrate (MD, ≥99%) were obtained from Fluka Analytical (Sigma-Aldrich). Double-distilled water was obtained from a Milli-Q Water System (Millipore, Billerica, MA, USA). All water was subsequently passed through a C_{18} Maxi-Clean cartridge (Alltech, Deerfield, IL, USA) to remove any potential organic contaminants.

HPLC operating parameters

A dual mobile phase gradient was used to achieve appropriate separation of all analytes of interest. Mobile phase A contained 0.52 mM sodium 1-pentanesulfonate and 0.20 M KH_2PO_4 monobasic at pH 3.5 using 85% phosphoric acid (HPLC-Grade, Fisher Scientific, Pittsburgh, PA, USA). Mobile phase B had the same final concentrations as mobile phase A, except for the addition of 10% acetonitrile (v/v). The gradient composition is shown in Fig. 2. The flow rate was 1.0 mL/min, and the system was allowed to equilibrate at that flow rate for 15 min prior to the first sample injection. The sample injection volume was 12 μL.

The detectors were linked in series, with the Model 528 UV–vis light spectroscopy spectrophotometer upstream of both electrochemical cells. UV–vis detection was set to a wavelength of 254 nm. The first electrode was set to −0.10 V and acted as a conditioning cell. The analytical electrodes 1 and 2 were set at +0.15 and +0.45 V, respectively. Data were collected using CoulArray Data Station 3.0 software (ESA Biosciences) with auto-range gain enabled.
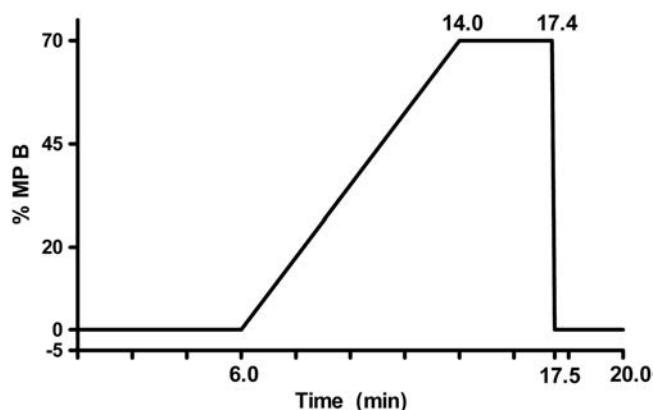

Figure 2. Mobile phase gradient paradigm. Mobile phase A: 0.2 M KH_2PO_4 monobasic, 0.52 mM sodium 1-pentanesulfonate, pH 3.5. Mobile phase B: 0.2 M KH_2PO_4 monobasic, 0.52 mM sodium 1-pentanesulfonate, 10% acetonitrile, pH 3.5. MP: Mobile phase.

Preparation of stock solutions and standards

Individual purine stock solutions were dissolved in double-distilled water that had been filtered through a C_{18} Maxi-Clean cartridge to a final concentration of 1.0 mg/mL except for UA, which was made at a stock concentration of 0.5 mg/mL owing to its solubility. Aliquots of the stocks were stored at −80°C until needed. A working mixed purine standard curve was created by serial dilutions of purine stocks in PE buffer containing 50 mM phosphoric acid, 0.1 mM EDTA, 50 μM MD and 1 μM DHBA (internal standards) from 1.0 mg/mL purine stocks. The working standard curve (except in limit of detection experiments) ranged from 1.0 to 0.001 mg/dL for all purines.

Preparation of mouse and human brain samples for purine analysis

Brain samples were weighed at −60°C and immediately homogenized on ice using a Teflon pestle in 20× volume (v:w) of PE buffer. Extracted samples were then centrifuged at 16,000***g*** for 15 min. The supernatant was then removed and filtered through a 0.22 μm Spin-X Cellulose Acetate filter tube (Corning, NY, USA) at 16,000***g*** for 5 min. Resulting filtrate was stored at −80°C until needed.

Preparation of mouse serum for purine analysis

Whole blood was collected and centrifuged at 16,000***g*** for 15 min. The serum was then transferred and stored at −80°C until needed. Serum deproteination was achieved by the addition of 30 μL of 0.4 M perchloric acid to 50 μL of serum and vortexing briefly. The solution was allowed to incubate on ice for 10 min prior to centrifugation at 1400***g*** for 15 min. The resulting supernatant was removed and added to 20 μL 0.2 M potassium phosphate (pH 4.75) with 1 μM DHBA (internal standard). The resulting solution was filtered through a 0.22 μm Spin-X Cellulose Acetate filter tube at 16,000***g***. The resulting filtrate was stored at −80°C until needed.

Enzyme degradation

To confirm peak identity, enzyme degradation was performed. Mixed purine standards and mouse brain samples were prepared in 0.2 M potassium phosphate monobasic (pH 7.75). Standards and samples were then incubated with the following individual enzymes: adenosine deaminase, purine nucleoside phosphorylase, xanthine oxidase and urate oxidase (all purchased from Sigma-Aldrich, St Louis, MO, USA). Reaction conditions were 25°C overnight for all enzymes, and concentration of each enzyme was predetermined to be sufficient to completely eliminate the target analyte over the overnight incubation period. The resulting mixtures were centrifuged for 15 min at 15,000 rpm, the supernatant was then filtered through a 0.22 μm Spin-X Cellulose Acetate filter tube (Corning, NY, USA) at 16,000***g*** for 5 min. The resulting filtrate was stored at −80°C until needed.

Spike recovery

Spike recovery experiments were performed to validate the accuracy of the method. Purine standards and mouse serum and brain samples were prepared. Baseline values of each analyte per sample were detected. Stock solutions were then

made at 5 times the basal concentrations. Each experiment consisted of a sample control, spike control and spiked sample, all of which were individually made to 60 μL to allow for triplicate runs at 20 μL each. Sample plus mobile phase A (in the amount of the spike) constituted the sample control. The spike control had a specified volume of stock that resulted in 5 times the basal analyte levels plus mobile phase A. The spiked sample included the necessary spike amount of stock and sample. Recovery percentage was calculated by comparing the spiked sample analyte values to the analyte values of the sample control plus spike control levels.

Results and discussion

The main goals of this method were to obtain suitable separation and high sensitivity of seven purine metabolites with a single injection and short run time, allowing for high-throughput analysis of biological samples. This reversed-phase chromatographic method was built upon previous isocratic methods using ECD of UA and Xan (Iriyama *et al.*, 1984; Liu *et al.*, 1995) and underwent optimization of pH and an ion-pairing agent parameters to ensure adequate separation of the analytes of interest. We also took advantage of the differential selectivity of UV and electrochemical detectors for the major purines in biological samples to achieve better signal separation than previously observed.

Determination of electrode potentials and UV–vis wavelength

Hydrodynamic voltammograms were obtained for UA, Xan and Gua to determine the optimum oxidizing potentials for each analyte (Fig. 3). The oxidation of UA increased with greater voltages, reaching a plateau near +0.1 V. A slightly higher potential of +0.15 V (P_1) was chosen to ensure that UA was being fully oxidized, while avoiding oxidation of other similarly retained analytes that might have obscured the UA signal. Oxidation of Gua and Xan reached a plateau at +0.45 V (P_2), at which no co-eluting UA peak interfered with the Gua measurement (data not

Figure 3. Hydrodynamic voltammogram curves for urate, guanine, and xanthine. Measurements were taken using a model 5011A coulometric cell. An analytical potential of +0.15 V (P_1) was selected for urate, and an analytical potential of +0.45 V (P_2) was selected for guanine and xanthine. A conditioning potential of −0.1 V (P_0) was chosen to minimize contaminant peaks.

shown), suggesting that the upstream electrode set at 0.15 V potential had fully oxidized UA. Thus, +0.15 and +0.45 V were chosen as the analytical potentials because they provided full oxidation of the analytes, while avoiding co-oxidation of Gua and UA, which have very similar retention times. A pre-analytical electrode was set to −0.1 V (P_0) to oxidize any potential contaminants that are more easily oxidized than UA. The −0.1 V potential was chosen because more positive potentials partially oxidize UA (Fig. 3), which would weaken the measurable signal at the analytical +0.15 V electrode.

Hyp, Ado, Ino and Guo were detected at a UV wavelength of 254 nm. UA, Xan and Gua were also detectable at this UV wavelength, but electrochemical detection provided a considerably lower limit of detection (LoD; Table 1). This advantage becomes apparent when measuring UA in brain tissue, in which UA concentrations are >5-fold lower than in serum (Cipriani *et al.*, 2010). UA and Gua also have very similar retention times, leading to co-elution and considerably overlapping peaks in UV detection that are easily avoided through electrochemical potential manipulation as described above.

Mobile phase and gradient development

Chromatographic baseline resolution of the analytes of interest was achieved through the manipulation of mobile phase composition and a gradient of organic solvent. The original mobile phase was adapted from Iriyama *et al.* (1984). Determination of the appropriate pH was performed through the measurement of retention times of all the analytes across a range of mobile phase pH values (Table 2). All other components of the mobile phase were kept constant through the pH calibration. pH dependencies of purine retention times were consistent with their respective values of pK_a in the pH range studied. For example, the greatest drop in the retention time of UA (pK_a at 5.4) occurred as pH was increased from 5 to 6, as expected given the increasing likelihood of the anionic urate form, which in contrast to neutral protonated form of urate is not retained on the hydrophobic interaction column. Conversely, Ado (pK_a 3.5) showed a markedly longer retention time as the pH was raised between 3 and 4, consistent with its loss of a proton to become neutral adenosine. Owing to poor separation between UA and Hyp below pH 3, and between Xan and Hyp at the higher pHs tested, a pH of 3.5 was selected for routine use.

After the optimal pH was determined, various concentrations of several ion-pairing agents were introduced into the mobile phase to manipulate retention time and individual peak shape. The retention times produced by the various ion-pairing agents and concentrations are shown in Table 2. It was determined that 0.5 mM 1-pentanesufonate produced the best peak symmetry and baseline separation of the variations.

In an attempt to keep analysis times short and throughput high, a gradient was introduced to elute Ado, Guo and Ino more quickly. Under the isocratic conditions these analytes eluted far later than any of the other analytes of interest. Their late elution led to excessive band spreading, contributing to a considerable loss of sensitivity. By using a gradient, these analytes were eluted sooner and with a sharper peak shape than was possible using an isocratic method (Fig. 4a). Optimization of the gradient percentage organic and ramp times was performed to produce the shortest run time possible with a clear chromatographic
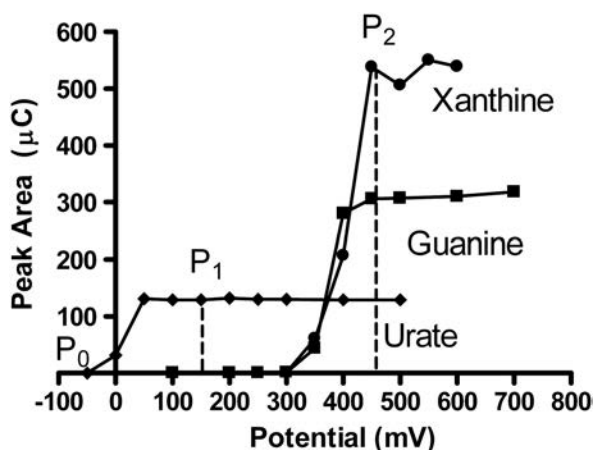
Table 1. Analytical parameters of merit for purine chromatographic peaks

Analytes	Retention time (min)	Method of detection	Limit of detection (mg/dL)	Standard range (mg/dL)	Slope-intercept	R^2
Guanine	4.52	EC (+0.45 V)	0.001	0.001–1.0	$y = 178.05x - 0.558$	0.9999
Urate	4.78	EC (+0.15 V)	0.0001	0.0001–5.0	$y = 131.76x + 0.5998$	1
Hypoxanthine	5.46	UV	0.001	0.001–5.0	$y = 10.928x - 0.0001$	1
Xanthine	6.90	EC (+0.45 V)	0.0005	0.0005–5.0	$y = 38.729x + 1.7033$	0.9977
Inosine	11.10	UV	0.001	0.001–5.0	$y = 7.8419x + 0.0708$	1
Guanosine	11.30	UV	0.001	0.005–1.0	$y = 5.3857x + 0.0048$	1
Adenosine	12.10	UV	0.001	0.001–5.0	$y = 9.9976x + 0.0725$	1
DHBA (IS)	3.38	EC (+0.15 V)	—	—	—	—
MD (IS)	8.76	EC (+0.15 V)	—	—	—	—

DHBA, 3,4-Dihydroxybenzylamine; IS, internal standard; MD, methyl-DOPA

Table 2. Retention times of purine metabolites vs pH and concentration of ion-pairing agent during method development

Chromatographic conditions	Retention time (min)					
pH[a]	Urate	Hypoxanthine	Xanthine	Inosine	Adenosine	MD
2.5	4.0	4.1	4.7	9.1	11.7	—
3.0	3.8	4.1	4.5	9.0	13.3	11.8
4.0	3.8	4.2	4.6	9.0	21.2	5.2
4.5	3.7	4.2	4.6	9.1	24.5	4.7
5.0	3.4	4.2	4.6	9.1	26.1	4.4
6.0	2.7	4.2	4.5	9.1	—	4.3
7.0	2.6	4.1	4.1	8.8	—	4.2
Ion-pairing agent[b]						
0.5 mM 1-Pentanesulfonate	4.3	4.6	5.3	11.8	14.4	—
1.5 mM 1-Pentanesulfonate	3.8	4.0	4.6	9.1	10.8	—
1.5 mM 1-Octanesulfonate	3.9	4.2	4.7	9.8	14.6	—

[a]Retention times with varying pH determined using 1.5 mM 1-pentanesulfonate.
[b]Retention times with varying ion-pairing agents/concentrations determined at pH 3.5.
MD, Methyl-DOPA

baseline resolution of the closely eluting Ado, Ino and Guo peaks, without affecting the resolution of earlier analytes.

Method validation

The validity of the method was assessed through determination of the limit of detection, calculation of the linearity and variation between separate standard curves, calculation of inter-/intra-day coefficient of variation (CV). Additionally peak identity and method accuracy were determined and are discussed together with biological sample results.

The LoD was defined as the lowest concentration of each analyte whose peak height exceeded 3 times the height of the average baseline noise. No analyte detected by UV was measurable below 0.001 mg/dL, while Xan and UA measured by electrochemical detection were measurable at 0.0005 and 0.0001 mg/dL, respectively (Table 1). Standard curves containing all the analytes of interest were then run in triplicate and the mean of these three curves was used to determine variation, the slope–intercept formula, and the R^2 for each analyte (Table 1). All standard curves had very little variation, with the

greatest deviation coming from the Xan curve measured by ECD with an R^2 of 0.998.

The method detection limit (MDL) was also determined for each purine analyte. Eleven sequential runs of freshly prepared 0.005 mg/dL concentration standards were analyzed. The MDL for the Ado values was the highest of the analytes, at 0.0018 mg/dL, with a standard deviation of 0.0006 mg/dL. The MDL for the method is set at that value to ensure that all other analytes can be assayed with at least 99% confidence. Therefore, the limit of quantification (LOQ) of our method is 0.006 mg/dL of analyte, which is 10 times the Ado SD value.

Intra- and interday coefficient of variance percentages (CV) were derived from standard solutions prepared at concentrations of 1.0, 0.1 and 0.005 mg/dL analyte. The mean, standard deviation and CV were calculated (Table 3). The intraday CV experiment was performed by running three standard samples of each concentration at three different time points a day. The intraday variation CV for all analytes was below 10%. Interday CV was assessed by repeating the intraday experiment over the subsequent 2 days, utilizing the same standard solutions and time points as on the first. The first day of CV experiments (intraday
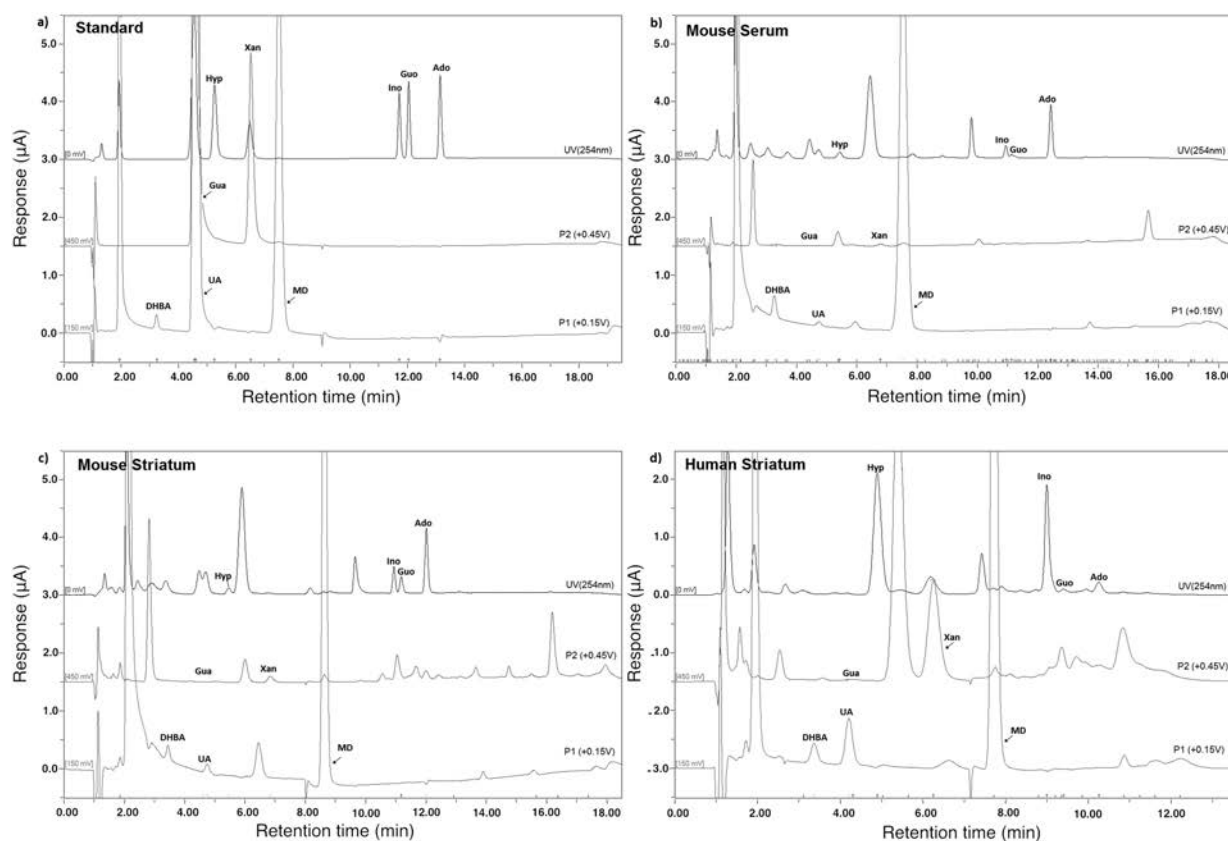
Figure 4. Chromatograms of 1 mg/dL standards mixture (a), mouse serum (b), mouse striatum (c) and human striatum (d). Detection of analytes was performed either by ECD at +0.15 V (P1), +0.45 V (P2), or UV–vis at 254 nm. Gua, Guanine; UA, urate; Hyp, hypoxanthine; Xan, xanthine; Ino: inosine; Guo: guanosine; Ado: adenosine. Internal Standards are 3,4-dihydroxybenzylamine (DHBA, 1 μm) and methyl-DOPA (MD, 50 μm).

assessment) was included in the interday CV calculations, totaling three days of data. Only Xan at 1.0 mg/dL had a CV that exceeded 10%. The increased variation in the Xan measurement is most likely due to variation in the baseline associated with the initiation of the gradient. The gradient begins at approximately the same time that Xan elutes, and causes a small artifact peak that introduces some variability into the Xan measurement that is not present in the measurements of all the other analytes (Table 3).

Measurement of purine metabolites in tissue and serum

The ultimate goal of this method was to obtain sufficient analyte separation and sensitivity to allow measurement of the purine metabolites of interest in mouse and human tissues, including brain and serum. After appropriate separation was achieved with standard mixtures, this method was applied to mouse serum and brain tissue (Fig. 4 b and c).

C57BL/6 mice were killed, and blood was collected. Serum was then analyzed after deproteination with perchloric acid. Mouse brains were extracted, their striatum were collected and purine analysis was performed. The concentrations were determined from the mean of 10 male mice. Except for guanine, all concentrations of metabolites of interest were considerably above their LoD (Table 1), allowing for accurate measurements of each analyte (Table 4). Routine application of this method later to measurement of purines in brain and serum in mice across different experiments has been consistently producing comparable basal level values, allowing direct comparison and data pooling.

This method was then applied to the analysis of post-mortem male human striatum ($n = 10$) processed in a similar manner to the mouse tissue (Table 4). We are aware that more work needs to be done to take postmortem interval into account when analyzing the final values. Nevertheless, the chromatogram of human tissue showed a satisfactory separation and sensitivity (Fig. 4d).

Two strategies were employed to further confirm peaks in biological samples and rule out peak contamination. First, we slightly altered the acetonitrile concentration in both mobile phases to change the analyte retention time in multiple runs of the same sample. Standards and sample analyte retention time changes matched, while analyte concentrations were held constant over different mobile phases (data not shown). Secondly, we performed enzyme degradation studies to eliminate analytes of interest. These studies once again confirmed analyte peaks and negligible underlying contamination. Incubation with urate oxidase, for example, eliminated 91% of urate peak value, and xanthine oxidase eliminated 98% of xanthine and 100% of hypoxanthine peak values.

To further validate the accuracy of our method, spike recovery was performed using mouse serum and brain samples. All recoveries fell within 80–120%. Mouse serum spike recoveries were 80.52% (UA), 103.83% (Xan), 96% (Hyp), 96.51% (Ino), 100.47% (Ado), 118.49% (Gua) and 89.8% (Guo). Mouse striatal spike recoveries were 95% (UA), 99% (Xan), 96% (Hyp), 86% (Ino), 93% (Ado), 84% (Gua) and 99% (Guo).

In conclusion, we have characterized an efficient method of separating seven purine metabolites using HPLC with dual-pump

Table 3. Intra- and inter-day coefficient of variation

Analytes	Intra-day (the area under the peak)						Inter-day (the area under the peak)					
	0.005 mg/dL		0.1 mg/dL		1.0 mg/dL		0.005 mg/dL		0.1 mg/dL		1.0 mg/dL	
	$x \pm$ SD	CV (%)	$x \pm$ SD	CV (%)	$x \pm$ SD	CV (%)	$x \pm$ SD	CV (%)	$x \pm$ SD	CV (%)	$x \pm$ SD	CV (%)
Urate	0.48 ± 0.03	6.5	9.41 ± 0.26	2.7	81.51 ± 0.93	1.1	0.48 ± 0.03	6.5	9.41 ± 0.29	3.0	82.77 ± 3.87	4.7
Xanthine	1.08 ± 0.07	6.6	23.27 ± 0.65	2.8	159.01 ± 1.21	0.8	1.08 ± 0.07	6.6	23.32 ± 0.72	3.1	167.57 ± 25.73	15.4
Hypoxanthine	0.11 ± 0.01	7.4	2.25 ± 0.04	1.6	21.79 ± 0.11	0.5	0.11 ± 0.01	7.4	2.25 ± 0.04	1.7	21.83 ± 0.17	0.8
Inosine	0.07 ± 0	2.9	1.41 ± 0.01	0.8	13.14 ± 0.11	0.9	0.07 ± 0	2.9	1.41 ± 0.01	0.8	13.27 ± 0.41	3.1
Adonosine	0.09 ± 0	3.6	1.93 ± 0.02	1.0	19.36 ± 0.09	0.5	0.09 ± 0	3.6	1.93 ± 0.02	0.8	19.29 ± 0.25	1.3
Guanine	0.73 ± 0.07	9.7	20.13 ± 1.96	9.7	218.89 ± 2.02	0.9	0.73 ± 0.07	9.7	19.53 ± 0.39	2.0	218.44 ± 2.31	1.1
Guonosine	0.07 ± 0	5.3	1.57 ± 0.02	1.1	16.86 ± 0.14	0.8	0.07 ± 0	5.3	1.57 ± 0.01	0.8	16.76 ± 0.33	2.0

CV, Coefficient of variation.

Table 4. Basal levels of purine metabolites in mouse serum and striatum, and human striatum

Tissue	Urate	Xanthine	Hypoxanthine	Inosine	Adenosine	Guanine	Guanosine
Mouse serum (mg/dL)	1.03 ± 0.001	0.32 ± 0.003	0.13 ± 0.004	0.22 ± 0.001	0.03 ± 0.001	$0.003 \pm 4.2 \times 10^{-5}$	0.08 ± 0.002
Mouse striatum (ng/mg wet tissue)	0.46 ± 0.07	1.54 ± 0.19	2.25 ± 0.33	33.98 ± 4.90	190.4 ± 25.63	0.044 ± 0.006	69.62 ± 8.95
Human striatum (ng/mg wet tissue)	7.76 ± 0.89	26.80 ± 2.43	167.9 ± 5.64	89.83 ± 11.18	1.61 ± 0.28	—	—

gradient and quantifying them using a combination of electrochemical and UV detection. This method has been validated to provide satisfactory sensitivity, specificity, accuracy and consistency for measurement in biological samples. The power of this method is its short run time and high sensitivity, both of which allow for high-quality and high-throughput analysis of biologically relevant tissue samples, with minimal variation between runs or days. The value of these technical refinements for neuroscience research is increasing with renewed interest in the neurobiology of purines in health and disease. Future efforts will include method development for measurement of allantoin, a nonezymatic oxidation product of UA and therefore an index of oxidative stress in humans (Marklund *et al.*, 2000; Zitnanová *et al.*, 2004) to advance our ability to assess the role of purine metabolic pathway in neurodegeneration and neuroprotection.

Acknowledgments

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MOVEMENT DISORDERS (SA FACTOR, SECTION EDITOR)

Urate in Parkinson's Disease: More Than a Biomarker?

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Abstract Parkinson's disease (PD) is a progressive neurodegenerative disease with characteristic motor manifestations. Although appreciation of PD as a multisystem disorder has grown, loss of dopaminergic neurons in the substantia nigra remains a pathological and neurochemical hallmark, accounting for the substantial symptomatic benefits of dopamine replacement therapies. However, currently no treatment has been shown to prevent or forestall the progression of the disease in spite of tremendous efforts. Among multiple environmental and genetic factors that have been implicated in the pathogenesis of PD, oxidative stress is proposed to play a critical role. A recent confluence of clinical, epidemiological, and laboratory evidence identified urate, an antioxidant and end product of purine metabolism, as not only a molecular predictor for both reduced risk and favorable progression of PD but also a potential neuroprotectant for the treatment of PD. This review summarizes recent findings on urate in PD and their clinical implications.

Keywords Urate · Parkinson's disease · Oxidative stress · Clinical trial · Antioxidant · Neuroprotectant · Neurodegeneration · Neuroprotection · Biomarker · Disease modifier · Neurodegenerative diseases · Purine · Risk factor

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Introduction

The past two decades have witnessed exciting advances in our understanding of Parkinson's disease (PD), one of the most common neurodegenerative disorders. With the identification and investigation of PD gene mutations, the pathogenesis of PD is beginning to unfold. Among molecular mechanisms that have been proposed to play a key role leading to the degeneration of nigrostriatal dopaminergic pathway, oxidative stress may represent a central common pathway in the complex convergence of genetic and environmental etiologic factors. Dopaminergic neurons in the substantia nigra (SN) pars compacta have high levels of basal oxidative stress likely due to enzymatic and nonenzymatic oxidation of dopamine [1–3]. This process is considered enhanced in PD due to early compensatory changes in dopamine turnover resulting from the initiation of nigral cell degeneration [4]. Furthermore, calcium influx through L-type calcium channels during autonomous pacemaking specific to these neurons impairs mitochondrial function and enhances dopamine synthesis and therefore dopamine oxidation [5, 6]. Oxidative stress intertwines with almost all other mechanisms that have been implicated in PD including protein misfolding and aggregation, mitochondrial dysfunction, cell cycle reactivation, apoptosis, and excitotoxicity [7, 8]. In particular, several PD-linked genes such as α-synuclein, DJ-1, PINK1, and Parkin have been demonstrated to interact with oxidative stress to promote or attenuate reactive oxygen species (ROS) and reactive nitrogen species [6, 7], and these interactions may contribute to the progressive neurodegeneration underlying PD. Markers of oxidative stress and damage, including lipid peroxidation, DNA, and protein oxidation, were found to be present in dopaminergic neurons in the SN of postmortem brain of PD patients [9–11].

Despite the compelling evidence supporting a pathogenic role of oxidative stress [12], agents with antioxidant properties studied to date, including selegiline, vitamin E, rasagiline, and mostly recently coenzyme 10 (CoQ10), have disappointingly failed to show clear benefits as disease-modifying treatment of PD in human trials [13–17]. To learn from the failure of these clinical trials and improve prospects for future tests of candidate neuroprotectants, difficult questions need to be answered [18]. At the far end of the translational pipeline, clinical investigators are asking whether our trial designs rely on the right treatment group structures and outcome measures. Do they test the right doses? In the right subpopulations? For optimal durations? On the near end of the translational pipeline, questions have been raised over the adequacy of our laboratory models of PD neurodegeneration; do they reflect the disease itself? Finally and perhaps most proximal, are we selecting the best drug candidates to enter the pipeline? In this setting, selection from among the many scientifically rational candidate neuroprotectants can be greatly enhanced when convergent epidemiological data are available for PD risk or progression [19–21].

Urate and Antioxidant Defense (Peripheral vs Central Nervous System)

Urate, the anionic form of uric acid, is a potent antioxidant. The antioxidant properties of urate include scavenging singlet oxygen, hydroxyl radicals, hydroxyl peroxide, and peroxynitrite [22, 23]. Urate also interacts and stabilizes other antioxidant systems including superoxide dismutase (SOD), ascorbate, and tetrahydrobiopterin [24–26]. Furthermore, urate displays the ability to chelate iron and block iron-dependent oxidation reactions [27]. Due to a series of mutations in the urate oxidase (UOx) gene and loss of UOx activity during primate evolution, urate in humans circulates, as the end product of purine metabolism, at high concentrations near the limits of its solubility and it accounts for most of the antioxidant capacity in human plasma (Fig. 1) [28]. Thus, urate may serve as one of our major endogenous defenses against oxidative and nitrosative damage. It has long been hypothesized that UOx mutations and the resulting urate elevation may have conferred an evolutionary advantage upon ancestors of higher primates through enhanced antioxidant function, possibly protecting cells from oxidative damage and mutagenesis, although suggestions of reduced cancer risk and longer life span [22, 29] remain speculative. Efforts in humans to directly assess the effects of manipulating urate levels on markers of oxidative stress and damage have produced mixed results [30–32].
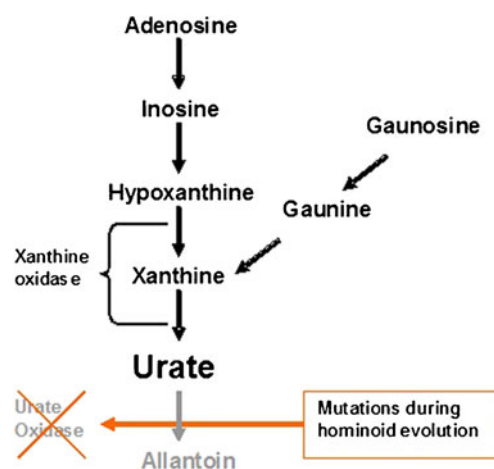

Fig. 1 Purine degradation pathway in humans. Urate is synthesized by xanthine oxidase from its purine precursors. Due to multiple mutations in the urate oxidase gene, urate circulates at high concentrations and it constitutes the end product of purine metabolism in humans

Nevertheless, a putative urate-based antioxidant defense has been proposed to be of particular importance in preventing oxidative damage in the more complex human brain [33]. Despite high blood urate, urate concentration in the central nervous system (CNS) is low, with cerebrospinal flood (CSF) urate consistently about 10 % of its peripheral concentration. This consistent gradient along with the close correlation between serum and CSF urate (despite the gradient) suggests that CNS (or at least CSF) urate concentration is dependent on blood urate and partial integrity of the blood–brain (or at least blood-CSF) barrier. Evidence that human brain has detectable activity of xanthine oxidase [34], the enzyme that catalyzes purine metabolism to urate, challenged the old hypothesis that urate is generated only peripherally in the liver and the small intestine. However, how local production contributes to CNS urate pool and how brain urate might be compartmentalized between neuron and glia, across cell membranes, and among different cellular organelles remains largely unknown. Nevertheless, recognition of the high oxygen consumption and high metabolic demands normally placed on CNS neurons and their particular susceptibility to oxidative damage led to the hypothesis that endogenous urate may serve as a protectant against neurodegenerative diseases [33]. Those with lower plasma urate levels and consequently even lower CNS urate may therefore be predisposed to neurodegenerative disorders such as PD where, as discussed above, oxidative stress is a major pathogenic mechanism [34].

Lower Urate in Patients with Parkinson's Disease

The first line of evidence supporting a link between urate and PD came from a postmortem study reporting reduced

levels of urate in SN from PD patients compared to age-matched controls [35]. Several case–control studies have since then consistently reported lower plasma or serum urate levels in idiopathic PD patients from Spain, Finland, Greece, the United States, and China, compared with their healthy controls [36–40]. Urine and CSF urate have also been studied but no clear differences were found, possibly due to limited sample size [37, 41, 42].

Urate, an Inverse Risk Factor for Parkinson's Disease

In addition to case–control studies, the initial pathological clue that urate is reduced in postmortem SN and striatum of PD patients [35] prompted a parallel series of epidemiological investigations in large prospectively followed populations. These studies consistently demonstrate that higher blood urate conveys a reduced risk for developing PD later in life [43–47]. A prospective study known as the Honolulu Heart Program first reported that among 7,968 men of Japanese or Okinawan ancestry, after adjusting for age and smoking, those with baseline urate concentrations higher than the median had a 40 % reduction in incidence of idiopathic PD during 30 years of follow-up [43]. In a larger prospectively followed cohort of 18,000 mostly Caucasian men, our neuroepidemiology group, led by Alberto Ascherio of the Harvard School of Public Health, found that those in the top quartile for plasma urate had a 55 % lower risk of PD than men in the bottom quartile. The decrease in risk was even greater in those with blood collected at least 4 years before diagnosis, suggesting that the lower urate in those with PD precedes symptom onset and is thus unlikely to be a consequence of changes in diet, behavior, or medical treatment early in the course of the disease [45]. Consistent with these findings, a recent community-based cross-sectional survey involving 69,000 subjects reported that participants with higher urate levels had lower odds of reporting PD with treatment compared to those with lower urate levels, indicating an association between higher urate levels and lower PD prevalence [47]. Another recent study in a community-based cohort demonstrated an association of low urate levels with higher PD risk but not high urate with lower PD risk, suggesting a more complex relationship between blood urate and PD risk in this particular population of older adults (≥65 years old) [48]. Other epidemiological studies also documented a relationship between urate-elevating diet [49] and gout [50, 51] and a lower risk of PD in prospectively followed men. In addition, variation in the urate transporter gene SLC2A9, which has been shown to be related to low serum urate levels, is associated with a lower age at onset of PD. These findings strengthen the link between urate and risk of developing PD [52•].

Interestingly, while robust and highly reproducible in men this inverse association between urate and PD risk is variably observed and weaker in women [46, 47, 53]. Whether the greater association in men reflects a true gender difference in the underlying biology is not clear. Alternatively, it may reflect the fact that men have substantially higher levels of urate than women and that the reduced risk is generally more robust for higher urate levels only above the median urate concentration. Of note, the gender difference in urate cannot explain the gender difference in PD risk (with men at greater risk than women) because the characteristically lower urate levels of women would have suggested that women should be at increased, not decreased, risk. The seeming paradox likely reflects the multiplicity of factors influencing PD risk, with potential factors other than urate (eg, estrogen) predominating in determining the reduced risk among women.

Urate, a Prognostic Biomarker of Favorable Progression in Parkinson's Disease

Remarkably, urate has also been linked to clinical progression of PD. Working with the Parkinson Study Group (PSG) and the Harvard School of Public Health, our group investigated two long-term, rigorously conducted clinical trials known as PRECEPT (Parkinson Research Examination of CEP-1347 Trial) and DATATOP (Deprenyl and Tocopherol Antioxidative Therapy of Parkinsonism), together comprising over 1,600 early cases of PD. We found that higher blood urate is strongly associated with a slower rate of clinical progression in both cohorts [54••, 55]. In the PRECEPT trial, serum urate was measured at a safety laboratory upon enrollment of 806 patients with early PD. The hazard ratio of reaching the primary study end point (ie, the development of disability warranting dopaminergic therapy) over nearly 2 years of follow-up declined with increasing serum urate. Similarly, the rate of Unified Parkinson's Disease Rating Scale (UPDRS) score worsening, a secondary end point in the PRECEPT study, was significantly higher in patients with lower urate levels [55]. The predictive association between higher urate at baseline and slower clinical progression in this cohort could be partially explained by the higher representation of subjects with a brain scan without evidence of dopaminergic deficit (SWEDD) among those with higher urate levels [56]. However, the inverse association between urate and clinical progression remained strong even after excluding all subjects with a baseline SWEDD from a secondary analysis (Unpublished data, by our and Dr. Alberto Ascherio's group with PSG investigators). Analysis of the independent 800-subject cohort of the DATATOP trial substantiated the inverse relationship between serum urate levels and subsequent disability progression early in PD as measured with the same primary outcome [54••].

Moreover, a similar robust inverse association was observed between baseline urate level and loss of striatal [123I] β-CIT uptake, a marker for the presynaptic dopamine terminal transporter [55]. Overall, the mean change among patients in the top quintile of serum urate was significantly lower than that of patients in the bottom quintile. Taking advantage of available stored CSF samples collected at baseline from 713 subjects of the DATATOP study, we measured CSF urate concentrations and found that like serum urate, lower CSF urate levels also predicted a slower rate of clinical disease progression in PD [54••]. The association of serum urate with the progression of PD has also been recently suggested in Chinese patients. Lower serum urate was found in patients with higher Hoehn and Yahr (H&Y) stages [40]. Furthermore, urate appears to be related to cognitive dysfunction, with higher urate predicting favorable neuropsychological performance in PD patients [57–59].

Urate and Links to Other Neurodegenerative Diseases

Consistent with the known antioxidant properties of urate and a common pathogenic mechanism of oxidative stress in neurodegeneration, increasing lines of evidence suggest relevance of urate to neurodegenerative diseases other than PD. A prospective population-based cohort study reported an inverse correlation between urate and cognitive function and risk of dementia later in life [60]. Reduced urate levels have also been implicated in Lewy body disease [61], Alzheimer's disease (AD) [62–64], Huntington's disease (HD) [65], and amyotrophic lateral sclerosis (ALS) [66, 67]. Furthermore, higher serum urate has been demonstrated to predict slower progression of HD [68], prolonged survival of ALS [69], and possibly lower rates of conversion to AD in untreated mild cognitive impairment [70]. These studies suggest that urate may play a general role across neurodegenerative disorders.

Neuroprotective Actions of Urate

Despite their statistical strength and reproducibility, these clinical and epidemiological findings do not settle the critical issue of whether urate is a primary pathogenic factor or a secondary disease marker. Although the question of causality is difficult to answer in humans without controlled clinical trials, evidence from experimental studies has to date provided valuable clues. For example, administration of urate is neuroprotective in both mechanical models of brain ischemia and thromboembolic models of autologous clot injection [71]. Urate not only reduced infarct volume in various models of ischemic brain injury, but also reduced ischemia-induced tyrosine nitration [72, 73]. In addition, inosine, a urate precursor,

was also shown to have a beneficial effect in stroke models, perhaps by inducing axonal rewiring and improving behavioral performance as well as by reducing cerebral infarction volume [74, 75]. In an experimental allergic encephalomyelitis model of multiple sclerosis, urate and inosine were found to delay the onset and improve the clinical symptoms of disease in mice [76–78], possibly through inhibiting peroxynitrite-mediated oxidation [77]. Urate has also been shown to protect embryonic rat spinal cord neuron cultures against glutamate toxicity [79], and it protected against secondary damage including general tissue damage, nitrotyrosine formation, lipid peroxidation, activation of poly(ADP-ribose) polymerase, and improved functional recovery after spinal cord injury in vivo [80]. Treatment with urate increased total glutathione (GSH) production in hippocampal CA1 pyramidal neurons in the slice culture, and it protected these cells from oxidant insult. The same study further demonstrated in vivo increased GSH synthesis after injection of uric acid intraperitoneally in mice [81]. Results from human studies have demonstrated that systemic administration of uric acid was not only safe in healthy volunteers but also increased their serum antioxidant capacity [31]. Administration of urate, which falls quickly in patients with acute stroke, has been shown to lessen several biomarkers of oxidative stress and provide neuroprotection synergistically with thrombolytic therapies [32].

Regarding PD models, it is reported that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment induced an increase in urate levels in mice [82], and in rats, which was antagonized by allopurinol, a xanthine oxidase inhibitor [83]. Increased extracellular urate levels were also found after acute infusion of N-methyl-4-phenylpyridinium (MPP+), 6-hydroxydopamine (6-OHDA), or iron chloride through microdialysis in the SN of guinea pigs [84]. Increased urate may reflect a higher oxidative metabolism rate in CNS, or a compensatory protective mechanism after insult, because older animals failed to boost urate in brain after brain injury [85]. Data on urate in PD models are relatively scarce considering the centrality of oxidative mechanisms in PD models. Nevertheless, protective effects of urate have indeed been demonstrated in cellular and animal models of PD. In differentiated PC12 cells, urate was shown to block dopamine-induced apoptotic cell death and oxidant production [86]. Also in PC12 cells, treatment with urate protected against 6-OHDA toxicity. It significantly reduced 6-OHDA–induced oxidative stress, malondialdehyde formation, 8-hydroxy-deoxyguanosine (8-OHdG) generation, and lactate dehydrogenase release, and it induced SOD activity and increased GSH levels [87]. In human neuroblastoma SK-N-MC cells, urate prevented death of the cells induced by rotenone or iron plus homocysteine, an agent that sensitizes dopaminergic neurons to environmental toxins both in vitro and in vivo. It completely suppressed oxidative stress and largely prevented membrane

depolarization in those cells exposed to homocysteine plus rotenone or iron [88]. Primary cultures of the ventral mesencephalon dopaminergic neurons undergo spontaneous degeneration in vitro, and urate exerted robust, long-term protection of these cells. Urate protected the cells by reducing intracellular ROS production. This protective effect was reproduced by iron-chelating agent desferrioxamine, H_2O_2 scavenger enzyme catalase, and lipid peroxidation inhibitor of Trolox (Hoffman-LaRoche, Piscataway Township, NJ), suggesting that urate prevented neurodegenerative changes induced by Fenton-type reactions in midbrain primary cell cultures. Furthermore, urate-mediated neuroprotection in these primary cells was enhanced substantially by high K^+-induced depolarization through a mechanism involving L-type Ca^{2+} elevation and subsequently extracellular signal-regulated kinases 1/2 activation [89]. In a 6-OHDA rat model of PD, injection of urate significantly improved related behavioral responses and dopamine depletion. This systemic injection paradigm was shown to elevate urate in the striatum [90]. By employing complementary pharmacological and genetic approaches, our group has demonstrated in preliminary studies that urate protects dopaminergic neurons in cellular and in vivo models of PD [91–93].

Urate Elevation as a Therapeutic Strategy for Parkinson's Disease

The clinical and epidemiological findings that urate levels are inversely correlated with development and progression of PD, in conjunction with experimental evidence that urate is neuroprotective, provided a solid foundation to support testing of urate as a potential neuroprotective treatment of PD. In pursuit of a rapid clinical translation of these convergent findings, we have launched a national clinical trial to assess therapeutic candidacy of inosine, an orally bioavailable, brain-penetrant urate precursor. The phase II placebo-controlled double-blind dose-ranging randomized trial known as SURE-PD (Safety and Ability to Elevate Urate in Early Parkinson Disease) is being conducted in individuals with early PD at 16 clinical sites. Tolerability, defined as the extent to which an assigned treatment can be continued without dose reduction for more than 4 weeks due to adverse experience(s), and safety, defined as absence of serious adverse experiences that collectively warrant terminating an inosine treatment dose or the trial, are being assessed in analyses of short-term (12-week) and long-term (up to 2-year) treatment periods. Levels of urate in blood and CSF and oxidative damage biomarkers will also be determined as secondary outcome measures. Results from this trial will determine whether and how to proceed with a larger phase III trial of inosine as a urate-elevating strategy to modify disease progression in PD [94].

High Urate and Health Risks

In the above-mentioned Honolulu Heart Program, men in the top quartile of serum urate had a lower risk of developing PD than those with lower urate concentrations. By contrast, after adjusting for age, the total mortality rate in this quartile was about 30 % higher than men in the bottom quartile, although the risk was reduced after further adjustment for several risk factors for major chronic disease [43]. Of note, in people with PD higher urate has not been linked to increased mortality and in men with PD it actually appeared to be a predictor of reduced mortality [54••]. Nevertheless, elevated urate clearly is a pathogenic factor in diseases of urate or uric acid crystallization such as gout and uric acid urolithiasis, and is positively correlated with many other conditions such as hypertension, cardiovascular disease, and metabolic syndrome [95, 96]. Therefore, a responsible question is whether potential neuroprotective benefits of elevating urate for PD outweigh expected and theoretical medical risks for individual patients. In the SURE-PD trial, known risks for gouty arthritis and uric acid kidney stones and possible risks for blood pressure elevation and other medical conditions will be carefully assessed. It is also recommended that clinicians and PD patients not attempt to raise urate to treat PD before a better understanding of the role of urate in PD and the benefit-risk ratio of elevating urate is achieved.

Conclusions and Future Directions

A convergence of clinical and epidemiological data has identified urate as a molecular predictor of both (reduced) risk and (slowed) progression of idiopathic PD. Evolutionary and laboratory evidence further support a role of urate as an antioxidant and neuroprotectant in the pathogenesis of neurodegeneration in PD. Collectively these findings have facilitated rapid translation to a phase II clinical trial of the urate precursor inosine as a potential neuroprotective strategy for PD. They are also stimulating mechanistic investigation and insight into our understanding of urate and neurodegeneration, an area that remains largely uncharted. Given the complex nature of PD and its heterogeneous genetic and environmental influences, it is unlikely that urate on its own is a sufficiently specific biomarker of PD outcomes to warrant clinical application as a prognostic test. However, it would be reasonable to expect urate will contribute to a composite prognostic biomarker of disease risk or progress (akin to a multifactorial "cardiac index" as is commonly employed clinically to predict heart disease risk [97]) in combination with other emerging predictive factors. More immediate is the application of serum urate's prognostic biomarker properties to the improvement of clinical

trial design and analysis [98]. For example, a clinical trial of another candidate neuroprotectant may require substantially fewer PD subjects to achieve statistical significance for a true disease-modifying benefit if calculated progression rates were adjusted for baseline serum urate in the study analysis. Urate is emerging as a novel biomarker for PD risk, diagnosis, and prognosis [99]. Whether higher urate concentration will be found to be more than a biomarker of favorable outcomes for PD and other neurodegenerative diseases remains to be determined. Specifically, whether it offers disease-modifying strategy for the treatment of PD is being investigated and could be elucidated in the years to come.

Future studies will need to focus on some of the as-yet unaddressed important issues regarding urate and PD. First, our knowledge about basic purine biology, particularly how urate homeostasis is regulated and how urate may work as an endogenous antioxidant and neuroprotectant in CNS is limited. Second, evidence that urate is linked to PD came from studies among men or both genders. Studies to date reported either absence of or uncertainty over a relationship between urate and PD risk and progression in women [46, 47, 53, 54••]. Whether urate is associated with PD in women is less clear. Third, the association between urate and PD has thus far been characterized primarily in blood. Exploring urate in other body fluids (especially CSF and urine) and correlations between these compartments will not only improve our understanding of urate regulation in the CNS versus the periphery, but will also refine the clinical application of urate as a biomarker for PD.

Furthermore, unlike the proverbial path from preclinical discovery to clinical development of a neuroprotective agent, the urate story has unfolded mostly through human studies. Given the fundamental genetic and metabolic differences in urate biology between humans and lower mammalian animals, caution should be taken when interpreting and translating results from animal studies. However, complementing laboratory studies will still be necessary and valuable for characterization of the neuroprotective actions of urate and the underlying mechanisms in toxin and genetic models of PD. Finally, despite compelling evidence for causality, possible alternative explanations for the urate-PD association such as a purine pathway metabolite upstream of urate or another urate determinant serving as a pathogenic factor for which urate is merely a marker, deserve further investigation.

Disclosure Conflicts of interest: X. Chen: is employed by Massachusetts General Hospital; G. Wu: is employed by Suzhou Municipal Hospital; M.A. Schwarzschild: has been a consultant for Harvard University; is employed by Massachusetts General Hospital; has received payment for lectures including service on speakers bureaus from Emory University; has received=travel/accommodations/meeting expenses unrelated to activities listed from Emory University, Columbia University.

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Urate and Its Transgenic Depletion Modulate Neuronal Vulnerability in a Cellular Model of Parkinson's Disease

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Abstract

Urate is a major antioxidant as well as the enzymatic end product of purine metabolism in humans. Higher levels correlate with a reduced risk of developing Parkinson's disease (PD) and with a slower rate of PD progression. In this study we investigated the effects of modulating intracellular urate concentration on 1-methyl-4-phenyl-pyridinium (MPP^+)-induced degeneration of dopaminergic neurons in cultures of mouse ventral mesencephalon prepared to contain low (neuron-enriched cultures) or high (neuron-glial cultures) percentage of astrocytes. Urate, added to the cultures 24 hours before and during treatment with MPP^+, attenuated the loss of dopaminergic neurons in neuron-enriched cultures and fully prevented their loss and atrophy in neuron-astrocyte cultures. *Exogenous* urate was found to increase intracellular urate content in cortical neuronal cultures. To assess the effect of reducing cellular urate content on MPP^+-induced toxicity, mesencephalic neurons were prepared from mice over-expressing urate oxidase (UOx). Transgenic *UOx* expression decreased *endogenous* urate content both in neurons and astrocytes. Dopaminergic neurons expressing UOx were more susceptible to MPP^+ in mesencephalic neuron-enriched cultures and to a greater extent in mesencephalic neuron-astrocyte cultures. Our findings correlate intracellular urate content in dopaminergic neurons with their toxin resistance in a cellular model of PD and suggest a facilitative role for astrocytes in the neuroprotective effect of urate.

Introduction

Urate (2,6,8-trioxy-purine; a.k.a. uric acid) is generated within cells from the breakdown of purines. In most mammals urate is converted to allantoin by uricase (urate oxidase; UOx) [1], an enzyme primarily expressed in the liver [2]. In humans and apes, uricase is not synthesized due to the sequential non-sense mutations of its gene (*UOx*) that occurred during hominoid evolution [3–5]. Thus, in humans urate is the end product of the purine catabolism and achieves concentrations approaching the limit of solubility, which are more than fifty times higher than those in other mammals [6]. Due its high levels and radical scavenging properties [7–9] urate is considered a major antioxidant circulating in humans. It may have played a facilitative role in human evolution as was initially proposed based on putative central nervous system benefits [10–12] and later based on its antioxidant properties – perhaps to have partially compensated for the lost of the capability of synthesizing ascorbate [7,13]. Urate's antioxidant proprieties have been extensively characterized *in vitro* where it was found to be a peroxynitrite scavenger [14] and to form stable complex with iron ions, reducing their oxidant potential [15].

Identification of these antioxidant proprieties of urate, together with evidence that oxidative damage plays a critical role in the neurodegeneration of PD, raises the possibility that urate may protect from the development of the disease. Prompted further by post-mortem evidence that the urate levels in midbrain and striatum of PD patients are reduced compared to those of control brains [16], epidemiological and clinical cohorts were investigated for a possible link between urate level and the risk of PD or the rate of its progression. Several studies found lower blood urate concentration in healthy individuals to be a reproducible risk factor for developing PD later in life [17–19]. Furthermore, among those already diagnosed with PD, lower serum levels were consistently associated with a more rapid clinical and radiographic progression of PD [20–22], suggesting urate may be a prognostic biomarker in PD. In addition, an inverse correlation between serum urate level and disease duration has been reported in PD and raises the possibility that urate may also be a marker of disease stage [23], though falling urate may simply reflect the weight loss that accompanies disease duration.

A causal basis for the link between urate and favorable outcomes in PD is supported by the neuroprotective properties of urate in models of PD. Presumably by reducing ROS levels, urate can prevent cellular damage and increase cell viability in *in vitro* models of toxicant-induced or spontaneous cell death [24–27]. Moreover, urate increased cell survival in MPP^+-treated cell cultures [28] and prevented dopaminergic neuron loss in a rodent model of PD [29].

MPP^+ (1-methyl-4-phenylpyridinium) is the toxic metabolite of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) [30], an agent shown to induce a parkinsonian condition in humans [31]. MPP^+ is generated in astrocytes and up-taken by dopamine transporter into dopaminergic neurons [32]. Within the cells,

MPP$^+$ can induce the irreversible inhibition of complex I activity, failure of ATP synthesis and cell death [33,34]. In this study we assessed whether modulating urate level in primary dopaminergic neurons affects their vulnerability to MPP$^+$ toxicity in the presence of a low or high percentage of astrocytes.

Results

Urate prevents dopaminergic neuron loss in MPP$^+$-treated cultures

To identify an MPP$^+$ concentration with selective toxicity for dopaminergic neurons, mesencephalic neuron-enriched cultures (Fig. 1A–D) were treated for 24 hours with increasing concentrations of MPP$^+$. Toxicant treatment reduced the number of dopaminergic neurons, which were identified by their immuno-reactivity for tyrosine hydroxylase (TH), in a concentration-dependent manner ($P<0.0001$). There was no change in the total number of neurons, which were scored as microtubule-associated protein 2-immunoreactive (MAP-2-IR) cells (Fig. 2A), due to the selectively toxic effect of MPP$^+$ on dopaminergic neurons and their low number in ventral mesencephalon cultures (2–3% of MAP-2-IR cells; see also Materials and Methods). To assess the effect of urate on dopaminergic neuron viability, neuron-enriched cultures were pretreated with urate 24 hours before and during exposure to 3 µM MPP$^+$. In MPP$^+$-treated cultures urate increased TH-IR viability over a concentration range of 0.1–100 µM ($P<0.0001$). The maximum effect was achieved at 100 µM with a 51% increase in TH-IR cell number in comparison to cells treated with MPP$^+$ only ($P<0.01$). Half-maximally effective concentration (EC$_{50}$) was achieved at a concentration of 1 µM [95% confidence interval (95%CI): 0.096–5.9] (Fig. 2B, D–G). Urate on its own produced no significant effect on dopaminergic neuron viability (Fig. 2C).

Previous data [35] have shown that urate's protective effect against toxin-induced neuronal cell death can be dependent on the presence of astrocytes in cultures. In our study urate treatment in neuron-enriched cultures only partially attenuated MPP$^+$ toxicity

on dopaminergic neurons. To assess whether astrocytes might potentiate the protective effect of urate in our cells, urate was tested in MPP$^+$-treated mixed neuron-astrocyte cultures (Fig. 3A–D). To obtain selective degeneration of dopaminergic neurons without toxic effect on non-TH-IR cells, cultures were treated with relatively low concentrations of MPP$^+$ for four days as previously described [36]. MPP$^+$ induced selective loss of TH-IR neurons in a concentration-dependent manner ($P=0.0005$) with no statistically significant effect on MAP-2-IR or glial fibrillary acid protein-immunoreactive (GFAP-IR) cells (Fig. 4A). To assess the effect of urate, neuron-astrocyte cultures were pretreated with urate 24 hours before and during exposure to 0.5 µM MPP$^+$. Urate increased the number of TH-IR neurons over a concentration range of 0.1–100 µM ($P<0.0001$). The maximum effect was seen at 100 µM with a 97% increase in the number of TH-IR neurons in comparison to cultures treated with MPP$^+$ only ($P<0.01$; Fig. 4B, F–I), corresponding to a complete blockade of MPP$^+$ toxicity. Urate on its own did not affect TH-IR cell number (Fig. 4C). No statistically significant difference was seen at the estimated EC$_{50}$'s for urate in neuron-enriched and neuron-astrocytes cultures ($\sim$1 µM in both; $F_{1,53}=0.01$, $P=0.9$).

Urate prevents MPP$^+$-induced atrophic changes in dopaminergic neurons

To assess whether the protective effect of urate on neuronal viability correlates with an improvement in toxin-induced cellular atrophy, neurite length and soma size were analyzed in neuron-astrocyte cultures. In MPP$^+$-treated cultures TH-IR cells showed shorter neurites (-32%, $P<0.01$) and smaller soma area (-20%, $P<0.001$) in comparison to control cells (Fig. 4D and E, respectively). The concentration that fully protected against dopaminergic neuron loss, 100 µM urate, prevented the decrease in neurite length ($P<0.01$) and soma size ($P<0.001$) in TH-IR neurons (Fig. 4D–E).

Exogenous urate raises its intracellular level

To assess whether urate's protective effects are associated with an increase in its intracellular content, neuron-enriched cultures were treated with exogenous urate for 0, 6 and 24 hours. In order to obtain the large number of neurons required for intracellular analyte measurements, cultures were prepared from the mouse cortex for this assay. Urate content in neurons increased in a time-dependent manner with about 4 fold increase at 24 hours of treatment ($P=0.002$) (Fig. 5A), the time at which MPP$^+$ would be added to the cultures. Exogenous urate did not affect the concentration of any measured urate precursor (adenosine, inosine, hypoxanthine and xanthine) within neurons (unpublished data). Similar results were obtained in astrocyte-enriched cultures (unpublished data).

Transgenic UOx expression lowers intracellular urate

To assess whether intracellular urate content affects dopaminergic neuron resistance to MPP$^+$ we prepared ventral mesencephalon cultures from a mouse line expressing transgenic uricase (UOx) [37], the enzyme that converts urate to allantoin. Intracellular urate content was measured in cortical neurons and astrocytes prepared from non-transgenic UOx (non-Tg), hemizygous transgenic UOx (Tg) and homozygous (double) transgenic UOx (Tg/Tg) mice. In Tg/Tg neurons UOx expression was about 6 times higher than in Tg neurons as assessed by western blotting; in non-Tg neurons UOx was not detected (Fig. 6A). UOx expression reduced intracellular urate content by 50% ($P<0.01$) and 60% ($P<0.01$) in Tg and Tg/Tg neurons, respectively
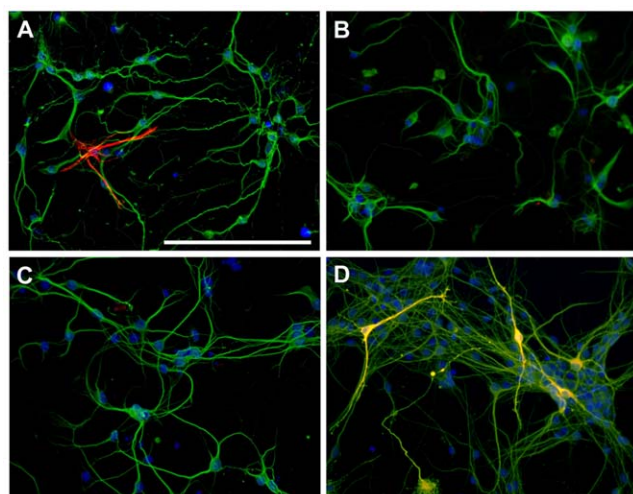

Figure 1. Cellular composition of neuron-enriched cultures. Composite fluorescence photomicrographs of neuron-enriched cultures that were immuno-stained with A–D) the neuronal marker MAP-2 (green) together with A) astrocyte marker GFAP (red) or B) the microglia marker CD11b (red, not detected) or C) the oligondendrocyte marker CNPase (red, not detected) or D) the dopaminergic neuron marker TH (yellow). Nuclei were counterstained with DAPI; scale bar length represents 100 µm.
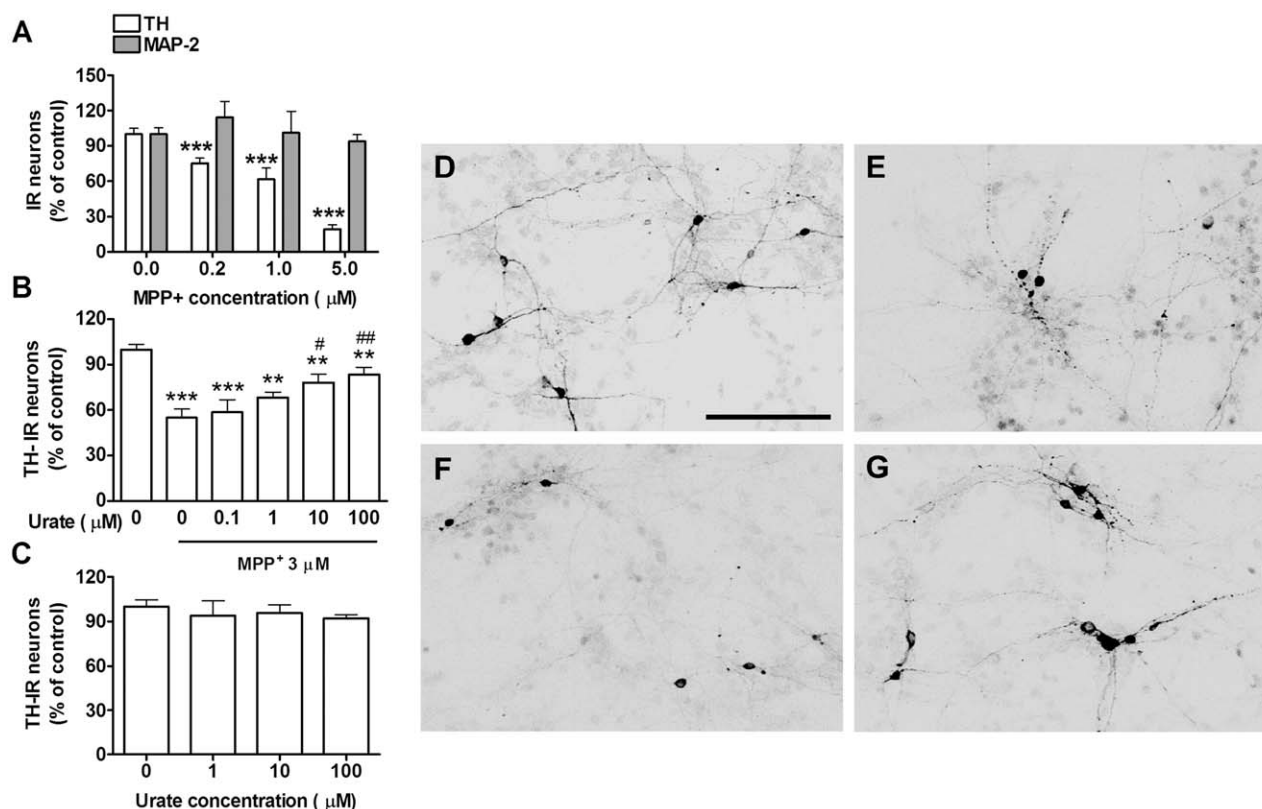doi:10.1371/journal.pone.0037331.g001

Figure 2. Urate's protective effect on dopaminergic neurons in neuron-enriched cultures. A) MPP$^+$ concentration-dependent effect on dopaminergic and total neuron viability expressed respectively as percentage of TH- and MAP-2-IR cell number in comparison to control cultures (n = 5). B) Urate concentration-dependent effect on TH-IR cell number in 3 μM MPP$^+$-treated cultures (n = 7). C) Lack of urate effect at any concentration on TH-IR neuron number in control (MPP$^+$-untreated) cultures (n = 5). Photomicrographs show TH-IR neurons in D) control cultures, E) MPP$^+$/0 urate-treated cultures, F) MPP$^+$/0.1 urate-treated cultures and G) MPP$^+$/100 μM urate-treated cultures. Scale bar = 50 μm. One-way ANOVA followed by Newman-Keuls test: **$P<0.01$, ***$P<0.001$ vs 0 MPP$^+$ value; #$P<0.05$, ##$P<0.01$ vs MPP$^+$/0 urate value.
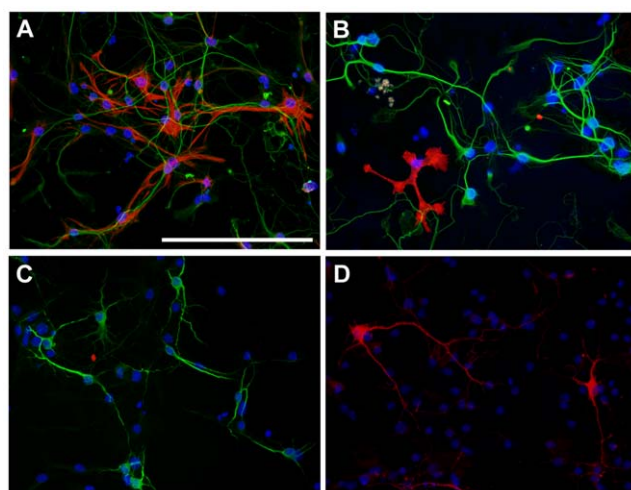doi:10.1371/journal.pone.0037331.g002

Figure 3. Cellular composition of neuron-astrocyte cultures. Composite fluorescence photomicrographs of neuron-astrocyte cultures that were immuno-stained with A–C) the neuronal marker MAP-2 (green) together with A) astrocyte marker GFAP (red) or B) the microglia marker CD11b (red) or C) the oligondendrocyte marker CNPase (red, not detected). D) Dopaminergic neurons were stained with the dopaminergic neuron marker TH (red). Nuclei were counterstained with DAPI; scale bar is 100 μm.
doi:10.1371/journal.pone.0037331.g003

(Fig. 6B). UOx activity was significantly increased in Tg/Tg (p<0.001) but not in Tg cell medium in comparison to non-Tg samples (Fig. 6C). In Tg/Tg astrocytes intracellular UOx expression was about 15 times higher than in Tg astrocytes; in non-Tg astrocytes UOx was not detected (Fig. 7A). UOx expression reduced intracellular urate content by 30% both in Tg and Tg/Tg (P<0.01) astrocytes (Fig. 7B). UOx activity was detected in the cell media of Tg and Tg/Tg astrocytes (Fig. 7C) where medium urate concentration was significantly reduced in comparison to that from non-Tg astrocytes (P<0.001) (Fig. 7D).

Transgenic UOx reduces neuronal resistance to MPP$^+$ toxicity

To determine whether the enzymatic reduction of intracellular urate exacerbates dopaminergic susceptibility to MPP$^+$, neuron-enriched ventral mesencephalon cultures from non-Tg, Tg and Tg/Tg mice were treated with increasing concentrations of toxin for 24 hours. Two-way ANOVA showed that both genotype ($F_{2,232} = 24.61$, $P<0.0001$) and MPP$^+$ concentration ($F_{2,232} = 312.64$, $P<0.0001$) affected the number of TH-IR neurons, and found significant interaction between these two factors ($F_{2,232} = 13.82$, $P<0.0001$). Dopaminergic viability was reduced in UOx expressing cultures in comparison to non-Tg cultures with a maximum effect at 1 μM MPP$^+$, which further reduced TH-IR neuron number by 10% and 18% in Tg and Tg/Tg cultures compared to non-Tg cultures, respectively (Fig. 8A).
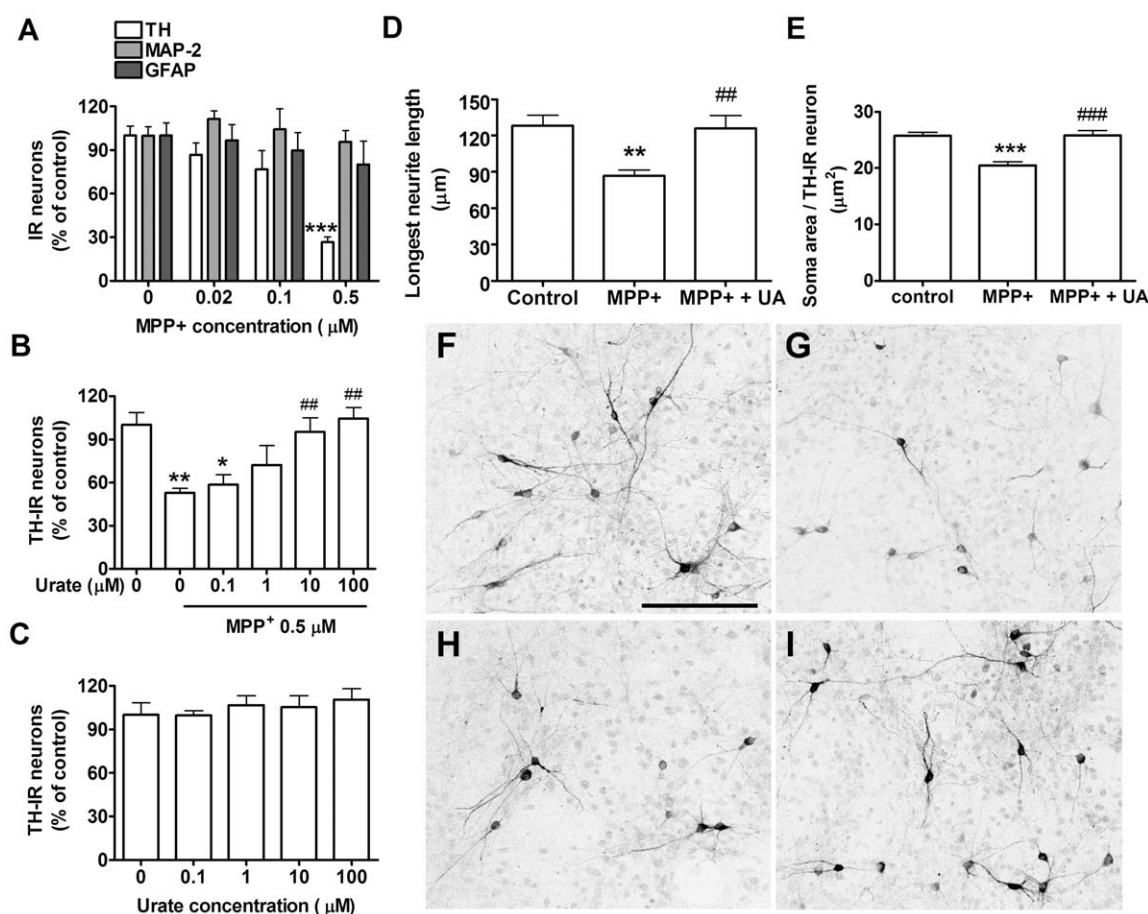
Figure 4. Urate's protective effect on dopaminergic neurons in mixed cultures. A) MPP$^+$ concentration-dependent effect on dopaminergic neuron, total neuron and astrocyte viability, expressed as percentage of TH-IR, MAP-2-IR and GFAP-IR cell number, respectively, in comparison to control cultures (n = 4). B) Urate concentration-dependent effect on TH-IR cell number in 0.5 μM MPP$^+$-treated cultures (n = 5). C) Lack of effect of urate at any concentration on TH-IR cell number (n = 5). Urate (100 μM) effects on reductions in D) longest neurite length and E) soma size in MPP$^+$ urate-treated TH-IR neurons. Photomicrographs show TH-IR neurons in F) control cultures, G) MPP$^+$/0 urate-treated cultures and H) MPP$^+$/0.1 urate-treated cultures and I) MPP$^+$/100 μM urate-treated cultures. Scale bar = 50 μm. One-way ANOVA followed by Newman-Keuls test: *$P<0.05$, **$p<0.01$, ***$P<0.001$ vs 0 MPP$^+$ value, $^{##}P<0.01$ and $^{###}P<0.001$ vs MPP$^+$/0 urate value.
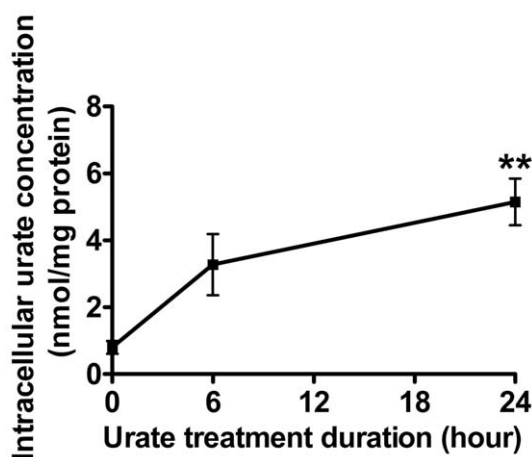doi:10.1371/journal.pone.0037331.g004

Figure 5. Urate accumulation in cortical neurons. A) Time-dependent effect of 100 μM *exogenous* urate on its intracellular content in primary cortical neurons. One-way ANOVA followed by Newman-Keuls test: **$P<0.01$.
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The EC$_{50}$ for MPP$^+$ was 5.2 μM (95%CI: 2.8–9.7 μM) in non-Tg cultures, 3.9 μM (95%CI: 2.4–6.4 μM) in Tg and 2.5 μM (95%CI: 0.9–6.8 μM) in Tg/Tg without statistically significant difference among genotypes (F$_{2,232}$ = 0.5612, P = 0.57).

Two-way ANOVA of MPP$^+$-toxicity on MAP-2-IR cell number revealed significant effect of MPP$^+$ concentration (F$_{2,199}$ = 28.47, $P<0.0001$), but neither a significant effect of genotype (F$_{2,199}$ = 1.64, P = 0.20) nor a significant interaction between these two factors (F$_{2,199}$ = 1.20, P = 0.31) (Fig. 8B).

To assess whether reducing basal urate levels in both, neurons and astrocytes, exacerbated the UOx effect on MPP$^+$-induced toxicity, we treated neuron-astrocyte cultures with MPP$^+$ for four days as mentioned above. Two-way ANOVA revealed significant effects of both genotype (F$_{2,284}$ = 10.09, $P<0.0001$) and MPP$^+$ concentration (F$_{2,284}$ = 96.36, $P<0.0001$) on the number of TH-IR neurons and a significant interaction between genotype and MPP$^+$ concentration (F$_{2,284}$ = 3.01, P = 0.007) (Fig. 9A). Dopaminergic viability was reduced in UOx expressing cultures in comparison to non-Tg cultures with a maximum effect at 0.1 μM MPP$^+$, which further reduced TH-IR neuron number by 39% and 49% in Tg and Tg/Tg cultures compared to non-Tg cultures, respectively. The EC$_{50}$ for MPP$^+$ was 0.11 μM (95%CI: 0.04–0.31 μM) in non-
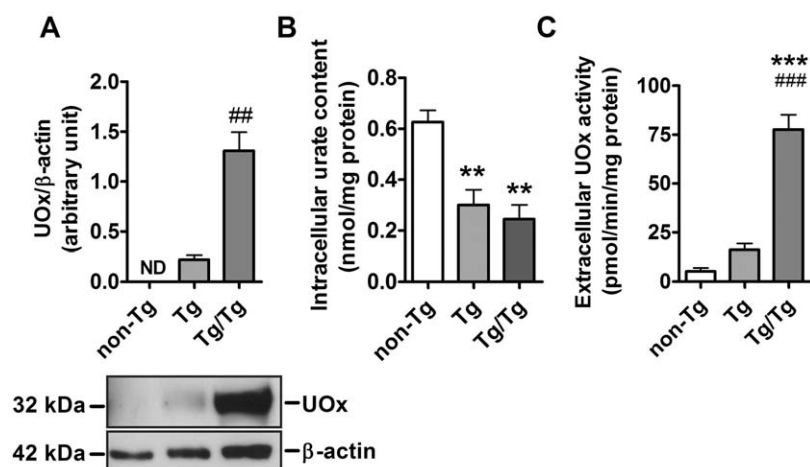
Figure 6. Characterization of non-Tg, Tg and Tg/Tg cortical neuron-enriched cultures. A) Western blot and graph showing UOx expression in wild-type (non-Tg) and UOx-expressing neurons (Tg and Tg/Tg) normalized to the β-actin level. Note that UOx was not detected in wild-type neurons (n = 3). B) Effect of UOx expression on intracellular urate content in neurons normalized to the protein level (n = 3). C) UOx activity in the media of non-Tg and UOx-expressing neurons (n = 6). Student's t test: $^{\#\#}P = 0.005$ vs Tg value; one-way ANOVA followed by Newman-Keuls test: **$P<0.01$, ***$P<0.001$ vs non-Tg value and $^{\#\#\#}P<0.001$ vs Tg value.
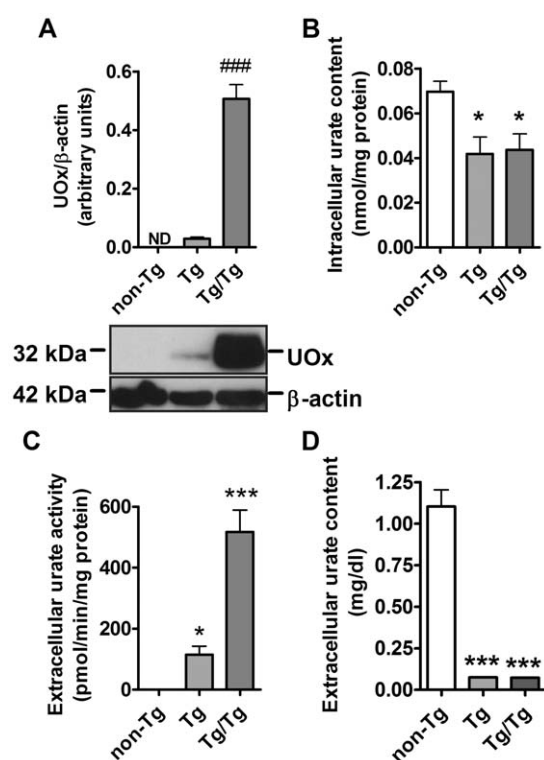doi:10.1371/journal.pone.0037331.g006

Figure 7. Characterization of non-Tg, Tg and Tg/Tg cortical astrocyte-enriched cultures. A) Western blot and graph showing UOx immunostaining in non-Tg and UOx-expressing astrocytes (Tg and Tg/Tg) normalized to the β-actin level. Note that UOx was not detected in non-Tg astrocytes (n = 7). B) Effect of UOx expression on the intracellular urate content normalized to the protein level (n = 5). C) UOx activity in the media of non-Tg and UOx-expressing astrocytes (n = 9). D) Effect of UOx expression on extracellular urate concentration in astroglial cultures (n = 6). Some error bars are not visible because of their small size. Student's t test: $^{\#\#\#}P<0.0001$ vs Tg value. One-way ANOVA followed by Newman-Keuls test: *$P<0.05$, ***$P<0.001$ vs non-Tg value.
doi:10.1371/journal.pone.0037331.g007

Tg cultures, 0.05 μM (95%CI: 0.02–0.12 μM) in Tg and 0.02 μM (95%CI: 0.01–0.04 μM) in Tg/Tg with a statistically significant difference among genotypes ($F_{2,284} = 5.66$, $P = 0.0039$).

Analysis of MPP+ effect on MAP-2-IR cell number revealed significant effect of MPP+ concentration ($F_{2,236} = 5.89$, $P<0.0007$), but neither a genotype effect ($F_{2,236} = 0.27$, $P = 0.76$) nor significant interaction between these two factors ($F_{2,236} = 0.06$, $P = 1$) (Fig. 9B).

These data indicate that dopaminergic tolerance to MPP+ was further reduced when basal urate content was reduced both in neurons and astrocytes.

Discussion

In our model we induced selective degeneration of dopaminergic neurons using the neurotoxin MPP+ in mouse ventral mesencephalon cultures. Urate, a known powerful antioxidant [7–9], added to cultures 24 hours before and during toxicant treatment, attenuated MPP+ toxicity in dopaminergic neurons. It increased the number of TH-IR cells both in neuron-enriched and neuron-astrocyte cultures containing respectively low and high percentage of astrocytes. In cultures with low percentage of astrocytes, urate only partially prevented dopaminergic neuron loss. On the other hand, in cultures prepared with a high percentage of astrocytes, urate completely prevented MPP+-induced toxicity. Moreover, in these mixed neuron-astrocyte cultures, urate fully prevented atrophic morphological changes in neurite length and soma size induced by MPP+. Both in neuron-enriched and neuron-astrocyte cultures, urate showed protective effects with an EC_{50} of about 1 μM, a concentration within the mouse physiological range where its CSF urate concentration is about 3 μM [38], ten-time lower than in humans [6].

Urate may have conferred protection against neuronal atrophy and death through its established antioxidant actions, as it has been shown to prevent ROS accumulation and oxidative damage in other neuronal populations [25,26,39] and to raise cysteine uptake and glutathione synthesis in mouse hippocampal slices [40]. Urate treatment might change the redox status of neurons, reducing their vulnerability to oxidative stress and preventing cellular degeneration. In fact, MPP+ toxicity may depend on the
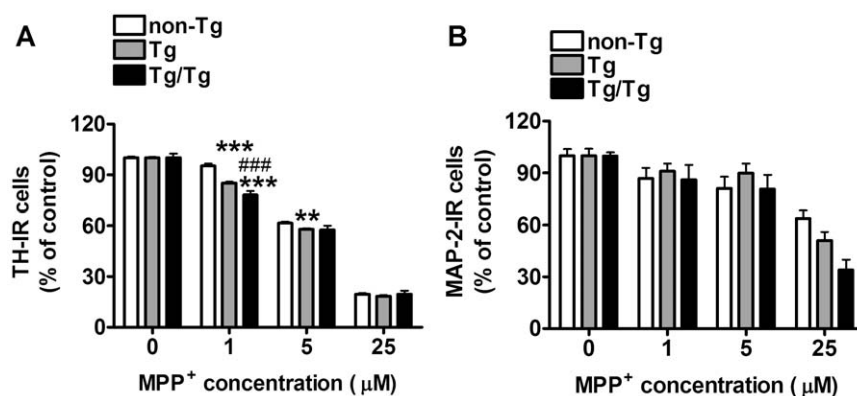
Figure 8. MPP$^+$ effect on non-Tg, Tg and Tg/Tg neuron-enriched cultures. A) MPP$^+$ effect on TH-IR cell number in non-Tg (n = 18), Tg (n = 35) and Tg/Tg (n = 8) neuronal cultures. B) MPP$^+$ effect on MAP-2-IR cell number in non-Tg (n = 18), Tg (n = 35) and Tg/Tg (n = 8) cultures. C) Two-way ANOVA followed by Bonferroni multiple comparison test: **$P < 0.01$, ***$P < 0.001$ vs respective non-Tg value; ###$P < 0.01$ vs respective Tg value.
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antioxidant status of neurons. Previous studies have shown non-toxic levels of iron and glutathione synthesis inhibition to enhance degeneration of dopaminergic neurons treated with MPP$^+$ [41] and antioxidant enzymes to prevent MPP$^+$-induced toxicity [41–44].

Although its antioxidant properties have been extensively described, a question remains to be answered: How does *exogenous* urate prevents oxidant toxicity? Ascorbate, an important antioxidant in the CNS [45–47], is present at high levels in neurons where its concentration is thought to be raised and maintained by the sodium-dependent vitamin C transporter-2 (SVCT2) [48,49]. Urate may protect through a similar mechanism that relies on the elevation of intracellular antioxidant content as was demonstrated here with *exogenous* urate substantially increasing intracellular urate in cortical neuronal cultures. By contrast, although Guerreiro et al. [25] reported a similar protective effect of urate on dopaminergic neurons in primary cultures, they did not find an associated increase in intracellular urate, possibly due to a greater sensitivity of our electrochemistry-based analytical methods or other differences between our studies.

To directly address the hypothesis that endogenous urate contributes to dopaminergic neuron resistance to toxicants, MPP$^+$ toxicity was assessed in cultures expressing the UOx enzyme, which catalyzes urate degradation to allantoin. UOx is not

normally synthesized in the mouse brain where, like in humans, urate is the enzymatic end product of the purine catabolism. Transgenic *UOx* expression reduced basal levels of urate both in cortical neurons and cortical astrocytes, even if this effect was not proportional to the increasing levels of UOx protein expression and enzyme activity observed with increasing transgene copy number. In neuron-enriched cultures, dopaminergic neurons expressing UOx were slightly more vulnerable to MPP$^+$ compared to wild-type neurons. In neuron-astrocyte cultures, transgenic *UOx* markedly exacerbated the toxicity and increased the potency of MPP$^+$ even though we did not see a greater decrease in intracellular urate concentration in astrocytes than in neurons. Because we were not able to measure urate content in ventral mesencephalic astrocytes and dopaminergic neurons due to their low number, we employed their cortical counterparts. Although urate transporter properties for each cell type are not expected to differ across brain regions, we cannot be sure that the changes in intracellular urate demonstrated in cortical cultures after both pharmacologic and genetic manipulations were achieved in ventral mesencephalic cells as well. Moreover, we cannot exclude that culturing neurons with astrocytes might affect the intracellular urate content in neurons. Nevertheless, our consistent observation of potentiated protection by astrocytes in cultures of dopaminergic
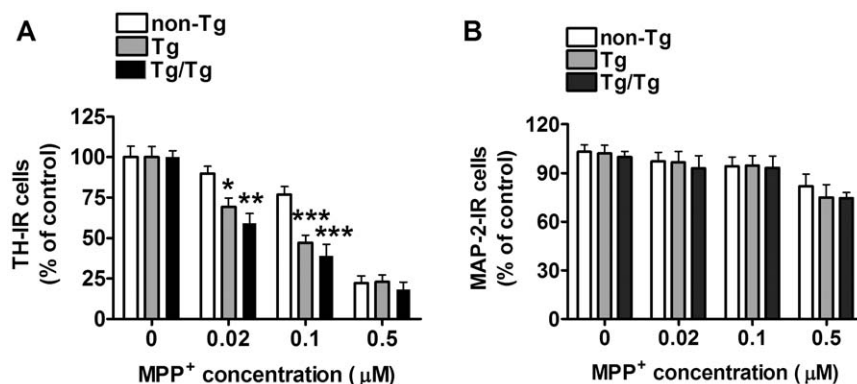

Figure 9. MPP$^+$ effect on non-Tg, Tg and Tg/Tg mixed neuron-astrocyte cultures. A) MPP$^+$ effect on TH-IR cell number in non-Tg (n = 18), Tg (n = 34) and Tg/Tg (n = 22) neuronal cultures. B) MPP$^+$ effect on MAP-2-IR cell number in non-Tg (n = 18), Tg (n = 34) and Tg/Tg (n = 22). Two-way ANOVA followed by Bonferroni multiple comparison test: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs respective non-Tg value.
doi:10.1371/journal.pone.0037331.g009

neurons strengthens the evidence for a facilitative role of astrocytes on the neuroprotective effect of urate [35].

The small protective effect of urate in neuron-enriched cultures containing few astrocytes and the far greater protection in neuron-astrocyte cultures may reflect the same astrocyte-dependent mechanism in both culture types. This interpretation is supported by the absolute astrocyte dependence previously observed for urate's protective effect in spinal cord cultures [35]. Although the content of astrocytes and other dividing glial cell populations was pharmacologically reduced in our preparation of neuron-enriched cultures, astroglia was not completely eliminated from these cultures. Indeed a small astrocyte-independent effect of urate acting directly on dopaminergic neurons cannot be excluded with the available data.

How physiological levels of urate in astrocytes might play an important role in dopaminergic neuron protection is not known. It has been suggested that urate may confer neuroprotection via astrocytes by stimulating their extracellular glutamate buffering capacity or their release of neurotrophic factors [35,50]. An intracellular antioxidant effect of urate on astrocytes might activate such glial functions. Indeed astrocytes were found to be susceptible to MPTP/MPP$^+$ treatment showing increased ROS level [51,52] and reduced glutamate buffering capacity [53,54]. Therefore, even though the toxicant concentration we employed was selected to be subthreshold for altering astroglial viability, reducing basal levels of urate in astrocytes might deplete their antioxidant reserves and indirectly enhance toxic MPP$^+$ effects on neurons. Although urate was found to protect neurons in association with the up-regulation of EAAT1 glutamate transporter expression in astrocytes [35], glutamate release was not detected in the striatum of MPP$^+$-perfused mice [55] and NMDA antagonism did not prevent MPP$^+$-induced dopaminergic cell death [56]. Further experiments will be needed to clarify the mechanism by which astrocytes play a facilitative role in the neuroprotective effect of urate and to confirm urate's protective effect in animal models of PD.

In conclusion, our data showed that intracellular urate may modulate dopaminergic neuron resistance to environmental toxins. This effect may be mediated by changes in astroglial urate content. A greater understanding of how urate protects neurons in models of PD may not only help elucidate its pathophysiology, it may also help accelerate or refine current urate-targeting strategies under investigation for their potential to slow or prevent PD (http://clinicaltrials.gov/ct2/show/NCT00833690).

Materials and Methods

Mice

UOx Tg mice [37] were obtained from Kenneth L. Rock at University of Massachusetts. Mice were backcrossed eight times on the C57BL/6 genetic background and phenotyped by measuring UOx activity in serum samples. Briefly, about two hundred µl of submandibular blood were collected from 1 month-old mice. Four µl of serum sample were added to 96 µl of 130 µM urate in 0.1 M borate (pH 8.5) and absorbance was read at 292 nm at the beginning of the assay and after 4–6 hours incubation at 37°C.

Ethics Statement

All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with approval from the animal subjects review board of Massachusetts General Hospital (Permit Number: 2006N000120).

Neuron-enriched cultures

Ventral mesencephalon was dissected from embryonic day E15–17 mouse embryos. Tissue was carefully stripped of their meninges and digested with 0.6% trypsin for 15 min at 37°C. Trypsinization was stopped by adding an equal volume of culture preparation medium (DMEM/12, N2 supplement 5%, fetal bovine serum (FBS) 10%, penicillin 100 U/ml and streptomycin 100 µg/ml) to which 0.02% deoxyribonuclease I was added. The solution was homogenized by pipetting up and down, pelleted and re-suspended in culture medium (Neurobasal medium (NBM), B27 supplement 2%, L-gluatamine (2 mM), penicillin 100 U/ml and streptomycin 100 µg/ml). The solution was brought to a single cell suspension by passage through a 40-µm pore mesh. Cells were seeded at a density of 220,000 cells/cm^2 onto 96 well plates or chamber-slides coated with poly-L-lysine (100 µg/ml)/DMEM/F12) and cultured at 37°C in humidified 5% CO2-95% air. On the third day half medium was replaced with fresh NBM containing the antimetabolite cytosine arabinoside (Ara-C,10 µM) to inhibit glial growth and glucose 6 µM. Medium was fully changed after 24 hours and then a half volume was replaced every other day. After 6 days in vitro (DIV) cultures were pretreated with urate or vehicle, and 24 hours later MPP$^+$ or vehicle was added. Cultures were constitute of >95% neurons, of which 2–3% were dopaminergic neurons and <5% astrocytes; microglia and oligodendrocytes were not detected (See Figure 1).

For Tg neuronal cultures, individual cultures were prepared from the ventral mesencephalon of individual embryos generated by crossing two Tg mice (with a resulting distribution of 23% non-Tg, 44% Tg and 33% Tg/Tg). The rest of the brain was used for phenotyping by western blotting. Brain tissue extracts negative for UOx staining were considered non-Tg; tissue, positive for UOx staining were considered Tg when UOx/actin value was >0.1 and ≤0.6, and Tg/Tg when UOx/actin value was >1.5. Cultured cell phenotypes were confirmed by measuring UOx activity in the cell medium.

Neuron-astrocyte cultures

Tissue was processed as described above but no Ara-C was added to the cultures. At 4 DIV cells were treated with urate or vehicle, 24 hour later MPP$^+$ or vehicle was added. Cultures comprised 45–60% neurons, of which 2–3% were dopaminergic neurons, 40–50% astrocytes and <1 microglia, oligodendrocytes were not detected.

Immunocytochemistry: After treatments cultures were fixed with 4% paraformaldehyde for 1 hour at room temperature. Then, cells were loaded with a blocking solution (0.5% albumin, 0.3% Triton-X 100 in phosphate buffer saline) for 30 min at room temperature and then incubated with a mouse anti-TH (1:200, Millipore, Temecule, CA) and a rabbit anti-MAP-2 antibody (1:200, Millipore, Temecule, CA), or a rabbit anti-GFAP antibody, overnight at 4°C to label dopaminergic neurons neurons and astrocytes, respectively. Cultures were loaded with a cy3-conjugated anti-mouse antibody (1:500, Jackson InnunoResearch Laboratories, Inc.; West Grove, PA) and a FITC-conjugated anti-rabbit antibody (1:300, Jackson InnunoResearch Laboratories, Inc.; West Grove, PA) 2 hours at room temperature. Cultures were imaged using an Olympus BX50 microscope with a 20×/0.50 objective and Olympus DP70 camera. Images were processed with DP Controller software (Olympus) and merged with ImageJ (NIH). Cells cultured in plates were observed with a Bio-Rad Radiance 2100 confocal laser-scanning microscope with krypton-argon and blue diode lasers. Images were acquired through a Plan Fluor DIC ELWD 20×/0.45 Ph1 DM ∞/0–2 WD 7.4 objective on an inverted Nikon Eclipse TE300 fluorescent microscope with

408/454 nm excitation/emission (blue), 485/525 nm excitation-emission (green) and 590/617 nm excitation-emission (red).

Neurite length was measured by the Simple Neurite Tracer tool of ImageJ software. In each sample, neurite length was determined by the average of the longest neurite of 100 TH-IR neurons randomly selected in the well. Neurons having neurites ending outside the optic field were excluded from the analysis. Values were expressed in μm.

High-Performance Liquid Chromatography

Cells were scraped in a solution of 150 mM phosphoric acid, 0.2 mM EDTA, and 1 μM 3,4-dihydroxybenzylamine (DHBA; used as internal standard), clarified by centrifugation and filtered through a 0.2 μm Nylon microcentrifuge filter (Spin-X, Corning). Samples were chromatographed by a multi-channel electrochemical/UV HPLC system with effluent from the above column passing through a UV-VIS detector (ESA model 528) set at 254 nm and then over a series of electrodes set at -100 mV, +250 mV and +450 mV. Urate was measured on the +250 mV electrode with a limit of detection at 0.0001 mg/dl. In order to generate a gradient, two mobile phases were used. Mobile phase B increased linearly from 0% to 70% between 6th and 14th min of the run and immediately reduced to 0% at 17.4 min and allowed to re-equilibrate for the final 3.6 min. Mobile phase A consisted of 0.2 M potassium phosphate and 0.5 mM sodium 1-pentanesulfonate; mobile phase B consisted of the same plus 10% (vol/vol) acetonitrile. Both mobile phases were brought to pH 3.5 with 85% (wt/vol) phosphoric acid.

Western blot assay

Cells were scraped in RIPA buffer (Sigma Co., St. Luis, MO) and loaded (50 μg of proteins per well) into a 10% SDS-PAGE gel. Proteins were then transferred electrophoretically onto 0.2 μ nitrocellulose membranes (Biorad Laboratories) and probed with a rabbit polyclonal antibody anti-UOx (1:200; Santa Cruz, CA) overnight at 4°C. After washing in Tris Buffer Saline containing 0.1% Tween20, membranes were incubated with a horseradish peroxidase-conjugated anti-rabbit IgG (1:2000; Pierce, Biotechnology, Rockford, IL, USA) for 2 hours at room temperature. Proteins were visualized using chemiluminescence (Immobilon, Millipore). In order to normalize the values of UOx staining, β-actin was detected in the same western blot run. Membranes were incubated for 2 hours at room temperature with an anti-β-actin antibody (1:2000; Sigma, St Louis, MO) and then with a horseradish peroxidase-conjugated anti-rabbit IgG (1:5000; Pierce, Biotechnology, Rockford, IL) for 2 hours. Membranes were developed as above. Bands were acquired as JPG files and densitometric analysis of bands was performed by ImageJ software. UOx/β-actin values were expressed as arbitrary units.

UOx activity assay

Cell medium was added to 0.5 mg/ml urate and absorbance was red at 292 nm before and after 24 hours incubation at 37°C. Activity was calculated as percentage of absorbance decrease in comparison to starting values.

Protein detection: Proteins were quantified in 4 μl of each sample using Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA, USA) and measured spectrophotometrically at 600 nm with Labsystems iEMS Analyzer microplate reader.

Author Contributions

Conceived and designed the experiments: SC KX MAS. Performed the experiments: SC CAD TCB. Analyzed the data: SC. Contributed reagents/materials/analysis tools: CAD TCB YX. Wrote the paper: SC MAS.

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ORIGINAL ARTICLE

Protection of dopaminergic cells by urate requires its accumulation in astrocytes

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Abstract

Urate is the end product of purine metabolism and a major antioxidant circulating in humans. Recent data link higher levels of urate with a reduced risk of developing Parkinson's disease and with a slower rate of its progression. In this study, we investigated the role of astrocytes in urate-induced protection of dopaminergic cells in a cellular model of Parkinson's disease. In mixed cultures of dopaminergic cells and astrocytes oxidative stress-induced cell death and protein damage were reduced by urate. By contrast, urate was not protective in pure dopaminergic cell cultures. Physical contact between dopaminergic cells and astrocytes was not required for astrocyte-dependent rescue as shown by conditioned medium experiments. Urate accumulation in dopaminergic cells and astrocytes was blocked by pharmacological inhibitors of urate transporters expressed differentially in these cells. The ability of a urate transport blocker to prevent urate accumulation into astroglial (but not dopaminergic) cells predicted its ability to prevent dopaminergic cell death. Transgenic expression of uricase reduced urate accumulation in astrocytes and attenuated the protective influence of urate on dopaminergic cells. These data indicate that urate might act within astrocytes to trigger release of molecule(s) that are protective for dopaminergic cells.

Keywords: cell viability, HPLC, MES 23.5 cells, transgenic, transporter, uricase.

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Currently, some 90% of Parkinson's disease (PD) cases are classified as sporadic, reflecting the uncertainty of their causes. A combination of genetic and environmental factors is thought to trigger pathogenic cascades that converge to increase oxidative stress or to reduce natural antioxidant defenses, leading to cellular impairment and the neurodegeneration characteristic of PD (Ross and Smith 2007). Dopaminergic neurons in the substantia nigra *pars compacta* are highly sensitive to oxidative stress and their selective degeneration is responsible for the progressive motor disability of PD.

Urate (2,6,8-trioxy-purine; a.k.a. uric acid) circulates in humans at concentrations that are near its limit of solubility and many fold higher than in most other mammals. In humans and apes, urate is the enzymatic end product of purine metabolism because of mutations of the uricase (a.k.a. urate oxidase) gene (*UOx*) that occurred during hominoid evolution (Oda *et al.* 2002). The resulting urate elevation has been hypothesized to have raised antioxidant levels in human ancestors and thereby lengthened their lifespans. Urate possesses antioxidant properties comparable to those of ascorbate (Ames *et al.* 1981) and forms stable coordination complexes with iron and other metal ions (Davies *et al.* 1986), accounting for its ability to reduce oxidative damage caused by reactive nitrogen and oxygen species (Whiteman *et al.* 2002).

Recently, epidemiological and clinical studies have found people with higher serum levels of urate to be less likely to develop PD (Weisskopf *et al.* 2007). Moreover, amongst PD patients those with higher urate in serum or CSF showed a slower rate of disease progression assessed clinically

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(Schwarzschild *et al.* 2008; Ascherio *et al.* 2009), or radiographically as a reduced rate of dopaminergic nerve terminal marker loss (Schwarzschild *et al.* 2008). In PD models urate attenuated motor and dopaminergic deficits in rodents (Wang *et al.* 2010). *In vitro*, urate reduced oxidative stress as well as cell death induced by toxicants in dopaminergic cell lines (Duan *et al.* 2002; Haberman *et al.* 2007), and rescued dopaminergic neurons in a model of spontaneous cell death (Guerreiro *et al.* 2009). Similarly, we reported that urate prevented dopaminergic neuron death induced by MPP$^+$ in ventral mesencephalon cultures, and conversely that enzymatically lowering urate levels exacerbated this neurotoxicity (Cipriani *et al.* 2012). Although the mechanism of neuroprotection by urate remains largely unknown, urate rescue of spinal cord neurons from excitotoxicity has been found to depend upon an astroglial mechanism (Du *et al.* 2007), consistent with our recent evidence that the neuroprotection conferred on cultured dopaminergic neurons by raising intracellular urate can be enhanced by co-culturing with astroglia (Cipriani *et al.* 2012).

In the present study, we assess the role played by astrocytes in the protective effect of urate on dopaminergic cells in a cellular oxidative stress model of PD.

Materials and methods

Mice

Transgenic (Tg) *UOx* mice (Kono *et al.* 2010) were obtained from Kenneth L. Rock and the University of Massachusetts. Mice were backcrossed eight times on the C57BL/6 genetic background and phenotyped by measuring UOx activity in serum samples (Cipriani *et al.* 2012). All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals with approval from the animal subjects review board of Massachusetts General Hospital.

MES 23.5 cell line

The rodent MES 23.5 dopaminergic cell line, which was derived from the fusion of a dopaminergic neuroblastoma and embryonic mesencephalon cells (Crawford *et al.* 1992), was obtained from Weidong Le at Baylor College of Medicine (Houston, TX, USA). Despite the inherent environmental (*in vitro*) and cellular (tumor cell) limitations in modeling dopaminergic neuron degeneration, the dopaminergic properties of MES 23.5 cells and their molecular responses to dopaminergic neuron toxins have been well characterized and support its relevance as a cellular model of the dopaminergic neuron degeneration in PD. The MES 23.5 cells were cultured on polyornithine-coated T75 flasks (Corning Co, Corning, NY, USA) in the Dulbecco modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA/Gibco, Rockville, MD, USA), which contained Sato components (Sigma Immunochemicals), supplemented with 2% newborn calf serum (Invitrogen), 1% fibroblast growth factor (Invitrogen), penicillin 100 U mL^{-1}, and streptomycin 100 µg mL^{-1} (Sigma, St Louis, MO, USA) at 37°C in a 95% air–5% carbon dioxide humidified incubator. The culture medium was changed every 2 days, MES 23.5 cells were subcultured either in new T-75 flasks or plated onto polyornithine-coated plates. The MES 23.5 cells were used at passage 10–20, at which we confirmed the persistence of their dopaminergic phenotype (Crawford *et al.* 1992) by quantifying the dopamine content (23 ± 3 pmol mg^{-1} protein) using HPLC with electrochemical detection (Xu *et al.* 2010).

Astroglia–enriched cultures

Astroglial cultures were prepared from the brain of 1- or 2-day-old neonatal mice with modifications to previously reported procedures (Saura *et al.* 2005). Cerebral cortices were carefully stripped of their meninges and digested with 0.25% trypsin for 15 min at 37°C. Trypsinization was stopped by adding an equal volume of culture medium DMEM, fetal bovine serum 10%, penicillin 100 U mL^{-1}, and streptomycin 100 µg mL^{-1} to which 0.02% deoxyribonuclease I was added. The suspension was pelleted, re-suspended in culture medium, and triturated to a single cell suspension by repeated pipetting followed by passage through a 70 µM-pore mesh. Cells were seeded at a density of 1,800 cells per mm^2 on poly-L-lysine (100 µg mL^{-1})/DMEM/F12-coated flasks and cultured at 37°C in humidified 5% CO$_2$-95% air. Medium was fully changed on the fourth day and then every other day. Cultures reached confluency after 7–10 days *in vitro*.

To remove oligodendrocytes and microglial cells, flasks were agitated at 200 × g for 20 min in an orbital shaker. Following shaking medium was changed and flasks were again agitated at 100 × g for 18–20 h. Floating cells were washed away and cultures were treated with 10 µM cytosine arabinoside (Ara-C) for 3 days. Our astroglial cultures comprised > 95% astrocytes, < 2% microglial cells, and < 1% oligodendrocytes. No neuronal cells were detected (Figure S1a–c).

To prepare astroglia-enriched cultures from UOx wild-type (WT) and Tg pups individual cultures were prepared from cortices of each pup. The rest of the brain was used for phenotyping by western blotting. Brain tissue extracts were considered WT when they were negative to UOx staining and Tg when they showed a band at 32 kDa corresponding to UOx. Cultured cell phenotypes were confirmed by measuring UOx activity in the cell medium.

Co-cultures

Astroglia-enriched cultures were prepared as described above. After Ara-C treatment astrocytes were detached from flasks by mild trypsinization (0.1% for 1 min) and re-plated on pre-coated plates in DMEM plus 10% fetal bovine serum. Astrocytes were allowed to grow for 2 days before MES 23.5 cells were seeded on top of them at a concentration of 600 cells per mm^2. MES 23.5 cells were detached from astrocytes by pipetting before processing for dopamine and protein carbonyl assays.

Co-cultures were imaged by an Olympus BX50 microscope with a 20X/0.50 objective and Olympus DP70 camera. Images were processed with DP controller software (Olympus, Center Valley, PA, USA) and merged with ImageJ (NIH).

Conditioned medium experiments

Enriched astroglial cultures were treated with 100 µM urate, or vehicle. Twenty-four hours later conditioned media were collected and filtered through a 0.2 µM membrane to remove cellular debris and immediately used for following experiments. The MES 23.5 cells were treated with increasing proportions of

conditioned medium 24 h before and during H_2O_2 treatment. In UOx experiments the enzyme was added to astrocytes for 15 h before conditioned medium collection.

Drug treatment

Urate was dissolved in DMEM as 20× concentrated stocks. H_2O_2 was dissolved in phosphate-buffered saline (0.1 M, pH 7.4) as 100× concentrated stocks. Probenecid and hydrochlorothiazide (HCTZ) were dissolved in ethanol and pyrazinoate (PZO) in DMEM 50× concentrated stocks. Drugs were obtained from Sigma.

Cell viability

In MES 23.5 cultures, cell viability was measured by the 3-(4,5-dmethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Sigma) (Hansen *et al.* 1989). MES 23.5 cells were seeded onto polyornithine-coated 96-well plates (600 cells per mm^2) and grown for at least 24 h until the cells became 70–80% confluent. The medium was changed to DMEM serum-free medium for 24 h before increasing concentrations (50–800 μM) of H_2O_2 were added to the culture medium. To assess protection by urate, increasing concentrations (0–100 μM) were loaded 24 h before and again during toxicant treatment. After three washes with DMEM, 100 μl of MTT solution (0.5 mg mL^{-1} in DMEM) was added for 3 h at 37°C. Then MES 23.5 cells were lysed with acidic isopropanol (0.01M HCl in absolute isopropanol) to extract formazan, which was measured spectrophotometrically at 490 nm with a Labsystems iEMS Analyzer microplate reader. The *n* for each treatment refers to the number of triplicate data points, which were usually obtained from separate 96-well plates.

In co-cultures, living MES 23.5 cells were quantified by immunocytochemistry. After treatments, astrocytes-MES 23.5 co-cultures were fixed with 4% paraformaldehyde for 1 h at 20°C. Then, cells were loaded with a blocking solution (0.5% albumin, 0.3% Triton-X in phosphate-buffered saline) for 30 min at 20°C and incubated with an Alexa 488-cojugated antibody specific for neuronal cells (1 : 200, overnight at 4°C; FluoroPan Neuronal Marker). The following day fluorescence was read at 535 nm by means of a microplate reader.

High–Performance Liquid Chromatography

Cells were scraped in a solution of 150 mM phosphoric acid, 0.2 mM EDTA, and 1 μM 3,4-dihydroxybenzylamine (DHBA; as internal standard) and chromatographed by a multi-channel electrochemical/ UV HPLC system as previously described (Burdett *et al.* 2012).

Western blot assay

Cells were scraped in ice-cold extraction buffer (RIPA, Sigma), boiled for 5 min in an appropriate volume of 6 × loading buffer, loaded (50 μg of proteins per well) into a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel and run at 120 mV. Proteins were then transferred electrophoretically onto 0.2 μ nitrocellulose membranes (Biorad, Hercules, CA, USA) and saturated for 1 h at 20°C with blocking buffer (5% non-fat dry milk in Tris buffered saline, 0.1% TWEEN-20). To detect urate transporter expression membranes were probed overnight with the following primary antibodies: rabbit glucose transporter 9 (GLUT9)-specific polyclonal antibody (1 : 2000; Abcam Inc, Cambridge, MA, USA), mouse organic anion transporter 1 (OAT1)-specific monoclonal antibody (1 : 200; Abbiotec, San Diego, CA, USA), goat urate transporter 1 (URAT1)-specific polyclonal antibody (1 : 200; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Kidney extract was used as positive control. Proteins were visualized using chemiluminescence (Immobilon; Millipore Millipore Corporation, Billerica, MA, USA). To detect UOx expression, samples were prepared as described above and loaded into a 10% SDS-PAGE gel. Membranes were probed overnight with a rabbit UOx-specific polyclonal antibody (1 : 200; Santa Cruz, Inc). Liver extract was used as positive control.

To normalize the values of stained bands β-actin was detected on the same blot run. Membranes were stripped by strong agitation with 0.2 N NaOH (10 min at 20°C), blocked in blocking buffer for 1 h and probed for 2 h at 20°C with anti-β-actin antibody (1 : 2000; Sigma). Membranes were incubated with horseradish peroxidase-conjugated rabbit-specific (1 : 2000; Pierce Biotechnology), mouse-specific (1 : 2000; Pierce Biotechnology; Rockford, IL), or goat-specific antibody (1 : 2000; Biorad Laboratories) and developed as above. Bands were acquired as JPG files; densitometric analysis was performed by ImageJ software (NIH).

Nitrite (NO_2^-) release

The NO_2^- is an indicator of free radical generation and it is a major unstable product of nitric oxide and molecular oxygen reactions. After treatment, 100 μl of supernatant was added to 100 μl of Griess reagent (Sigma) and spectrophotometrically read at 540 nm with a microplate reader. Blanks were prepared by adding medium containing toxicants and/or protectants to Griess solution.

Protein carbonyl protein assay

Oxidized proteins were detected using the Oxyblot assay kit (Chemicon) according to the manufacturer's instructions. Briefly, protein carbonyl groups were derivatized with 2,4-dinitrophenylhydrazine, subjected to 10% SDS-PAGE and transferred electrophoretically onto 0.2 μ nitrocellulose membranes. Membranes were loaded with an antibody specific to the dinitrophenylhydrazone moieties of the proteins and developed using chemiluminescence.

Protein detection

Protein concentration was measured in 4 μl of each sample using Bio-Rad Protein Assay reagent (Biorad Laboratories) and reading the absorbance at 600 nm with a microplate reader.

Statistical analysis

Statistical analyses were performed by GraphPad Prism version 4.00 (GraphPad Software Inc., San Diego, CA, USA). Unpaired Student's *t*-test was used when only two group samples were compared. ANOVA analysis, followed by Newman Keuls or Bonferroni *post-hoc* test, was used when more than two group samples were compared. Values were expressed as mean ± SEM. Differences at the $p < 0.05$ were considered significant and indicated in figures by symbols explained in legends.

Results

Astrocyte–dependent protection of dopaminergic cells by urate

To reproduce an oxidative stress model of PD (Sherer *et al.* 2002; Anantharam *et al.* 2007) we incubated MES 23.5 cells

with increasing concentrations of H_2O_2. Treatment for 24 h with H_2O_2 decreased cell viability in a concentration-dependent manner (Fig. 1a) with about 60% of cell death at 200 μM, which was the H_2O_2 concentration chosen for following experiments.

To evaluate the effect of urate on H_2O_2-induced cell death urate was added to cultures 24 h before and during H_2O_2 application. Urate treatment tended to decrease H_2O_2-induced cell death over a concentration range of 0–100 μM (Fig. 1b) without a statistically significant effect.

Du and coworkers (Du et al. 2007) reported that urate's protective effect on primary spinal cord neurons was dependent on the presence of astrocytes in cultures. To assess whether urate protects dopaminergic cells cultured with astrocytes against oxidative stress, its effect was tested on MES 23.5-astrocytes co-cultures treated with H_2O_2. To minimize confounding effects by astroglial established inherent protection on dopaminergic cells (Yu and Zuo 1997), H_2O_2 toxicity was assessed in co-cultures established at astrocytes/MES 23.5 cells ratios of 0 : 1, 1 : 1, and 1 : 5. Astrocytes cultured with MES 23.5 cells at a ratio of 1 : 5 did not prevent H_2O_2-induced death in MES 23.5 cells (Fig. 1c); the same ratio was employed in following experiments. The H_2O_2 did not affect astrocyte viability up to the highest tested concentration of 200 μM (data not shown). Urate added to co-cultures 24 h before and during H_2O_2 application conferred significant, dose-dependent protection on H_2O_2-treated dopaminergic cells (Fig. 1d, e–g).

Urate decreased reactive oxygen species (ROS) production and protein oxidation

To determine if protection is associated with reduced oxidative stress and protein damage, we measured reactive oxygen species in cell media from H_2O_2-treated co-cultures of MES 23.5 cells and astrocytes. H_2O_2 raised the concentration of NO_2^- (nitrite) in the medium over time (Fig. 2a). Urate significantly decreased medium NO_2^- concentration in H_2O_2-treated cultures at 24 h of treatment (Fig. 2b). As an index of oxidative damage, protein carbonyl levels were measured in MES 23.5 cells (after removal from astrocytes) and found to be increased by H_2O_2 over time (Fig. 2c). Urate attenuated the increase in protein oxidation at 3 h of treatment with H_2O_2 (Fig. 2d).

Astrocytes mediate protection by urate without physically contacting dopaminergic cells

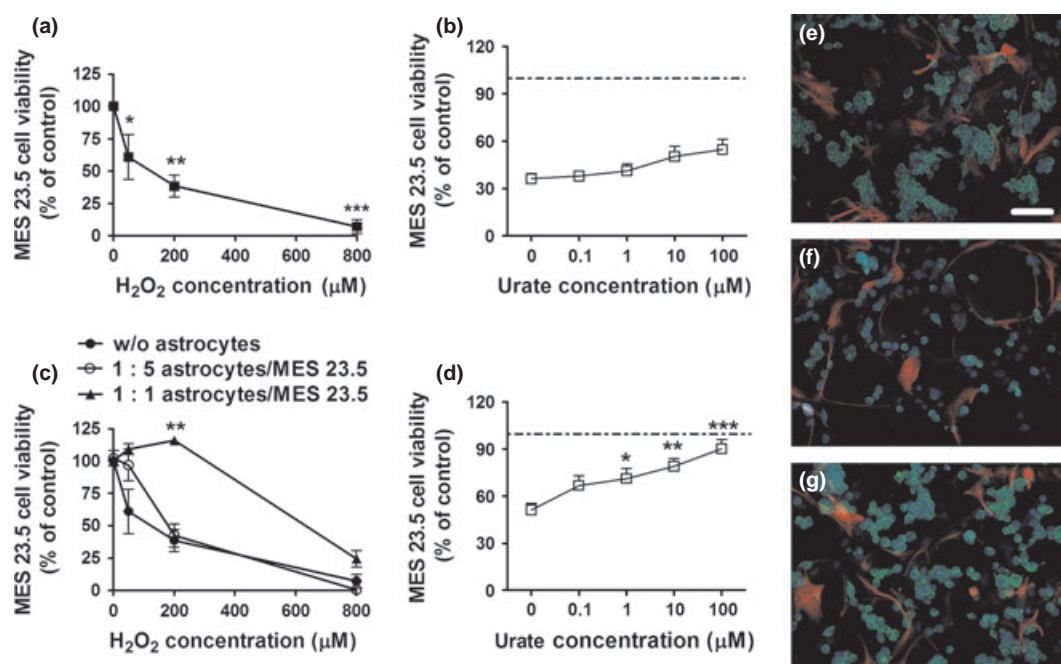The MES 23.5 cells were treated with increasing percentages of medium collected from vehicle-treated (control) or

Fig. 1 Protection of MES 23.5 cells by urate is mediated by astrocytes. (a) Cell viability in MES 23.5 cultures after 24 h of H_2O_2 treatment at indicated concentrations. One-way ANOVA: $n = 3$, *$p < 0.05$, **$p < 0.001$, ***$p < 0.001$ versus 0 value. (b) Effect of urate ($n = 4$) treatment on 200 μM H_2O_2-induced cell death in MES 23.5 cultures. (c) Cell viability of MES 23.5 cells cultured for 24 h with increasing H_2O_2 concentrations and astrocyte densities. Ratio between astrocytes and MES 23.5 cells is shown in symbol key. Two-way ANOVA: $n = 3$; **$p < 0.01$ versus 0 and 1 : 5. (d) Effect of urate treatment on 200 μM H_2O_2-induced cell death in co-cultures (1 : 5::astrocytes/MES 23.5). One-way ANOVA: $n = 13$; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus respective 0 value. Photomicrograph of (e) untreated, (f) H_2O_2-treated, (g) H_2O_2 + 100 μM urate-treated and MES 23.5 cells (green) cultured on astrocytes (red), DAPI staining was used to label nuclei (blue). Scale bar is 50 μm. The dashed line indicates the control value (100%) against which the other values were measured.
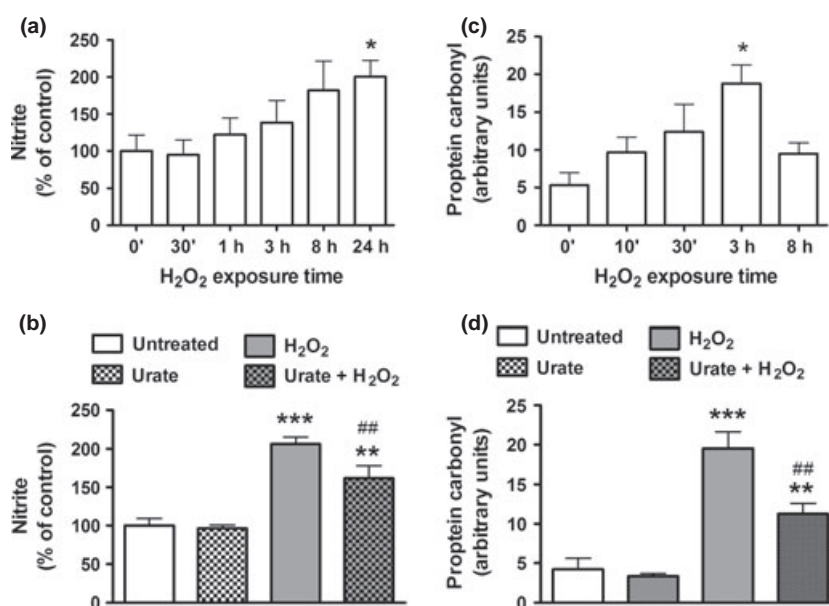
Fig. 2 Urate reduced reactive oxygen species and protein oxidation in MES 23.5 cells cultured with astrocytes. (a) NO_2^- release in co-culture medium after 200 μM H_2O_2 treatment for the indicated times. One-way ANOVA: $n = 15$; *$p < 0.05$ versus 0′ value. (b) Effect of urate treatment on H_2O_2-induced NO_2^- release at 24 h of treatment. One-way ANOVA: $n = 16$; **$P < 0.01$ and ***$P < 0.001$ versus control and urate values,

$^{##}p < 0.01$ versus H_2O_2 value. (c) Protein carbonyl content in MES 23.5 cells cultured with astrocytes after 200 μM H_2O_2 treatment for the indicated times. One-way ANOVA: $n = 5$; *$p < 0.05$ versus 0 value. (d) Effect of urate treatment on H_2O_2-induced protein carbonylation at 3 h of treatment with H_2O_2. One-way ANOVA: $n = 7$; **$p < 0.01$ and ***$p < 0.001$ versus control and urate values; $^{##}p < 0.05$ versus H_2O_2 value.

urate-treated astrocytes. Medium from control astrocytes did not increase viability of H_2O_2-treated MES 23.5 cells at any concentration, whereas conditioned medium from astrocytes treated for 24 h with 100 μM urate significantly increased MES 23.5 viability in a concentration-dependent manner.

To address a possible direct effect of carry-over urate on MES 23.5 cells, we added UOx or vehicle to astroglial cultures after 24 h of treatment with 100 μM urate. UOx-catalyzed (> 99.9%) elimination of urate from the conditioned medium was confirmed by HPLC measurements of 0.020 ± 0.003 μM versus 98 ± 12 μM urate 15 h after addition of UOx versus vehicle, respectively ($p < 0.0001$).

The protective effect of conditioned medium was only slightly attenuated by UOx, indicating that carry-over urate could not account for most of the protection conferred by urate-treated astrocyte-conditioned medium (Fig. 3b). The finding is consistent with our earlier observations that urate alone had no appreciable effect on dopaminergic cell viability.

Exogenous urate treatment increased intracellular urate content

Although intracellular antioxidant actions of urate might explain its observed attenuation of H_2O_2-induced oxidative
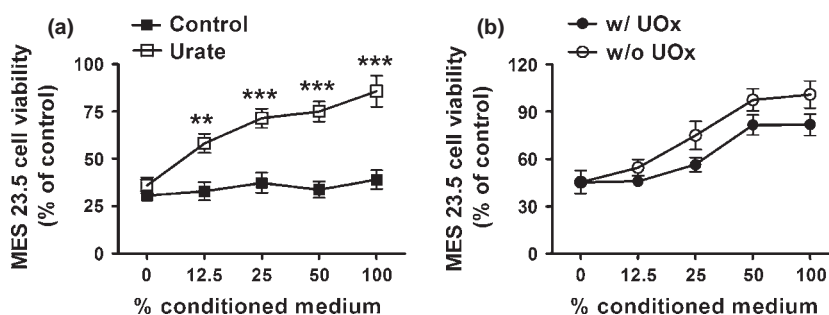

Fig. 3 (a) Effect of increasing percentages of conditioned medium from control or urate-treated astrocytes on MES 23.5 cell viability. Two-way ANOVA: $n = 15$; **$p < 0.01$ and ***$p < 0.001$ versus respective control value. (b) Effect of uricase (UOx; 0.12 U/l) on conditioned

medium from urate-treated astrocytes on MES 23.5 cell viability ($n = 4$). The UOx or vehicle was added to astroglial cultures after 24 h of urate treatment; conditioned media were collected after 15 more hours.

damage, Guerreiro *et al.* (Guerreiro *et al.* 2009) concluded that urate may act as an extracellular antioxidant to protect dopaminergic neurons. Similarly, the astrocyte-dependence of protection found in the present study leaves uncertain the site targeted by urate.

To determine whether urate entered MES 23.5 cells and astrocytes, intracellular urate was measured in dopaminergic and astroglial cells treated with vehicle or urate for 24 h. *Exogenous* urate raised intracellular urate content from 0.81 ± 0.30 to 5.09 ± 0.44 nmol/mg of protein ($p < 0.01$) and from 0.14 ± 0.06 to 0.38 ± 0.04 nmol/mg of protein ($p < 0.01$) in MES 23.5 cells and astrocytes, respectively (see Table S1 and S2), at the time when toxicant treatment would have been initiated. No statistically significant effect on its precursors was found either intracellularly (See Table S1 and S2) or extracellularly (data not shown).

To assess if urate was metabolized by UOx in MES 23.5 cells we treated the cell line with increasing concentrations of oxonate, a UOx selective inhibitor. Oxonate did not affect urate content in MES 23.5 cells at any given concentration (Figure S2a). This result was supported by western blotting analysis, which detected no staining for UOx in both MES 23.5 cells and astrocytes (Figure S2b).

Intracellular urate increase is required for dopaminergic protection

To determine whether urate accumulation into MES 23.5 cells and astrocytes is transporter-mediated, protein expres-sion of urate transporters known to be key regulators of urate levels in rodents (Hosoyamada *et al.* 2004; Preitner *et al.* 2009) was investigated. Immunostaining for URAT1 and GLUT9 was positive in MES 23.5 cells and astrocytes, while immunostaining for OAT1 was negative in both cell types (Fig. 4a). To investigate whether any of these transporters played a role in increasing intracellular urate levels, cells were loaded with urate immediately after one of the following drugs: PZO, the active metabolite of pyrazinamide (a URAT1 inhibitor), probenecid (a URAT1 and GLUT9 inhibitor), and HCTZ (a URAT1 and OAT1 inhibitor).

The HPLC determinations showed that HCTZ signifi-cantly reduced urate accumulation in a concentration-dependent manner in MES 23.5 cells, whereas probenecid and PZO had no effect (Fig. 4b). By contrast, PZO, probenecid and HCTZ markedly reduced urate accumula-tion in astrocytes in a concentration-dependent manner (Fig. 4c).

To determine if intracellular urate accumulation was required for urate's protective effect, we conducted viability experiments pretreating mixed cultures with HCTZ, PZO, or probenecid together with urate 24 h before toxicant treat-ment. The PZO and HCTZ prevented dopaminergic protec-tion induced by urate in a concentration-dependent manner; a similar effect was seen with 0.5 mM probenecid (Fig. 4d). PZO, HCTZ and probenecid did not affect susceptibility of MES 23.5 cells to H_2O_2 in urate-untreated cultures (data not shown).
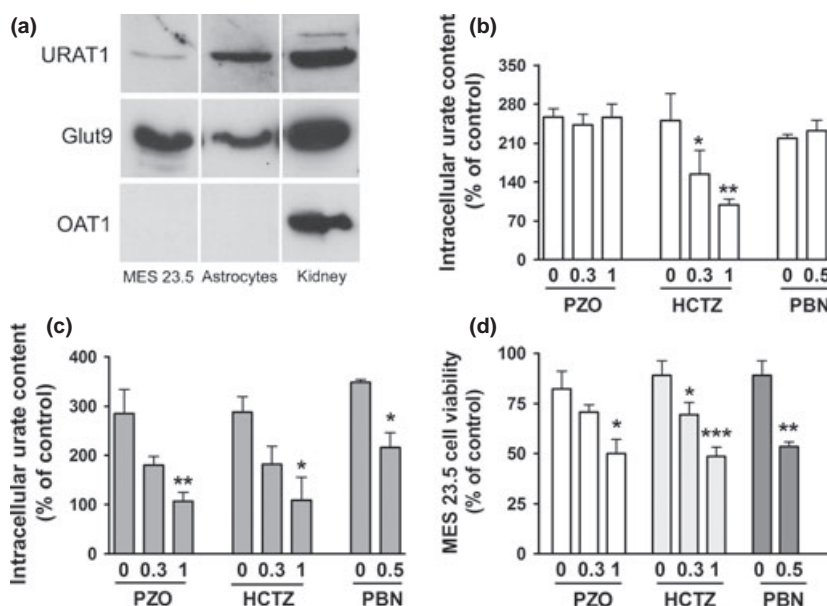

Fig. 4 Urate's protective effect depends on its accumulation in as-trocytes. (a) Western blotting shows urate transporter 1 (URAT1), GLUT9 and organic anion transporter 1 (OAT1) urate transporter expression in extracts of MES 23.5 cells and astrocytes. Kidney extract was used as positive control. (b) Urate concentration in MES 23.5 cells and (c) astrocytes treated with urate transporter inhibitors: pyrazinoate (PZO), hydrochlorothiazide (HCTZ), or probenecid (PBN), at the indicated concentrations (mM) ($n = 4$–7). (d) Effects of urate transporter inhibitors: PZO, HCTZ and PBN, at the indicated concentrations (mM), on MES 23.5 cell viability in H_2O_2-treated co-cultures expressed as percentage of control ($n = 4$–6). One-way ANOVA: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus 0 value.
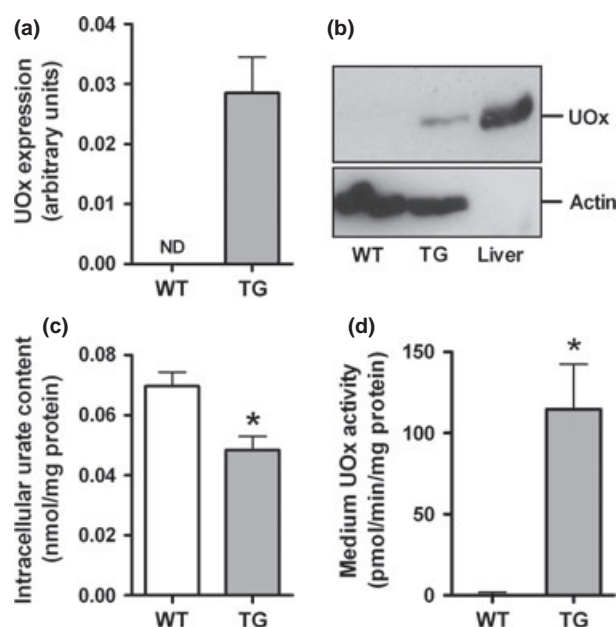
Fig. 5 UOx expression in transgenic (Tg) astrocytes reduced urate intracellular content. (a) Uricase (UOx) expression in Tg *UOx* astrocytes with no detection (ND) in wild-type (WT) astrocytes. (b) Western blot of UOx immunoreactivity in 50 μg of WT and Tg astrocytes; liver extract (10 μg) was used as positive control. Actin was used as loading control. (c) Basal intracellular urate content in WT and Tg astrocytes. Student's *t*-test: $n = 5$; *$p = 0.02$. (d) Basal UOx activity in media from WT and Tg astrocytes. Student's *t*-test: $n = 10$, *$p = 0.015$.

Transgenic urate degradation in astrocytes reduces protection by conditioned medium

To exclude possible secondary pharmacological effects of urate-lowering transport inhibitors, we also took a genetic approach to reduce urate content through enzymatic degradation within astrocytes. To that end, astrocytes were prepared from mice over-expressing *UOx* (*UOx* Tg) (Kono *et al.* 2010). Cultured Tg astrocytes expressed UOx protein, which was undetectable in WT astrocytes (Fig. 5a and b). The UOx expression reduced urate basal levels in Tg compared with WT astrocytes (Fig. 5c). Because the UOx transgene we employed (Kono *et al.* 2010) can lead to secretion of the enzyme (Fig. 5d), we assessed the extent to which extracellular urate was catabolized in Tg and WT astrocyte cultures after addition of medium containing 100 μM urate. We found that urate was not altered for at least 8 h in the medium, although by the end of the 24 h treatment period a small but significant (21%) reduction was appreciated (Fig. 6a). The intracellular urate content in Tg astrocytes was reduced compared with WT astrocytes after 8 h of exposure to urate (Fig. 6b). Thus transgenic *UOx* expression in astrocytes produced marked and rapid reduction primarily of intracellular urate. To assess whether reduced urate accumulation affected the protective effect of conditioned medium on MES 23.5 cells, medium was collected from urate-treated WT and Tg astrocytes and immediately used to pretreat MES 23.5 cells. Cell viability of MES 23.5 cells pretreated with medium collected from urate-treated Tg astrocytes was reduced in comparison to cell viability of MES 23.5 cells pretreated with medium collected from urate-treated WT astrocytes, suggesting a critical role for intracellular urate in the release of a soluble astrocyte-derived protective factor (Fig. 6c).

Discussion

In cultures, urate markedly enhanced survival of dopaminergic neurons in a model of spontaneous cell death (Guerreiro *et al.* 2009) and reduced oxidative stress as well as cell death induced by toxicants (Duan *et al.* 2002; Haberman *et al.* 2007; Zhu *et al.* 2011; Cipriani *et al.* 2012). Urate's protective effects have been also found *in vivo*, where urate prevented dopaminergic cell death in a rodent model of PD (Wang *et al.* 2010).
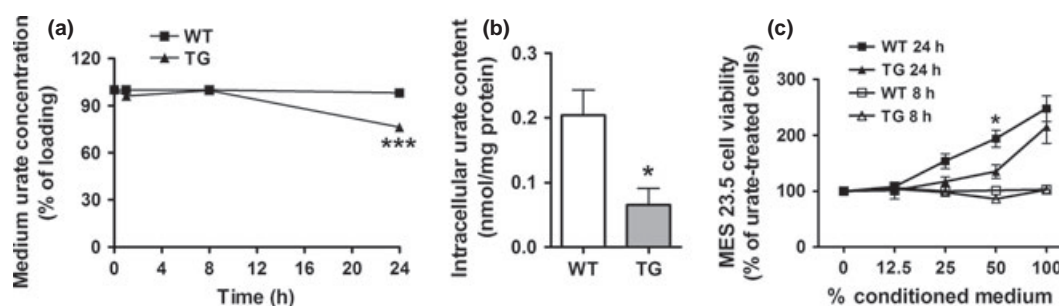

Fig. 6 UOx expression in transgenic astrocytes reduced astrocyte-mediated protection of H_2O_2-treated MES 23.5 cells. (a) Medium urate concentration in 100 μM urate-treated wild-type (WT) and transgenic (Tg) astroglial cultures over the time ($n = 3$; ***$P < 0.001$ vs. respective WT value). Error bars were smaller than symbols for all data points. (b) Intracellular urate content in WT and Tg astrocytes after 8 h of treatment with 100 μM urate; Student's *t*-test ($n = 4$, *$p = 0.02$). (c) Effect of conditioned medium collected from urate-treated WT and Tg astrocytes on viability of 200 μM H_2O_2-treated cells ($n = 4$; *$p < 0.05$ vs. respective Tg value).

Although considerable evidence indicates that urate is a powerful antioxidant few studies have been investigated alternative mechanisms of its protective effect. Du and coworkers (Du *et al.* 2007) reported that the protective effect of urate on primary spinal cord neurons was dependent on the presence of astrocytes in cultures. They showed that urate induced up-regulation of the EAAT-1 glutamate transporter in astrocytes, suggesting that urate may enhance the ability of astrocytes to reduce extracellular glutamate levels around nearby neurons. Moreover, our previous studies showed that the effect of modulating intracellular urate content on the susceptibility of dopaminergic neurons to MPP$^+$ treatment was amplified in cultures containing a high percentage of astrocytes in comparison to cultures were glial growth was inhibited (Cipriani *et al.* 2012). Of note, the data reported in the present study argue against an important direct antioxidant action of urate in protecting stressed dopaminergic cells, or at least that this putative antioxidant action of urate is not sufficient to account for its benefits in this model.

In the present study, the protective role of urate on dopaminergic cells was demonstrated not only in co-cultures but also in conditioned medium experiments. The inability of UOx to prevent completely urate's effect confirmed that protection of dopaminergic cells is not induced by carry-over urate on its own, but more likely by the release of soluble protective factor(s) by astrocytes in response to urate. This finding also excludes a direct interaction between H_2O_2 and urate as an explanation for how urate attenuates H_2O_2 toxicity. Similarly, the previously reported ability of urate to increase EAAT-1 expression on astrocytes and thereby reduce local glutamate buffering (Du *et al.* 2007) could not directly explain urate's protective effect in the present study, in which the astrocyte-dependence does not require proximity between astrocytes and dopaminergic cells. Of note, the capacity of astrocytes to mediate neuroprotection by urate is not likely to be restricted to the cortical astrocytes- which we employed in this study based on their abundance relative to those in stratum or mesencephalon- being consistent with enhanced neuroprotection achieved with spinal cord and ventral mesencephalon astrocytes as well (Du *et al.* 2007; Cipriani *et al.* 2012).

Although the identity of a putative protective factor released by urate-stimulated astrocytes remains to be determined, there is ample precedent for the inducible release of neuroprotectants from astrocytes. For example, protective effects of pramipexole on a human dopaminergic cell line were found to be mediated by astroglial release of the brain-derived neurotrophic factor (Imamura *et al.* 2008). Similarly, grape seed extract was found to protect primary neurons against H_2O_2-induced cell death inducing IL-6 release from astrocytes (Fujishita *et al.* 2009; Li *et al.* 2009).

In the present study, we investigated whether the protective effect of urate could be mediated by elevation of its intracellular content in dopaminergic cells and astrocytes. In agreement with our previous findings (Cipriani *et al.* 2012), we found that *exogenous* urate elevated intracellular urate content in dopaminergic cells and astrocytes. Thus urate may have a protective effect on dopaminergic cells not only by modulating the redox status of cellular membranes, as previously suggested by (Guerreiro *et al.* 2009), but also by acting on intracellular targets. Increasing intracellular content by *exogenous* urate might better explain the effect induced in astroglial cultures where it was found to up-regulate protein expression (Du *et al.* 2007).

An intracellular conversion of urate to allantoin, a possible active metabolite of urate, was largely excluded by the absence of UOx expression in dopaminergic cells and astrocytes and by the lack of oxonate effect in dopaminergic cells. These data are in agreement with previous studies that reported low UOx activity in the brain (Truszkowski and Goldmanowna 1933; Robins *et al.* 1953). Moreover, if allantoin were the active, protective metabolite of urate then one would have expected transgenic *UOx* expression to have enhanced the protective effect of urate (by increasing it conversion to allantoin), rather than attenuating it as observed.

To investigate whether cellular urate accumulation was dependent on membrane carriers, transporter inhibitors were employed. The increase in intracellular urate content of MES 23.5 cells and astrocytes treated with urate was markedly reduced by these transport inhibitors, indicating that urate accumulation in cells was likely because of the uptake of *exogenous* urate rather than a modulation of the purine pathway. This hypothesis was also supported by the finding that *exogenous* urate did not significantly affect, intracellularly or extracellularly, the content of any other purine measured. In mixed cultures, all three of the urate transporter inhibitors tested – HCTZ, probenecid, and PZO – prevented urate's protective effect. The correlation of these protective effects with the blockade of urate accumulation in astrocytes but not in MES 23.5 cells, strengthens the evidence that urate increase in astrocytes is a critical first step in its protective effect on cultured dopaminergic cells. This hypothesis is supported by the finding that transgenic expression of UOx in astrocytes attenuated urate's protective effect on dopaminergic cells. Of interest, the loss of UOx enzyme during hominoid evolution (Oda *et al.* 2002) has increased urate levels in the human body and it has been proposed to have raised antioxidant levels in human ancestors and thereby lengthened their lifespans. Verisimilarly, loss of UOx expression may have enhanced cellular antioxidant defenses by not only increasing circulating levels of urate in the human body but also presumably its intracellular content.

Urate transporters are highly expressed in the kidney where they control urate secretion and reabsorption. Urate transporters have also been found in the human and rodent

brain at the level of choroid plexus and blood-brain barrier (Alebouyeh *et al.* 2003; Mori *et al.* 2003) and localized in neuronal and endothelial cells (Ohtsuki *et al.* 2004; Bahn *et al.* 2005). The presence of urate transporters suggests a possible role for these carriers in regulating urate homeostasis in the brain, although their function there is unknown. Interestingly, an allelic variation in the *GLUT9* gene, associated with lower serum uric acid levels, was reported to correlate with a lower age at onset in PD (Facheris *et al.* 2011).

Translational significance

A compelling convergence of epidemiological, clinical, and initial cellular studies has suggested a potential neuroprotective effect of higher urate levels on dopaminergic neurons (Cipriani *et al.* 2010; Shoulson 2010) and expedited development of a phase II randomized clinical trial of inosine to elevate urate in PD (http://clinicaltrials.gov/ct2/show/NCT00833690). In parallel, efforts to gain mechanistic insight into protection by urate might be of considerable therapeutic as well as biological value as they could impact both the rationale and the pace of advancing to phase III clinical investigation. The present findings, in a cellular oxidative stress model of PD, provide evidence of a novel urate mechanism, possibly independent of its established antioxidant properties and support its candidacy as a neuroprotective agent for PD. They also suggest a more intricate mechanism of action that involves an astroglial intermediate, consistent with a growing appreciation of the critical pathophysiological role for astrocytes in the cellular microenvironment of degenerating neurons in PD (Rappold and Tieu 2010).

In addition, our findings that urate transporters can modify purine uptake and dopaminergic cell death extend the range of translational strategies for targeting urate levels in PD. Although initial human trials aiming to raise CNS urate elevation in PD are conservatively focused on a precursor (inosine) approach, a drawback is the increased risk of gout and uric acid urolithiasis that accompanies the associated systemic rise in urate levels. Our demonstration that urate transport inhibitors commonly employed in clinical practice (e.g., probenecid and HCTZ, which lower and raise serum urate, respectively) can block urate uptake and dopaminergic cell death *in vitro* suggests that transport-targeted therapeutics may provide an alternative or adjunct to urate precursors. Thus they may avoid peripheral complications of hyperuricemia. Because the directionality of urate transport at the tissue (e.g., blood-brain barrier) as well as the cellular levels are not easily addressed in culture models, *in vivo* preclinical studies of urate transport pharmacology in the CNS and in whole animal models of PD will be an important next step.

In conclusion, we found that protection of dopaminergic cells by urate depends on its accumulation in astroglial cells that in turn release soluble protective factors. The data bolster the rationale for targeting urate elevation as a therapeutic strategy for PD and indicate that urate transporters on astrocytes might also be a pharmacological target to modulate urate levels in PD brain.

Acknowledgements

Supporting information

Additional supporting information may be found in the online version of this article:

Figure S1. Cellular composition of astroglia-enriched cultures.

Figure S2. Undectable uricase expression in MES 23.5 cells and astrocytes.

Table S1. Intracellular urate accumulation in vehicle (control) and urate-treated MES 23.5 cells.

Table S2. Intracellular urate accumulation in vehicle (control) and urate-treated astrocytes.

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Disrupted and transgenic *urate oxidase* alter urate and dopaminergic neurodegeneration

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Urate is the end product of purine metabolism in humans, owing to the evolutionary disruption of the gene encoding urate oxidase (UOx). Elevated urate can cause gout and urolithiasis and is associated with cardiovascular and other diseases. However, urate also possesses antioxidant and neuroprotective properties. Recent convergence of epidemiological and clinical data has identified urate as a predictor of both reduced risk and favorable progression of Parkinson's disease (PD). In rodents, functional UOx catalyzes urate oxidation to allantoin. We found that *UOx* KO mice with a constitutive mutation of the gene have increased concentrations of brain urate. By contrast, *UOx* transgenic (Tg) mice overexpressing the enzyme have reduced brain urate concentrations. Effects of the complementary *UOx* manipulations were assessed in a mouse intrastriatal 6-hydroxydopamine (6-OHDA) model of hemiparkinsonism. *UOx* KO mice exhibit attenuated toxic effects of 6-OHDA on nigral dopaminergic cell counts, striatal dopamine content, and rotational behavior. Conversely, Tg overexpression of *UOx* exacerbates these morphological, neurochemical, and functional lesions of the dopaminergic nigrostriatal pathway. Together our data support a neuroprotective role of endogenous urate in dopaminergic neurons and strengthen the rationale for developing urate-elevating strategies as potential disease-modifying therapy for PD.

U rate, the anionic component of uric acid, predominates at physiological pH. As an apparent consequence of mutations in the *urate oxidase* (*UOx*) gene during primate evolution, urate constitutes the enzymatic end product of purine metabolism in humans (1). There remains controversy over how the loss of UOx activity and the resultant high urate concentrations in hominoids may have been beneficial and whether it still is. On one hand, urate is considered a pathogenic factor in gout, urolithiasis, and nephropathy, and hyperuricemia is associated with other medical conditions, such as hypertension, cardiovascular disease, and metabolic syndrome (2). On the other hand, the loss of UOx activity through multiple independent mutations in hominoids presumably conferred evolutionary advantages. Urate possesses potent antioxidant properties. High urate levels may have provided an antioxidant defense against aging and cancer, thereby contributing to a prolonged hominoid life span (3). In addition, increased urate may mediate blood pressure homeostasis in low-salt environments. Furthermore, higher urate has been suggested to enhance human intelligence or motivational behaviors or promote neuronal integrity and function (4).

Recently a series of population and clinical epidemiology studies have lent support to a potential neuroprotective effect of urate (5, 6). These studies demonstrated a robust inverse link between urate levels and both the risk and clinical progression of Parkinson's disease (PD), one of the most common neurodegenerative diseases. Given the putative role of oxidative stress in the pathogenesis of PD (7), these studies have identified urate as not only a unique biomarker for PD risk and progression but also a potential new target for treatment of PD (5, 6). A clinical trial of a urate precursor in PD has been launched as part of an effort to explore urate elevation as a possible disease-modifying strategy for PD (8).

To better understand the biological basis for a role of urate in PD and better gauge the therapeutic potential of urate in the treatment of neurodegenerative disease, we investigated the effects of urate manipulation in a well-established mouse 6-hydroxydopamine

(6-OHDA) model of PD. Urate concentrations were altered by modifying the *UOx* gene, which in rodents encodes a functional enzyme catalyzing the degradation of urate to allantoin. Comprehensive pathological, neurochemical, and behavioral outcome measures were evaluated to determine nigrostriatal dopaminergic pathway deficits after unilateral intrastriatal 6-OHDA infusion in *UOx* KO and transgenic (Tg) mice, in which respective elevations and reductions were achieved in brain concentrations of urate.

Results

Altered Urate but Not Its Precursors in *UOx* KO and Tg Mouse Brain. Western blotting was performed to confirm deletion and overexpression of UOx in peripheral tissues and brain of adult mice from *UOx* KO and Tg lines. As expected, there was no detectable UOx in liver, heart, or brain in *UOx* KO mice. In contrast, UOx was expressed in all organs examined in *UOx* Tg mice. Littermate WT animals did not have detectable UOx in brain and heart, consistent with a previous report that UOx is a liver-specific enzyme (Fig. 1*A*) (9).

Urate and its purine precursors—adenosine, inosine, hypoxanthine, and xanthine—in serum and brain tissue (striatum) were quantified by HPLC coupled with UV and electrochemical detection. In *UOx* KO mice, serum urate reached 5.2 mg/dL, more than 10-fold greater than in their WT littermates ($P < 0.01$, t test) (Fig. 1*B*). Despite the absence of UOx in the brain of naïve mice and presumably minimal local central nervous system (CNS) effects of *UOx* disruption, the increase of urate in the periphery was accompanied by a significant increase in urate in brain. Striatal urate in *UOx* KO animals was four times higher than in WT littermate controls ($P < 0.01$, t test) (Fig. 1*C*). Disruption of *UOx* did not result in changes in purine precursors of urate in brain. Similarly, striatal levels of the urate metabolite allantoin in *UOx* KO mice, which was quantified by LC-MS, was not different from WT mice, in agreement with undetectable UOx activity in WT brain (9) and therefore minimal local enzymatic contribution to CNS levels of allantoin (Fig. 1*D*).

In *UOx* Tg animals, HPLC analysis revealed a more than fivefold reduction in serum urate when compared with WT non-Tg littermates ($P < 0.01$, t test) (Fig. 1*E*). Striatal urate was also significantly lower in the Tg mice ($P < 0.05$, t test) but to a lesser extent than in serum despite broad expression of UOx transgene driven by a β-actin promoter (10) (Fig. 1*F*). The similar gradient and yet tight correlation in urate concentrations between blood and brain in both *UOx* KO and Tg mice may reflect a role of blood–brain barrier in regulating brain concentrations of urate (6). No significant differences in adenosine, inosine, hypoxanthine, or xanthine were observed between *UOx* Tg mice and their WT non-Tg littermates. However, striatal allantoin was elevated in *UOx* Tg mice, consistent with locally increased UOx activity in these mice (Fig. 1*G*).
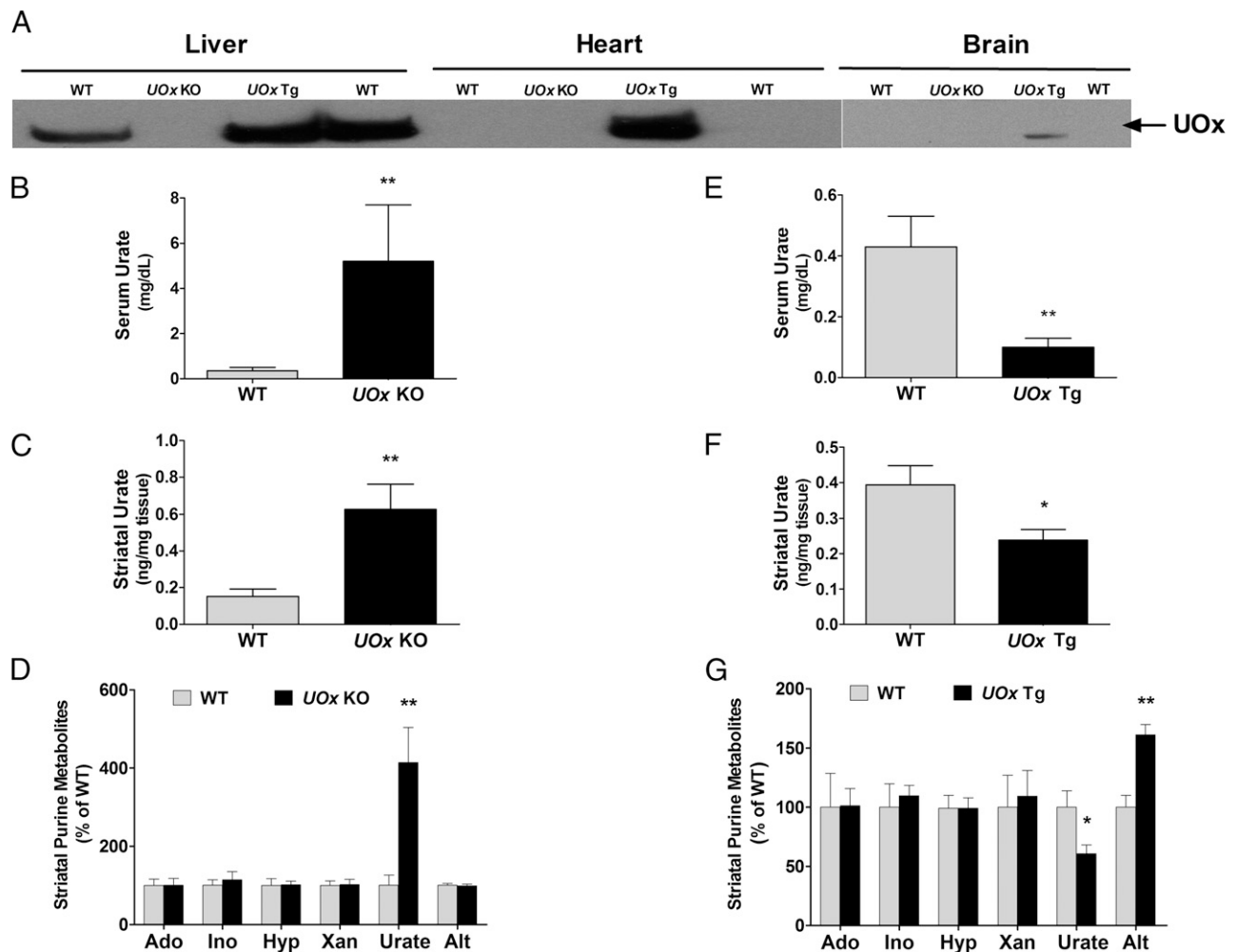
Fig. 1. Altered urate in serum and brain in *UOx* KO and Tg mice. (*A*) Western blot of UOx showing the absence of UOx in liver, heart, and brain in a *UOx* KO mouse (10 mo old). UOx is expressed in liver, heart, and brain in a *UOx* Tg mouse (12 mo old), and it is not detectable in heart and brain in WT mice. HPLC analysis indicates elevated urate levels in blood (*B*) and brain (*C*) in *UOx* KO mice. (*D*) Changes in urate levels are not accompanied by changes in concentrations of urate precursors adenosine (Ado), inosine (Ino), hypoxanthine (Hyp), xanthine (Xan), or urate metabolite allantoin (Alt) in brain in *UOx* KO mice. Conversely, *UOx* Tg mice have lower urate levels in blood (*E*) and brain (*F*). Overexpression of *UOx* also leads to an increase in striatal Alt in the Tg mice (*G*). There are no significant differences in striatal Ado, Ino, Hyp, or Xan between *UOx* Tg mice and the non-Tg WT littermate controls (*G*). Data are expressed as mean ± SEM. n = 6, WT and *UOx* KO (8–10 mo old); n = 9, WT and *UOx* Tg (4–5 mo old). *P < 0.05 vs. WT; **P < 0.01 vs. WT.

Impaired Renal Function in UOx KO Mice but Not *UOx* Tg Mice. Given that altered urate levels are often associated with renal dysfunction and that urate nephropathy has been reported in *UOx* KO mice (11), we monitored kidney and body weights and urea levels, an indicator of kidney function, in both the KO and Tg mice. As shown in Fig. 2*A*, adult (4 mo old) *UOx* KO had 30% lower kidney to body weight ratio, compared with WT littermates (P < 0.01, t test). Body weight in *UOx* KO mice was slightly lower but not statistically different from that in WT littermates during the entire experimental course. Brain urea levels in the KO animals were more than twice as high as in WT littermates (P < 0.01, t test) (Fig. 2*B*). *UOx* Tg mice had normal gross renal morphology, as well as kidney to body weight ratio, compared with WT littermates (Fig. 2*C*). Brain urea was not changed in these mice (Fig. 2*D*).

UOx Disruption or Overexpression Changes Levels of Protein Carbonyls. Urate is known to have antioxidant properties; altered urate levels resulting from *UOx* gene manipulation may therefore change susceptibility to oxidative stress. To evaluate oxidative stress status, levels of protein carbonyls, a general marker of oxidative damage, were assessed by Western blotting of immunoreactivity to derivitized protein carbonyl groups (Oxyblot) with tissues from adult

UOx KO and Tg mice. Band densities were normalized with Ponceau staining of the blots. The results did not demonstrate decreased levels of protein carbonyls, as one might expect, but instead a trend toward increased levels of protein carbonyls in liver of the KO animals (Fig. 3*A*) and significantly higher levels in brain (P < 0.01, t test) (Fig. 3*B*) compared with those in WT littermates. Overexpression of *UOx* also increased protein carbonyl content in both liver and brain, as shown in Fig. 3 *C* and *D*; relative band densities in liver and brain tissues from *UOx* Tg mice were higher than in WT non-Tg littermates (P < 0.05, both liver and brain, t test).

UOx KO Mice Are Resistant to 6-OHDA Neurotoxicity. To evaluate the effects of *UOx* disruption and urate elevation in a standard mouse model of PD, young adult *UOx* KO mice (average age, 3 mo) and their WT littermates received unilateral intrastriatal infusion of 6-OHDA, a dopaminergic toxin. Spontaneous and amphetamine-induced rotational behaviors were recorded 3 and 4 wk after lesioning, as behavioral indices of ipsilateral dopaminergic deficit. Animals were killed at 5 wk after lesion (Fig. 4*A*). *UOx* KO mice showed markedly reduced spontaneous net ipsilateral rotations (P < 0.05, t test) and a trend toward reduced amphetamine-induced rotations ipsilateral to the lesion (Fig. 4*B*). Neurochemical
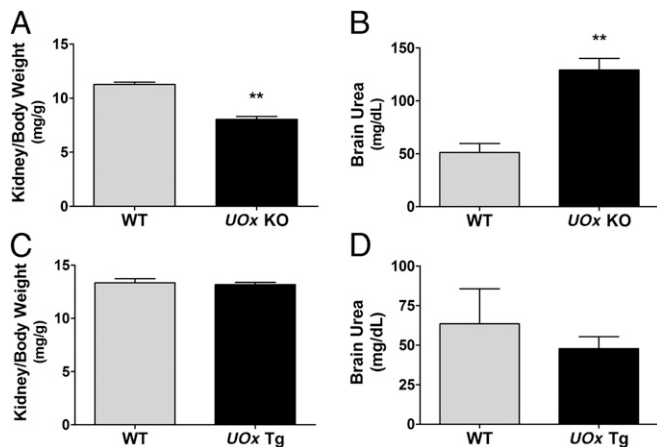
Fig. 2. Kidney to body weight ratios and urea levels in *UOx* KO and Tg mice. (*A*) *UOx* KO have significantly lower kidney to body weight ratio than WT mice (both kidneys from each animal; 4 mo old; $n = 11$ and 8 WT and *UOx* KO, respectively). (*B*) HPLC demonstrates a marked increase in brain urea level in *UOx* KO mice (3–4 mo old; $n = 5$, both WT and *UOx* KO). (*C*) Kidney to body weight ratio in UOx Tg mice (both kidneys from each animal. 6 mo old; $n = 11$ and 14 for WT and *UOx* Tg, respectively). (*D*) Brain urea is not changed in *UOx* Tg mice (4–5 mo old; $n = 5$, WT and *UOx* Tg). Data are expressed as mean ± SEM. **$P < 0.01$ vs. WT.

analysis demonstrated significantly higher levels of residual dopamine (DA) ($P < 0.05$, Tukey's post hoc test) and its metabolite homovanillic acid (HVA) on the lesion side of *UOx* KO animals
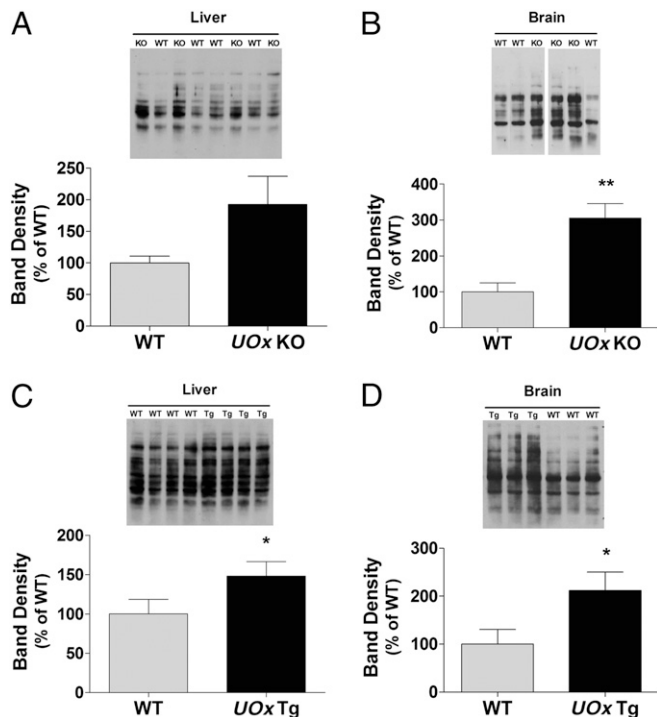

Fig. 3. *UOx* disruption or overexpression changes levels of protein carbonyls in mice. Protein carbonyls were assessed by Oxyblot. Band density was normalized with Ponceau staining of the proteins. (*A*) A trend toward increased levels of protein carbonyls in liver in *UOx* KO animals. (*B*) Protein carbonyls are higher in brain in *UOx* KO mice. Overexpression of *UOx* leads to increased protein carbonyl content in both liver (*C*) and brain (*D*) in Tg mice. Data are expressed as mean ± SEM $n = 6$, WT and *UOx* KO (3–4 mo old); $n = 5$, WT and *UOx* Tg (4–5 mo old). *$P < 0.05$; **$P < 0.01$ vs. WT.

compared with their WT littermate controls ($P < 0.01$, Tukey's post hoc test) (Fig. 4*C*). Increased striatal levels of urate were confirmed in the KO animals compared with WT controls ($P < 0.01$, Tukey's post hoc test), in which the intrastriatal 6-OHDA infusion produced a long-lasting local increase in urate content compared with that of the unlesioned striatum (Fig. 4*D*). This 6-OHDA–induced increase in urate may reflect persistent changes in oxidative stress status or energy metabolism after 6-OHDA. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), another commonly used parkinsonian toxin, has also been reported to increase striatal urate in mice (12). A representative set of sections stained for tyrosine hydroxylase (TH), a marker for dopaminergic neurons, showed few remaining TH-positive neurons in the substantia nigra (SN) on the lesion side in a WT mouse and preservation of TH positive neurons in a *UOx* KO mouse (Fig. 4*E*). Finally, stereological quantification of TH-positive nigral neurons indicated 46% survival of TH-positive neurons on the lesioned vs. unlesioned side in KO mice, a twofold increase over the percentage of surviving neurons in WT littermates (Fig. 4*F*) ($P < 0.05$, Tukey's post hoc test).

UOx Tg Mice Are Susceptible to 6-OHDA Neurotoxicity. The asymmetric turning behavior reflecting the extent of ipsilateral dopaminergic deficits was significantly exacerbated in *UOx* Tg mice at 3 and 4 wk after 6-OHDA infusion for both spontaneous ($P < 0.01$, t test) and amphetamine-induced ($P < 0.05$, t test) rotations in *UOx* Tg mice (average age, 5 mo) compared with WT non-Tg littermates (Fig. 5*A*). Consistent with the neurochemical phenotype of *UOx* Tg mice shown in Fig. 1*F*, their unlesioned striata had a significantly lower urate content than in WT non-Tg littermates. 6-OHDA lesioning induced an increase in local urate in WT mice, and even in the presence of excess UOx, in their Tg counterparts (Fig. 5*B*). DA and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) in the striatum decreased by ~70% after 6-OHDA in WT non-Tg mice. In *UOx* Tg mice, DA content on the lesion side was further reduced to 13% of that of the nonlesion control side, a significant difference from WT littermates. DOPAC in the Tg mice was reduced to 20% of control nonlesion side, significantly lower than in WT mice ($P < 0.05$, DA and DOPAC, Tukey's post hoc test) (Fig. 5*C*). Quantitative stereological analysis demonstrated a significant decrease in the number of residual TH-positive nigral neurons on the lesion side in *UOx* Tg mice, compared with WT non-Tg littermates ($P < 0.01$, Tukey's post hoc test) (Fig. 5*D*). The difference was still significant statistically when expressed as percentage of control (CON) to normalize for the small difference on the control (unlesioned) side between the two groups of animals ($P < 0.01$, Tukey's post hoc test). The subtle reduction in TH-positive neurons but not in DA content in *UOx* Tg remains to be further characterized. Representative sections of the ventral mesencephalon stained for TH depicted the extensive disruption of dopaminergic neurons in the SN in a *UOx* Tg mouse at 5 wk after intrastriatal 6-OHDA (Fig. 5*E*).

Discussion

In contrast to the established and hypothesized deleterious effects of urate on human health, its putative protective effects against disease have taken on particular relevance for CNS function and disorders. Among neurodegenerative diseases, PD has been most closely linked to low urate by convergent epidemiological and clinical findings (5, 6). In pursuing their translation toward therapeutics it is important to understand whether and how urate may have disease-modifying effects in preclinical models of PD. By using complementary genetic approaches disrupting and overexpressing *UOx*, we have demonstrated that disruption of the *UOx* gene with a resultant rise in urate protects the nigrostriatal dopaminergic system, and conversely that transgenic overexpresssion of *UOx* with a resultant fall in urate exacerbates dopaminergic neurodegeneration and resultant neurochemical and behavioral deficits in a 6-OHDA mouse model of PD.
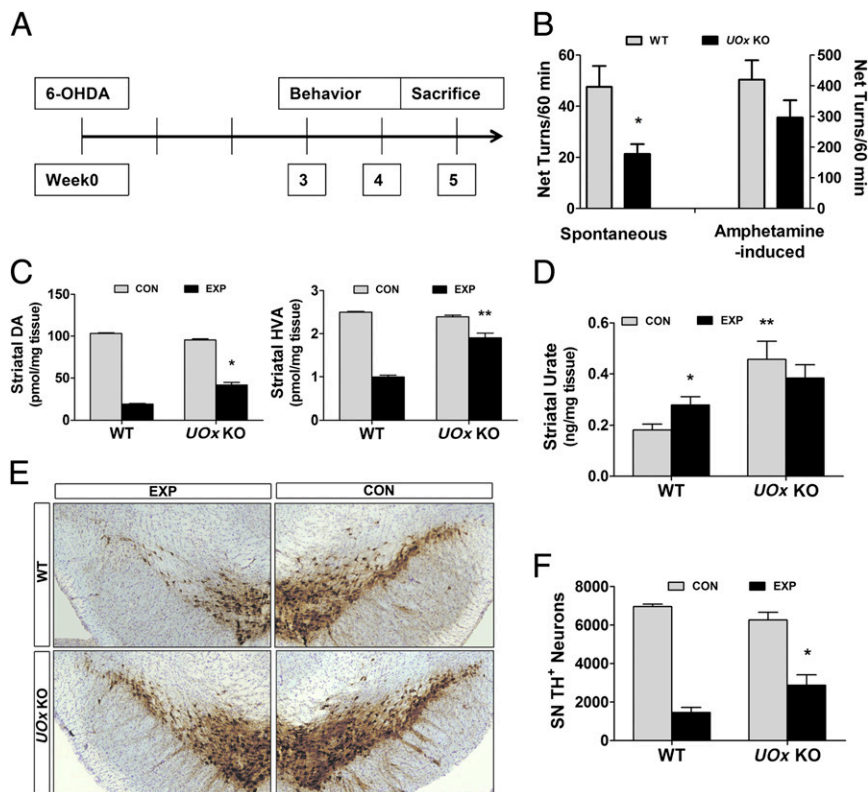
Fig. 4. *UOx* KO mice are more resistant to 6-OHDA neurotoxicity. (*A*) 6-OHDA (15 μg) was infused into the left striatum of *UOx* KO mice (average age, 3 mo). Spontaneous and 5 mg/kg amphetamine-induced rotational behavior were recorded at 3 and 4 wk after the lesion. Animals were killed at 5 wk after 6-OHDA lesion. (*B*) Spontaneous net ipsilateral rotations in *UOx* KO mice are attenuated (*$P < 0.05$ vs. WT), with a similar trend for attenuated amphetamine-induced net ipsilateral turns (WT $n = 11$; *UOx* KO $n = 9$). (*C*) *UOx* KO animals have significantly higher levels of DA and HVA on the experimental (lesion) side compared with their WT littermates (WT $n = 11$; *UOx* KO $n = 8$). *$P < 0.05$; **$P < 0.01$ vs. WT experimental side. (*D*) HPLC-electrochemical detection (ECD) confirms an increased level of urate in the *UOx* KO group. Injection of 6-OHDA induces an increase in urate in WT mice ($n = 11$ and 9 WT and *UOx* KO, respectively). *$P < 0.05$; **$P < 0.01$ vs. WT control side. (*E*) Preservation of SN dopaminergic neurons (TH positive) on the lesion side in a *UOx* KO mouse and disruption of SN TH neurons in a WT mouse. (*F*) Stereological quantification of TH neurons in the SN demonstrates more surviving dopaminergic neurons in the KO mice ($n = 8$ both WT and *UOx* KO groups). *$P < 0.05$ vs. WT experimental side. Data are expressed as mean ± SEM. CON, control nonlesion side; EXP, experimental lesion side.

Neuroprotective effects of urate have been reported in various in vitro and in vivo experimental models of neurological disorders, including ischemic brain injury (13, 14), multiple sclerosis (15), and spinal cord injury (16, 17). However, evidence regarding urate in PD models is sparse and largely restricted to cellular models of the disease. Urate blocked DA-induced apoptotic cell death, and it protected against 6-OHDA toxicity in PC12 cells (18, 19). In dopaminergic neurons, it reduced mitochondrial dysfunction and cell death occurring spontaneously in culture or induced by pesticides rotenone or iron ions (20, 21). We report here that *UOx* KO mice are more resistant to 6-OHDA neurotoxicity. *UOx* disruption with elevated urate levels can prevent DA loss, promote long-term survival of dopaminergic neurons, and preserve functional performances after 6-OHDA lesion.

Fig. 5. *UOx* Tg mice are more susceptible to 6-OHDA neurotoxicity. *UOx* Tg mice (average age, 5 mo) received intrastriatal 6-OHDA infusion. Behavioral tests were performed and animals killed at time points indicated in Fig. 4*A*. (*A*) Marked increases in both spontaneous and amphetamine-induced net ipsilateral rotations in *UOx* Tg mice after 6-OHDA infusion compared with WT non-Tg mice ($n = 11$ WT; $n = 14$ *UOx* Tg). *$P < 0.05$; **$P < 0.01$ vs. WT. (*B*) *UOx* Tg mice had lower urate levels in the striatum, and 6-OHDA induces local increases in urate in both WT and Tg mice ($n = 11$ and 14 WT and *UOx* Tg, respectively). **$P < 0.01$ vs. WT nonlesion control side; #$P < 0.05$ vs. WT experimental side, and vs. *UOx* Tg control side. (*C*) Significant further reductions in DA and DOPAC on experimental side in *UOx* Tg animals after 6-OHDA lesion ($n = 11$ and 14 WT and *UOx* Tg, respectively). *$P < 0.05$ vs. WT experimental side. (*D*) A significant decrease in the number of nigral TH-positive neurons on the experimental side in *UOx* Tg mice, compared with WT littermates. The difference is still significant statistically when expressing as percentage of CON to normalize for the difference on control side between the two genotypes ($n = 6$ and 7 WT and *UOx* Tg, respectively). *$P < 0.05$ vs. WT nonlesion control side; ##$P < 0.01$ vs. WT experimental side. (*E*) Few remaining TH-positive neurons in the SN in a *UOx* Tg mouse after intrastriatal 6-OHDA. Data are expressed as mean ± SEM. CON, control nonlesion side; EXP, experimental lesion side.
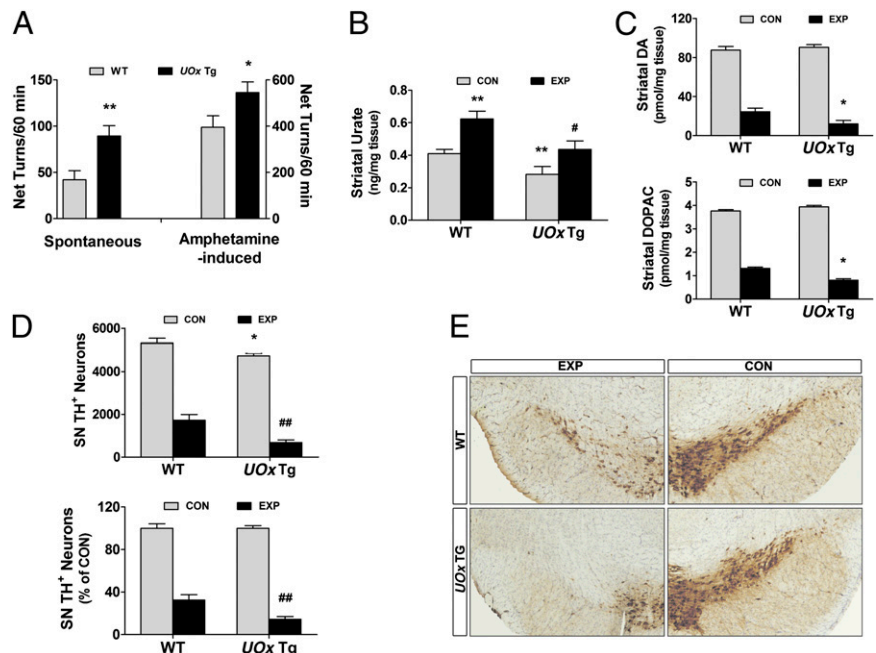
Conversely, Tg mice overexpressing *UOx* demonstrate enhanced susceptibility to 6-OHDA neurotoxicity. Lower urate levels have been associated with higher risk of PD, as well as more rapid clinical progression of PD (5, 6) and possibly other neurodegenerative diseases (22, 23). Similarly, lower urate levels have been consistently reported in PD patients compared with control subjects (24, 25). However, no experimental evidence has directly linked hypouricemia to neurodegeneration in vivo. Overexpression of *UOx* in mice leads to significant reduction in urate both in blood and in brain. The exacerbated neurotoxicity of 6-OHDA on the nigrostriatal dopaminergic pathway in *UOx* Tg mice entails greater DA depletion, more extensive neuron loss, and exacerbated asymmetry of movement. These findings in vivo are consistent with our recent report that this *UOx* transgene exacerbates dopaminergic neuron degeneration in a cellular model of PD (26).

Our findings thus provide a demonstration that genetic modulation of *UOx* modifies brain concentrations of urate and neurodegeneration in an established model of PD, supporting a contention that the known neuroprotective effects of urate itself may account for the complementary phenotypes of these opposing genetic manipulations in the 6-OHDA model of PD. However, altering *UOx* may have had other effects, particularly on purine metabolism, that could provide alternative explanations for the observed phenotypes. Blocking or accelerating purine catabolism at the level of UOx might also be expected to alter steady-state levels of its product allantoin as well as the multiple precursors of urate, including adenosine and inosine, which are themselves capable of modifying neuronal viability (27, 28). However, we have found that brain concentrations of major purine precursors upstream of urate from adenosine to xanthine are unaltered in *UOx* KO and Tg mice, arguing strongly against a proximal metabolic alteration as the basis of their phenotypes. Similarly, in *UOx* KO mice levels of brain allantoin were unaltered, confirming the absence of functional endogenous UOx in WT brain and supporting the hypothesis that the increased brain concentration of urate in *UOx* KO mice is the basis of their neuroprotective phenotype. By contrast, in *UOx* Tg mice allantoin was significantly increased in brain, raising the possibility of an alternative explanation for their exacerbated neurotoxicity other than the commensurate reduction in brain urate. However, the possibility that elevated allantoin mediates the *UOx* Tg phenotype presumes that allantoin can act as a neurotoxicant. However, the only available data of relevance indicate that allantoin actually displays neuroprotective properties in a 6-OHDA model of PD (29). Collectively, these data suggest that alterations in urate, rather those in allantoin or another purine metabolite, are the basis for the observed *UOx* KO and Tg phenotypes.

Urate is a potent antioxidant, and antioxidant properties of urate have been proposed to mediate its neuroprotective effects in most aforementioned studies (13–21). We investigated oxidative stress status in *UOx* KO and Tg mice and found higher protein carbonyls, one of the most commonly used markers of oxidative stress, in both. Although an increase in basal levels of oxidative protein modification in *UOx* Tg animals is consistent with their lower levels of antioxidant urate, the converse hypothesis of lower levels of protein carbonyls in *UOx* KO mice is not supported. It is uncertain why protein carbonyls changed in the same direction despite urate level modulation in opposite directions. However, we are not the first to observe dissociation between urate and oxidative stress indices, protein carbonyl levels in particular. Clinical studies have revealed higher or unchanged protein carbonyls in patients with high urate, including refractory gout patients (30–32). Furthermore, urate has the capacity to act as a prooxidant under some circumstances (33–35).

In addition to this possible dual role of urate, it is particularly noteworthy that *UOx* KO mice develop nephropathy early in their lives despite pre- and perinatal allopurinol treatment. The severe kidney damage we and the others have documented (11, 36) in *UOx* KO mice may have confounded the testing of our hypothesis that elevated urate could confer antioxidant protection under basal conditions. Excessive oxidative stress has been linked to various renal pathologies (37), and urea, specifically, has been shown both in vitro and in vivo to induce reactive oxidative species (38). It is possible that an offsetting systemic effect of chronic nephropathy may predominate in determining the basal levels of oxidative stress in *UOx* KO mice. Therefore, despite the known oxidative mechanisms of 6-OHDA neurotoxicity (39) and antioxidant properties of urate, the basis for attenuated and exacerbated neurodegeneration in *UOx* KO and Tg mice, respectively, remains to be established.

Our complementary genetic approaches targeting *UOx* effectively manipulated urate in mice both peripherally and, perhaps more importantly in this study, in their brains. The findings that *UOx* KO (with higher urate) are more resistant to local 6-OHDA lesioning and that *UOx* Tg (with lower urate) are more susceptible to this neurotoxin support the possibility of a neuroprotective role for urate and PD. Together with previous epidemiological and clinical evidence (5, 6), these findings strengthen the rationale for investigating urate-elevating agents as potential therapeutic approaches to PD and possibly other neurodegenerative diseases. As proof-of-concept, our genetic study together with newly published pharmacological data (40) provides critical experimental evidence in vivo that urate may have beneficial CNS actions in the context of PD, and it provides a basis and justification for further mechanistic investigation. Nevertheless, further efforts to investigate the therapeutic potential of urate elevation—even within what is considered a "normal range"—must be tempered by known and potential risks of excessive urate.

Methods

Experimental Animals. *UOx* KO mice, originally constructed by Wu et al. (11) by homologous recombination disrupting exon 3 of the *UOx* gene, were obtained from the Jackson Laboratory. Our initial characterization indicated that whereas homozygous mice demonstrated significantly elevated urate in both serum and brain (Fig. 1), heterozygous *UOx* KO animals did not have a urate elevation phenotype (11). We therefore used only homozygous mice (generated by heterozygote × heterozygote crosses) for this study. Allopurinol (150 mg/L) was provided in the drinking water of breeders and pups until weaning for rescue from perinatal lethality of hyperuricemia (11). *UOx* Tg mice were obtained from Kenneth L. Rock, Department of Immunology, University of Massachusetts, Worcester, MA. *UOx* transgene expression is driven by a strong constitutive (β-actin) promoter (10). Hemizygous *UOx* Tg mice were used. Both *UOx* KO and Tg mice had been back-crossed to C57BL/6 (Jackson Laboratory) for at least eight generations. *UOx* KO, *UOx* Tg mice and their littermate controls were maintained in home cages at constant temperature with a 12-h light/dark cycle and free access to food and water. All animal protocols were approved by the Massachusetts General Hospital Animal Care and Use Committee.

Measurement of Urate and Urate Precursors. Animals were killed at indicated times via cervical dislocation. Whole blood was collected, and striatal tissues were dissected. Samples were prepared, and adenosine, inosine, hypoxanthine, xanthine, and urate concentrations were determined simultaneously using an HPLC method that we developed and recently reported (41).

Measurement of Allantoin. The urate metabolite allantoin was determined by Bioanalytical Systems. Animals were killed via cervical dislocation. Fresh frozen striatal tissues were weighed and homogenized in water. A volume of 100 µL was taken for extraction. Calibrator, quality control standard, and sample homogenates were extracted with acetonitrile in a 96-well plate after adding isotope-labeled allantoin as internal standard. LC-MS was used for detection.

Western Blot. For Western blot analysis of UOx, liver, heart, or brain tissues were obtained. Proteins were extracted and electrophoresed. After transferring, the membrane was treated with rabbit anti-UOx antibody (Santa Cruz Biotechnology, catalog no. SC33830) at 1:200, followed by secondary antibody (Thermo Scientific, catalog no. 32460). Densitometric analysis of band intensity was performed by using the Image J system (National Institutes of Health).

Protein Carbonylation. Protein carbonlys in liver and brain were detected using the Oxyblot Protein Oxidation Detection Kit (Millipore, catalog no. S7150) according to the manufacturer's instructions. Densitometric analysis of band intensity was performed by using the Image J system.

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6-OHDA Lesion. Mice received a unilateral intrastriatal injection of 6-OHDA (42). Animals were pretreated with desipramine (Sigma-Aldrich). A total dose of 15 µg 6-OHDA was infused into the left striatum at the following coordinates: anterior–posterior (AP), +0.09 cm; medial–lateral (ML), +0.22 cm; dorsal-ventral (DV), −0.25 cm relative to bregma.

Rotational Behavior Assessment. Spontaneous and 5 mg/kg amphetamine-induced rotational behavior in mice was tested at 3–4 wk after the 6-OHDA lesion using an automated rotometry system (San Diego Instruments) as previously described (42). Results were expressed as ipsilateral net turns (net difference between ipsilateral and contralateral turns) per 60 min.

Measurement of DA and Metabolite. Five weeks after 6-OHDA lesion, mice were killed by rapid cervical dislocation, and their striata were dissected. DA and metabolites DOPAC and HVA were determined by standard HPLC with electrochemical detection, as previously described (42).

TH Immunohistochemistry and Stereological Cell Counting. Immunostaining for TH was performed as described previously (43). Five weeks after 6-OHDA lesioning mice were killed by rapid cervical dislocation. The hinder brain block containing midbrain was immediately fixed and cryoprotected. Every fourth section from a complete set of coronal midbrain sections was processed. The primary antibody was mouse monoclonal anti-TH (Sigma-Aldrich, catalog no. T1299) at 1:800. Stereologic analysis was performed with the investigator blinded to genotypes using the Bioquant Image Analysis System (R&M Biometrics) (43). For each animal, the SN on both sides of the brain was analyzed. For each section, the entire SN was identified and outlined as the region of interest. The number of TH-positive neurons in each counting frame (50 µm × 50 µm) was then determined under 40× objective by focusing down through the section using the optical dissector method. Our criterion for counting an individual TH-positive neuron was the presence of its nucleus either within the counting frame or touching the right or top frame lines (green), but not touching the left or bottom lines (red). The total number of TH-positive neurons for each side of the SN was calculated: total number = raw counts × 4 (every fourth section) × 6.25 (area of grid 125 µm × 125 µm/ area of counting frame 50 µm × 50 µm).

Statistic Analysis. All values are expressed as mean ± SEM. The difference between two groups was analyzed by Student t test. Multiple comparisons among groups were performed by one-way ANOVA and Tukey's post hoc test. All statistical analyses were performed using SigmaStat software (SPSS). $P < 0.05$ is considered statistically significant.

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Conditional neural knockout of the adenosine A₂ₐ receptor and pharmacological A₂ₐ antagonism reduce pilocarpine-induced tremulous jaw movements: Studies with a mouse model of parkinsonian tremor

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Abstract
Tremulous jaw movements are rapid vertical deflections of the lower jaw that resemble chewing but are not directed at any particular stimulus. In rats, tremulous jaw movements can be induced by a number of conditions that parallel those seen in human parkinsonism, including dopamine depletion, dopamine antagonism, and cholinomimetic drugs. Moreover, tremulous jaw movements in rats can be attenuated using antiparkinsonian agents such as L-DOPA, dopamine agonists, muscarinic antagonists, and adenosine A₂ₐ antagonists. In the present studies, a mouse model of tremulous jaw movements was established to investigate the effects of adenosine A₂ₐ antagonism, and a conditional neuronal knockout of adenosine A₂ₐ receptors, on cholinomimetic-induced tremulous jaw movements. The muscarinic agonist pilocarpine significantly induced tremulous jaw movements in a dose-dependent manner (0.25–1.0 mg/kg IP). These movements occurred largely in the 3–7.5 Hz local frequency range. Administration of the adenosine A₂ₐ antagonist MSX-3 (2.5–10.0 mg/kg IP) significantly attenuated pilocarpine-induced tremulous jaw movements. Furthermore, adenosine A₂ₐ receptor knockout mice showed a significant reduction in pilocarpine-induced tremulous jaw movements compared to littermate controls. These results demonstrate the feasibility of using the tremulous jaw movement model in mice, and indicate that adenosine A₂ₐ receptor antagonism and deletion are capable of reducing cholinomimetic-induced tremulous jaw movements in mice. Future

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studies should investigate the effects of additional genetic manipulations using the mouse tremulous jaw movement model.

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1. Introduction

Resting tremor is a cardinal symptom of Parkinson's disease (Deuschl et al., 2001). Moreover, tremor and other parkinsonian symptoms can be induced by various drugs, including dopamine (DA) antagonists (Bezchlibnyk-Butler and Remington, 1994) and cholinomimetics (Song et al., 2008). Adenosine A_{2A} antagonists have emerged as a potential treatment of parkinsonian symptoms, including tremor (Schwarzschild et al., 2006; LeWitt et al., 2008). Adenosine A_{2A} receptors are highly expressed in neostriatum, and A_{2A} antagonists exert effects in animals that are consistent with antiparkinsonian actions (Ferré et al., 2008; Chen et al., 2001; Simola et al., 2004; Salamone et al., 2008; Collins et al., 2010). Clinical reports have indicated that adenosine A_{2A} antagonists significantly improve motor deficits, reduce OFF time, and increase ON time in parkinsonian patients (LeWitt et al., 2008).

One animal test that is useful for assessing the role of adenosine A_{2A} receptors in motor function is tremulous jaw movements (TJMs), an extensively validated rodent model of parkinsonian resting tremor (Simola et al., 2004; Miwa et al., 2009; Collins et al., 2010, 2011; for reviews, see Salamone et al., 1998; Collins-Praino et al., 2011). TJMs are rapid vertical deflections of the lower jaw that are not directed at any stimulus (Salamone et al., 1998), and occur in phasic bursts of repetitive jaw movement activity. TJMs have many of the neurochemical, anatomical, and pharmacological characteristics of parkinsonism, and meet a reasonable set of validation criteria for use as an animal model of parkinsonian tremor (Salamone et al., 1998; Collins-Praino et al., 2011). These movements are induced by conditions associated with parkinsonism, including neurotoxic or pharmacological depletion of striatal DA (Jicha and Salamone, 1991; Salamone et al., 2008), and DA antagonism (Ishiwari et al., 2005; Salamone et al., 2008). TJMs also are induced by cholinomimetic drugs, including muscarinic agonists such as pilocarpine and oxotremorine (Salamone et al., 1986, 1998; Collins et al., 2010), and anticholinesterases (Salamone et al., 1998; Simola et al., 2004; Collins et al., 2011). TJMs occur largely within the 3–7 Hz frequency range that is characteristic of parkinsonian resting tremor (Ishiwari et al., 2005; Collins et al., 2010), and can be attenuated by several classes of antiparkinsonian drugs, including DA agonists and anticholinergics (Salamone et al., 1998, 2005; Betz et al., 2009). Adenosine A_{2A} antagonists attenuate the TJMs induced by DA depletion, DA antagonism and cholinomimetics (Correa et al., 2004; Simola et al., 2004; Salamone et al., 2008; Betz et al., 2009; Collins et al., 2010, 2011; Pinna et al., 2010).

With the rising importance of genetic manipulations in mice (i.e. transgenic, knockout, knockin, etc.), it is necessary to investigate whether it is possible to extend well-validated behavioral paradigms currently being used in rats to mouse models. Although one previous study showed that muscarinic M4 receptor knockout mice showed significantly fewer cholinomimetic-induced TMMs than wild-type mice (Salamone et al., 2001), every other study of TJM activity has employed rats. Given the putative antiparkinsonian properties of adenosine A_{2A} receptor antagonists, it is of great interest to determine if adenosine A_{2A} receptor antagonism or genetic deletion reduces levels of TJM activity in mice. In order to investigate this research question, several experiments were necessary. The first experiment studied the ability of the muscarinic agonist pilocarpine to induce TJMs in the specific strain of mice being used for the knockout study (C57/BL6). The second experiment studied the local frequency range of the TJM "bursts" induced by pilocarpine using freeze-frame video analysis. Experiments 3 and 4 investigated the effects of the adenosine A_{2A} antagonist MSX-3 and genetic deletion of the adenosine A_{2A} receptor on pilocarpine-induced TJMs. It was hypothesized that A_{2A} knockout mice would show fewer TJMs than their wild-type littermates.

2. Experimental procedures

2.1. Animals

Male C57BL/6 mice (25; Harlan Laboratories, Indianapolis, IN, USA) were used for the first three studies. For the final study, a total of 24 neuronal A_{2A} receptor conditional knockout mice and their littermate controls (12 CaMKIIα-cre, A_{2A} flox/flox and 12 non-transgenic [no cre] A_{2A} flox/flox mice) congenic for the C57BL/6 background and with no prior drug experience were obtained from Massachusetts General Hospital (Boston, MA, USA; see Bastia et al., 2005 for details on the generation of these mice). Mice, weighed 15–40 g throughout the course of the experiment, had ad libitum access to lab chow and water, and were group-housed in a colony maintained at 23 °C with a 12-h light/dark cycle (lights on at 0700 h). Studies were conducted according to the University of Connecticut and NIH guidelines for animal care and use.

2.2. Drugs and selection of doses

Pilocarpine (Sigma Aldrich Chemical, St. Louis, MO, USA) was dissolved in 0.9% saline. The adenosine A_{2A} antagonist MSX-3 ((E)-phosphoric acid mono-[3-[8-[2-(3-methoxyphenyl)vinyl]-7-methyl-2,6-dioxo-1-prop-2-ynyl-1,2,6,7-tetra-hydropurin-3-yl]propyl] ester) was synthesized at the Pharmazeutisches Institut (Universität Bonn; Bonn, Germany), and dissolved in 0.9% saline. MSX-3 is a pro-drug of the active adenosine A_{2A} antagonist, MSX-2. Extensive pilot work was performed to determine doses, and the dose of 1.0 mg/kg pilocarpine used in experiments 2–4 was based upon the results of the first experiment.

2.3. Behavioral procedure: tremulous jaw movements

Observations took place in a $11.5 \times 9.5 \times 7.5$ cm clear glass chamber with a mesh floor, which was elevated 26 cm from the table top. TJMs were defined as rapid vertical deflections of the lower jaw

that resembled chewing but were not directed at any particular stimulus (Salamone et al., 1998). Each individual deflection of the jaw was recorded using a mechanical hand counter by a trained observer, who was blind to the experimental condition of the mouse being observed. Separate studies with two observers demonstrated an inter-rater reliability of $r = 0.98$ ($p < 0.001$) using these methods in mice.

2.4. Experiments

Experiment 1: ability of pilocarpine to induce tremulous jaw movements
Eleven male C57BL/6 mice were used to assess the effect of pilocarpine on TJMs. Mice received IP injections of either 1.0 ml/kg saline or 0.25, 0.5, 0.75, or 1.0 mg/kg pilocarpine in a within-groups design, with all mice receiving all treatments in a randomly varied order (once per week; no treatment sequences were repeated). Five minutes after injection, mice were placed in the observation chamber and allowed 5 min to habituate, after which TJMs were counted for 10 min.

Experiment 2: freeze-frame video analysis of local frequency of the tremulous jaw movements induced by pilocarpine
Three male C57BL/6 mice received an IP injection of 1.0 mg/kg pilocarpine. After five minutes, mice were placed in a flat bottomed mouse restrainer (myNeuroLab.com, Richmond, IL) so that a consistent view of the orofacial area could be achieved. After habituating for 5 min, each mouse was recorded for 15 min using a FlipVideo UltraHD (Cisco Systems, Farmington, CT). The sections of video that allowed for clear observation of the orofacial area were subjected to a freeze-frame analysis (1 frame = 1/30 s), in which the observer went frame-by-frame through each burst of jaw movements (i.e. each group of at least two jaw movements that were within 1.0 s of each other). The observer recorded the inter-movement interval for each pair of jaw movements within bursts, which was defined as the number of frames between each point at which the jaw was fully open during successive jaw movements. This information was used to determine the local frequency within bursts of jaw movements.

Experiment 3: ability of the adenosine A_{2A} antagonist MSX-3 to attenuate the tremulous jaw movements induced by pilocarpine
Eleven male C57BL/6 mice were used to assess the effects of the adenosine A_{2A} antagonist MSX-3 on the TJMs induced by 1.0 mg/kg pilocarpine. A within-groups design was utilized for this study, with all mice receiving all drug treatments in a randomly varied order (one treatment per week; no treatment sequences were repeated). On the test day each week, each mouse was given an IP injection of either 1.0 ml/kg saline or 2.5, 5.0, or 10.0 mg/kg MSX-3. After Ten minutes, all mice received an IP injection of 1.0 mg/kg pilocarpine. Five minutes after injections, mice were placed in the observation chamber and allowed 5 min to habituate, after which TJMs were counted for 10 min.

Experiment 4: ability of pilocarpine to induce tremulous jaw movements in mice with a knockout of the adenosine A_{2A} receptor
A total of 24 male C57BL/6 mice ($n = 12$ postnatal neuronal A_{2A} receptor conditional KO mice (A_{2A} $-/-$); $n = 12$ littermate controls (A_{2A} $+/+$)) were used to assess the effect of the knockout of the adenosine A_{2A} receptor on pilocarpine-induced TJMs. For this experiment, only homozygous A_{2A} KO mice and littermate controls were used. All mice received an IP injection of 1.0 mg/kg pilocarpine. Five minutes after IP injection, mice were placed in the observation chamber and allowed 5 min to habituate, after which TJMs were counted for 10 min by an observer blind to the condition of the mouse (i.e. littermate control vs. A_{2A} KO).

2.5. Data analyses

The data for experiments 1 and 3 were analyzed using a repeated measures analysis of variance (ANOVA). Average TJMs over the two five-min observation periods were calculated and then used in the ANOVA calculations (SPSS 12.0 for Windows). When there was a significant ANOVA, planned comparisons using the overall error term were used to assess the differences between each dose and the control condition; the total number of comparisons was restricted to the number of treatments minus one. The behavioral data from the knockout experiment (Experiment 4) was analyzed using Student's t-test for independent samples.

3. Results

3.1. Experiments 1 and 2: ability of pilocarpine to induce tremulous jaw movements.

There was a significant overall effect of pilocarpine treatment on TJM activity (Fig. 1A; $F(4, 40) = 24.46$; $p < 0.001$). All doses of pilocarpine significantly induced TJMs (planned comparisons, $p < 0.001$) compared to the vehicle condition. Fig. 1B displays the results of the freeze-frame video analyses. A total of 509 jaw movements were analyzed. About 83.69% of these jaw movements took place within "bursts," defined as a group of at least two jaw movements that were within 1.0 s of each other. Data are shown as the number of inter-movement intervals (i.e. the number of 1/30 s frames that elapsed from one jaw movement to another) from jaw movements in bursts, assigned to four frequency bins. To interpret these data in terms of frequencies (i.e. jaw movements per second), the reciprocal of the inter-movement interval was calculated (e.g. 10/30 frames per second corresponds to 3 Hz; 4/30 frames per second to 7.5 Hz, etc.) The majority (77.60%) of the TJMs took place in the 3.0-7.5 Hz frequency range. There were no jaw movements in the 1-3 Hz or > 10 Hz bins.

3.2. Experiments 3 and 4: ability of adenosine A_{2A} receptor antagonism and knockout attenuate the tremulous jaw movements induced by pilocarpine

The adenosine A_{2A} antagonist MSX-3 attenuated the TJMs induced by 1.0 mg/kg pilocarpine (Fig. 2A). There was a significant overall effect of MSX-3 treatment on pilocarpine-induced TJMs ($F(3,30) = 35.88$; $p < 0.001$), and the 2.5, 5.0 and 10.0 mg/kg doses of MSX-3 significantly reduced the pilocarpine-induced TJMs (planned comparisons, $p < 0.05$). Fig. 2B shows that adenosine A_{2A} receptor neuronal knockout mice (A_{2A} $-/-$) showed significantly fewer pilocarpine-induced TJMs than their littermate controls ((A_{2A} $+/+$); $t = 2.45$, df = 22; $p < 0.05$).

4. Discussion

These studies describe the development of a mouse model of TJM activity. Pilocarpine has consistently been shown to induce TJMs in rats (Salamone et al., 1986, 1998; Finn et al., 1997; Betz et al., 2007; Collins et al., 2010), so the first experiment investigated the ability of the pilocarpine to induce TJMs in C57BL/6 mice. Pilocarpine induced TJM
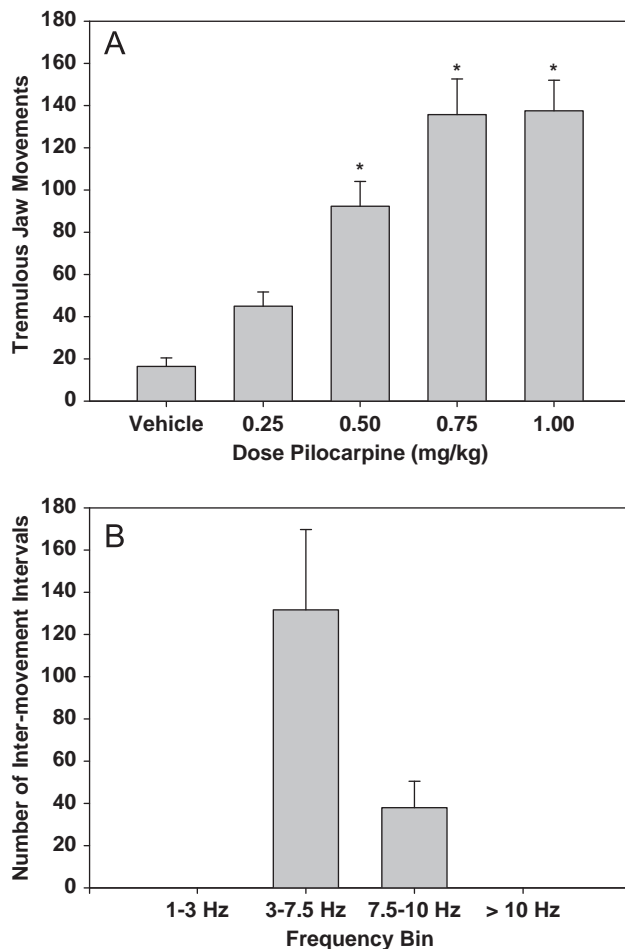
Fig. 1 (A) Effects of different doses of pilocarpine (IP) on tremulous jaw movements. Mean ($\pm$SEM) number of jaw movements in mice ($n=11$) treated with either saline vehicle or pilocarpine. **Significant difference from vehicle control ($p<0.05$). (B) This figure shows the results of the freeze-frame analysis of inter-movement intervals using the video analysis methods described above. Inter-movement times were determined by freeze-frame analysis of video obtained from three mice treated with 1.0 mg/kg pilocarpine, and were assigned to one of four local frequency bins. Distribution of the mean ($\pm$SEM) number of inter-movement intervals within each frequency bin is shown.

Fig. 2 (A) Effect of the adenosine A_{2A} antagonist MSX-3 on the tremulous jaw movements induced by 1.0 mg/kg pilocarpine. Mean ($\pm$SEM) number of jaw movements in mice ($n=11$) treated with pilocarpine plus vehicle (Veh/Pilo), and pilocarpine (Pilo) plus various doses (2.5, 5.0 and 10.0 mg/kg IP) of MSX-3. *Significant difference from pilocarpine plus vehicle control ($p<0.05$). (B) Effect of neuronal adenosine A_{2A} receptor knockout on the tremulous jaw movements induced by 1.0 mg/kg pilocarpine. Mean ($\pm$SEM) number of jaw movements in knockout mice ($n=12$) and littermate controls ($n=12$) treated with pilocarpine. *Significant difference from littermate controls ($p<0.05$).

activity in C57BL/6 mice at all doses tested (i.e. 0.25–1.0 mg/kg). This is consistent with the previous research indicating that the administration of pilocarpine induced TJMs in 129SvEv (50%) $\times$ CF1 (50%) mice (Salamone et al., 2001). Local frequency analysis of the pilocarpine-induced TJMs in mice indicated that pilocarpine-induced TJMs occurred largely in the 3–7.5 Hz frequency range, which is consistent with the findings from previous studies of the local frequency of TJMs induced by DA depletion, D2 antagonism, and administration of cholinomimetic drugs in rats (Ishiwari et al., 2005; Collins et al., 2010; Collins-Praino et al., 2011). Moreover, this 3–7.5 Hz frequency range is similar to that reported during resting tremor in parkinsonian patients (Deuschl et al., 2001). These findings are consistent with the hypothesis that pilocarpine-induced
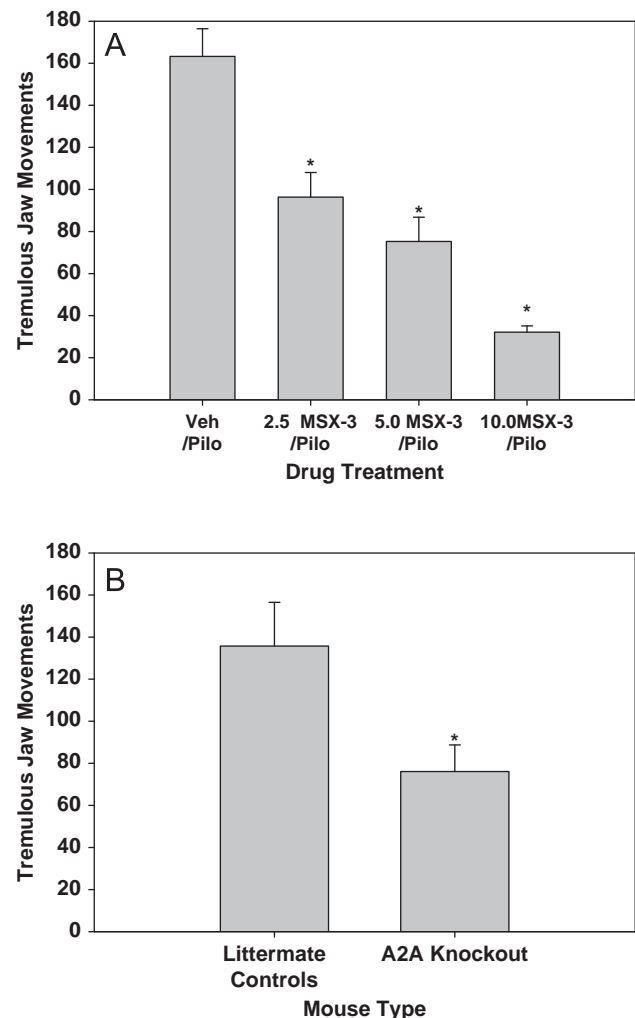
TJMs pilocarpine are potentially a useful mouse model of parkinsonian resting tremor. Also, the finding that pilocarpine is capable of significantly inducing TJMs in mice highlights the role that ACh plays in striatal motor functions related to parkinsonism. Cholinomimetic drugs, such as muscarinic agonists and anticholinesterases used for the treatment of Alzheimer's disease, have been shown to induce or exacerbate parkinsonian symptoms, including tremor, in humans (Song et al., 2008; Collins-Praino et al., 2011). Furthermore, muscarinic receptor antagonists have been used as treatments for the motor symptoms of parkinsonism (Bezchlibnyk-Butler and Remington, 1994).

Adenosine A$_{2A}$ antagonists have emerged as a potential treatment of parkinsonian motor impairments. One clinical report suggested that tremor was particularly sensitive to the effects of adenosine A$_{2A}$ antagonism (Bara-Jimenez et al., 2003). Adenosine A$_{2A}$ receptors are highly expressed in neostriatum, and A$_{2A}$ antagonists exert motor effects in rodents and primates that are consistent with antiparkinsonian actions (Ferré et al., 2008; Chen et al., 2001; Salamone et al., 2008; Collins et al., 2010). For that reason, the final two experiments investigated the ability of adenosine A$_{2A}$ receptor antagonism or genetic deletion to attenuate pilocarpine-induced TJMs. The adenosine A$_{2A}$ antagonist MSX-3 significantly attenuated pilocarpine-induced TJMs in mice, which is consistent with previous findings in rats (Correa et al., 2004; Simola et al., 2004; Salamone et al., 2008; Pinna et al., 2010; Collins et al., 2010, 2011). Furthermore, deletion of the adenosine A$_{2A}$ receptor also resulted in significantly lower levels of pilocarpine-induced TJMs compared to wild-type mice. This is consistent with previous research showing that knockout of the adenosine A$_{2A}$ receptor is capable of reversing the catalepsy induced by the DA D1 antagonist SCH 23390, the D2 antagonist haloperidol, and the muscarinic agonist pilocarpine (El Yacoubi et al., 2001). Moreover, genetic deletion of the adenosine A$_{2A}$ receptor in mice has been shown to alter the locomotor response to adenosine antagonists (Yu et al., 2008), and to affect amphetamine sensitization (Chen et al., 2003), self-administration of cocaine and MDMA (Ruiz-Medina et al., 2011), aspects of cognition (Wei et al., 2011), and effort-related choice behavior (Pardo et al., 2012). Furthermore, mice lacking striatal adenosine A$_{2A}$ receptors showed an absence of motor stimulation in response to adenosine A$_{2A}$ antagonists (Yu et al., 2008; Wei et al., 2011).

The present results demonstrate the feasibility of using the TJM model in mice, and indicate that adenosine A$_{2A}$ receptor antagonism and deletion are capable of reducing cholinomimetic-induced TJMs in mice. These findings add to growing evidence demonstrating that adenosine A$_{2A}$ function is involved in regulating motor functions in animals that are potentially related to parkinsonism. Additional studies should further characterize the effects of adenosine A$_{2A}$ receptor deletion on motor function, and should investigate the effects regionally-specific knockout of A$_{2A}$ receptors (e.g. Lazarus et al., 2011).

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Contributors

All authors contributed significantly to this manuscript. The work is part of the Ph.D. dissertations of L. Collins and M. Pardo. L. Collins, M. Pardo, S. Podurgiel and M. Correa performed the behavioral studies. Y. Baqi and C.E. Müller provided the MSX-3, and M. Schwarzschild provided the kockout mice. J. Salamone and M. Correa supervised the entire project.

Conflict of interest

There are no conflicts of interest connected to this work. In addition to the income received from my primary employer, compensation has been received from Merck-Serono and Pfizer within the last 3 years. There are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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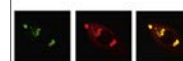
Research Report

Allopurinol reduces levels of urate and dopamine but not dopaminergic neurons in a dual pesticide model of Parkinson's disease

CrossMark

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ARTICLE INFO

ABSTRACT

Robust epidemiological data link higher levels of the antioxidant urate to a reduced risk of developing Parkinson's disease (PD) and to a slower rate of its progression. Allopurinol, an inhibitor of xanthine oxidoreductase (XOR), blocks the oxidation of xanthine to urate. The present study sought to determine whether lowering levels of urate using allopurinol results in exacerbated neurotoxicity in a dual pesticide mouse model of PD. Although oral allopurinol reduced serum and striatal urate levels 4-fold and 1.3-fold, respectively, it did not alter the multiple motor deficits induced by chronic (7 week) intermittent (biweekly) exposure to intraperitoneal Paraquat (PQ) plus Maneb (MB). However, striatal dopamine content, which was unaffected after either allopurinol or chronic pesticide exposure alone, was significantly reduced by 22% in mice exposed to the combination. Stereological assessment showed that the numbers of dopaminergic nigral neurons were significantly reduced by 29% and the tyrosine hydroxylase (TH) negative neurons unaffected after PQ+MB treatments. This reduction in TH-positive neurons was not affected by allopurinol treatment. Of note, despite the expectation of exacerbated oxidative damage due to the reduction in urate, protein carbonyl levels, a marker of oxidative damage, were actually reduced in the presence of allopurinol. Overall, allopurinol lowered urate levels but did not exacerbate dopaminergic neuron degeneration, findings suggesting that basal levels of urate in mice do not appreciably protect against oxidative damage and neurotoxicity in the PQ+MB model of PD, and/or that allopurinol produces an antioxidant benefit offsetting its detrimental urate-lowering effect.

1. Introduction

Epidemiological studies have identified both positive and negative risk factors for the incidence of Parkinson's disease (PD). Amongst environmental positive risk factors, pesticide exposures have been linked to an increased risk in developing PD, with Paraquat (PQ) and Maneb (MB) (Costello et al., 2009) among those implicated. Negative risk factors for PD include purine-based compounds, urate and caffeine. In fact, robust epidemiological data have linked higher levels of urate to

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a reduced risk of developing PD (Weisskopf et al., 2007; Chen et al., 2009) and of its progression (Schwarzschild et al., 2008; Ascherio et al., 2009). Urate accounts for most of the anti-oxidant capacity in human plasma (Yeum et al., 2004); with its antioxidant properties as powerful as those of ascorbic acid (Ames et al., 1981). Urate has been shown to specifically confer protection in cellular (Jones et al., 2000; Duan et al., 2002; Guerreiro et al., 2009) as well as in animal models of disease such as multiple sclerosis (Scott et al., 2002), stroke (Yu et al., 1998) and PD (Wang et al., 2010).

While it has been proposed that higher serum urate levels may be of selective advantage in the evolution of the hominids because of its antioxidant effects; hyperuricemia is associated with multiple diseases in humans and points to the deleterious effects of high concentrations of urate. Current approved pharmacological approaches to lower urate levels in patients with gout rely on allopurinol to reduce urate production (Bieber and Terkeltaub, 2004; Pea, 2005). Allopurinol, an inhibitor of xanthine oxidoreductase (XOR) blocks the successive oxidations of hypoxanthine to xanthine, and xanthine to urate. The enzyme XOR is widely distributed throughout various organs including the liver, heart, lung, brain as well as plasma and can exist in either one of 2 forms, xanthine dehydrogenase (XDH), predominating in healthy tissues or xanthine oxidase (XO) which plays an important role in injured cells and tissues (Harrison, 2002). Both forms are interconvertible with one another, with the XO subtype causing reduction of molecular oxygen leading to generation of reactive oxygen species (Berry and Hare, 2004).

The present study sought to determine whether pharmacologically lowering urate levels in mice using chronic allopurinol treatment alters the pesticide-induced neurotoxic phenotype in an environmental toxin model of PD.

2.   Results

2.1.   Allopurinol and not PQ+MB significantly attenuates serum and striatal urate levels

Serum urate levels of mice exposed to allopurinol in the drinking water were significantly decreased by approximately 4-fold ($p < 0.0001$) compared to their unexposed water-drinking counterparts. PQ+MB treatment had no effect (Fig. 1A). Striatal urate levels were found to be significantly reduced ($p = 0.0024$) in allopurinol treated mice though only by 1.3-fold compared to non-allopurinol treated mice (Fig. 1B). Measurement of additional purines included in the purine metabolism pathway such as hypoxanthine and xanthine was consistent with those published by Enrico et al. (1997) with no demonstrated differences after allopurinol treatment. The data values (ng/mg tissue units) for striatal hypoxanthine levels for the groups: Tap water–Saline, Tap–PQ+MB, Allopurinol–Saline and Allopurinol–PQ+MB were 0.04 ± 0.005; 0.04 ± 0.004; 0.04 ± 0.004; 0.04 ± 0.002, respectively. For the same groups striatal xanthine levels were 0.10 ± 0.02; 0.12 ± 0.03; 0.11 ± 0.01; 0.08 ± 0.01. The data values (mg/dL units) for serum hypoxanthine levels for the groups: Tap water–Saline, Tap–PQ+MB, Allopurinol–Saline and Allopurinol–PQ+MB were 0.041 ± 0.003; 0.042 ± 0.004; 0.04 ± 0.003;
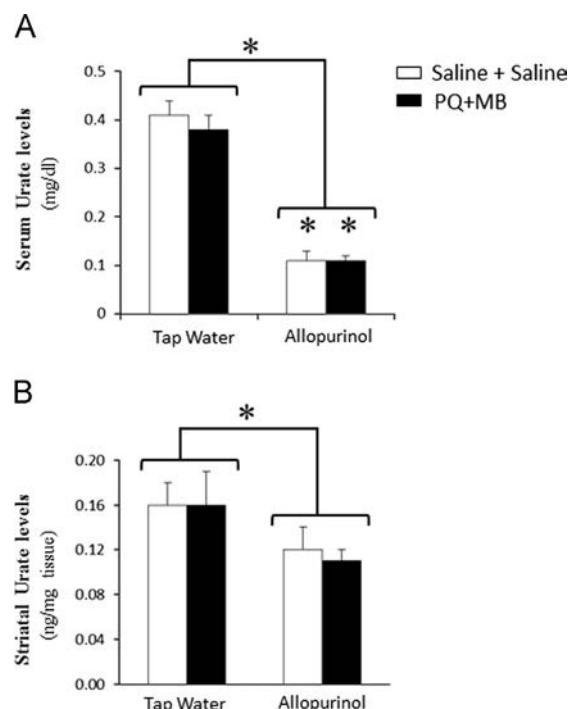

Fig. 1 – Evidence that Allopurinol and not PQ+MB significantly attenuates serum and striatal urate levels in mice. (A) Serum urate level, *$p < 0.0001$ vs respective tap water treatment groups; *$p < 0.0001$ (combined allopurinol vs combined tap water treatment groups); unpaired t-test. (B) Striatal urate level, *$p = 0.024$ (comparison between combined allopurinol vs combined tap water treatment groups). Groups are: Tap water [Saline control ($n = 8$); PQ+MB ($n = 12$)]; Allopurinol [Saline control ($n = 8$); PQ+MB ($n = 11$)]. Combined treatment groups are: Tap water group ($n = 20$); Allopurinol group [Saline control ($n = 19$)].

0.06 ± 0.02, respectively. For the same groups serum xanthine levels were 0.0179 ± 0.0014; 0.014 ± 0.0006; 0.02 ± 0.0010; 0.01 ± 0.001. The lack of a change in the levels of both hypoxanthine and xanthine may be due to the fact that allopurinol increases the conversion of hypoxanthine to inosinic acid and the inhibition of the rate of de novo purine biosynthesis.

2.2.   Allopurinol does not potentiate PQ+MB-induced motor dysfunction

The pole test and the beam traversal task were used to detect any motor dysfunction that may reflect toxin-induced dopaminergic deficit. Specifically, MPTP-treated mice have been shown to display slower times in the descent time parameter of the pole test compared to controls, impairments reversed by L-dopa (Matsuura et al., 1997). In addition, Huntington disease knock-in mice displayed significant impairments in the pole test (Hickey et al., 2003), indicating it is a useful test for basal ganglia dysfunction. The beam traversal task is used to specifically assess fine-motor initiation, coordination and postural balance. Hwang et al. (2005) highlighted transgenic mouse models of PD as significantly slower in traversing

a narrow, raised beam as well as taking an increased number of steps, compared to wild-type control animals. These test parameters mimic the slower movements and shorter steps observed in PD patients. PQ+MB treated animals exposed to only water demonstrated a significant increase in duration required to descend the pole, reported as descent time (Fig. 2A); in addition to increased time required to cross the beam (beam latency) (Fig. 2B), as well as the number of steps required to do so (Fig. 2C). Allopurinol exposure did not seem to affect either of these measures.
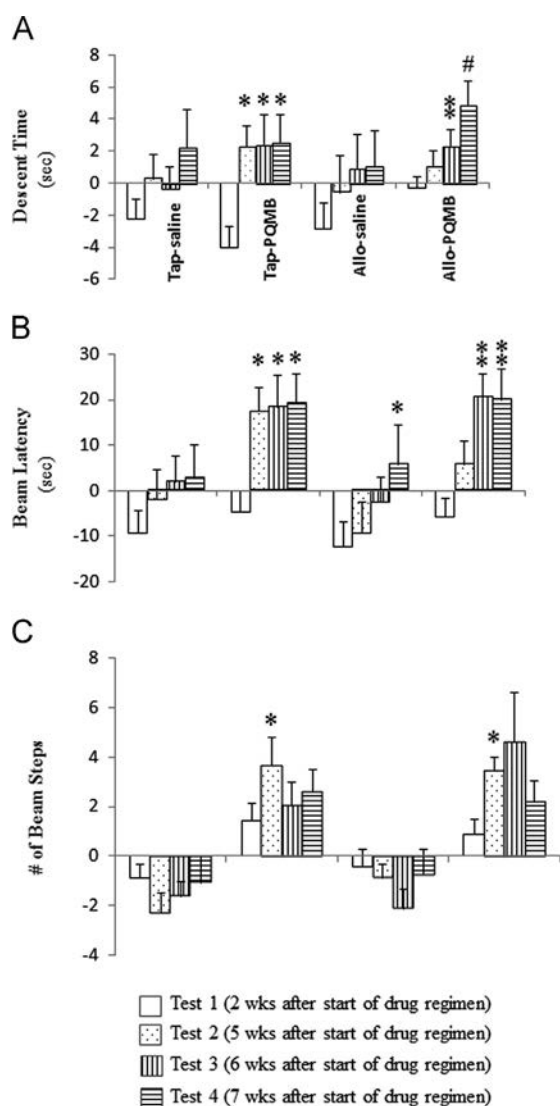
2.3. Allopurinol potentiates striatal DA loss in combination with PQ+MB exposure

Chronic intermittent administration of PQ+MB on its own did not reduce striatal DA content, consistent with prior studies (Thiruchelvam et al., 2000a, 2000b). However, in the presence of urate-lowering allopurinol treatment PQ+MB significantly reduced striatal dopamine by approximately 22% (Fig. 3A).

2.4. Allopurinol does not further exacerbate PQ+MB-induced loss of TH+ neuronal cells

By contrast, PQ+MB treatment on its own significantly decreased the number of TH+ neurons in the substantia nigra pars compacta (SNpc) by approximately 28% compared to the corresponding saline-treated group (Fig. 3B). Allopurinol treatment did not alter dopaminergic neuron cell counts and did not potentiate pesticide-induced dopaminergic neuron cell loss. Direct assessment of TH− (negative) neurons confirmed the specificity of pesticide-induced neurotoxicity for dopaminergic neurons of the SNpc, as previously shown (Kachroo et al., 2010) (Fig. 3C). Allopurinol, either alone, or in

Fig. 2 – PQ+MB treatment significantly (A) increases time to descend the pole. *$p < 0.01$, **$p < 0.05$, #$p < 0.001$ vs Testing 1; RMANOVA. (B) increases beam latency. *$p < 0.01$, **$p < 0.001$ vs Testing 1; RMANOVA. (C) increases number of beam steps. *$p < 0.01$ vs Testing 1; RMANOVA. Data reported as Test–Baseline scores. Water [Saline control ($n = 8$); PQ+MB ($n = 12$)]; Allopurinol [Saline control ($n = 8$); PQ+MB ($n = 11$)]. Tests 1–4 correspond to behavioral assessments performed at 2, 5, 6 and 7 weeks respectively, after the start of the toxin injections.
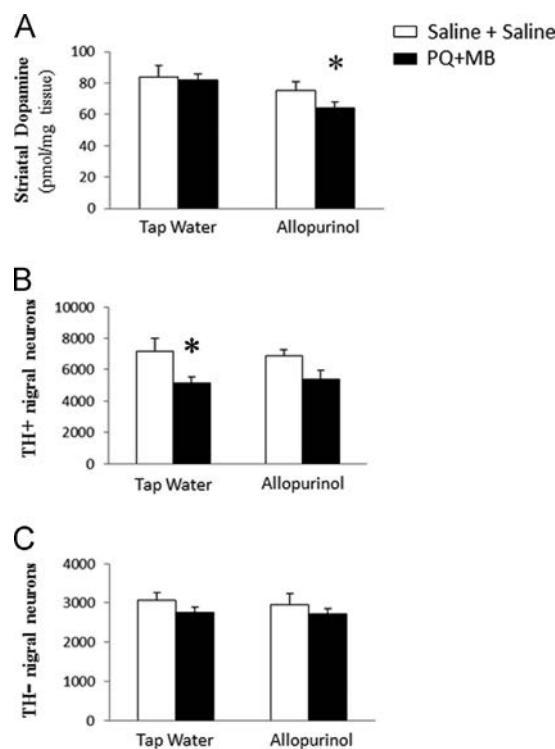

Fig. 3 – Allopurinol potentiates striatal DA loss in combination with PQ+MB exposure, but does not further exacerbate loss of total TH+ neuronal cells (bilateral) in the substantia nigra pars compacta (A) *$p = 0.0038$ vs Water-PQ+MB group; unpaired t-test. (B) *$p = 0.03$ vs Water–Saline group; unpaired t-test. (C) No significant differences within the Tap water group (Saline vs PQ+MB); Allopurinol group (Saline vs PQ+MB); between Tap water and Allopurinol groups (Saline vs Saline; PQ+MB vs PQ+MB). Water [Saline control ($n = 8$); PQ+MB ($n = 12$)]; Allopurinol [Saline control ($n = 8$); PQ+MB ($n = 11$)].
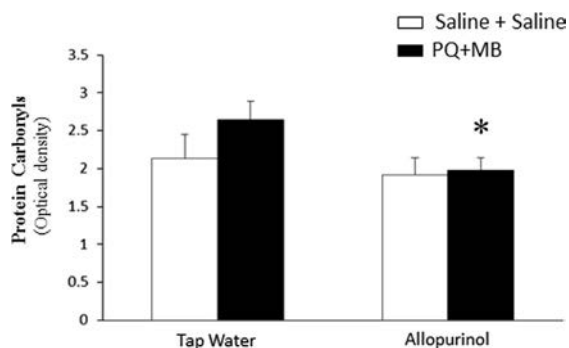
Fig. 4 – Allopurinol attenuates protein carbonyl levels in brains of PQ+MB-lesioned mice. *$p = 0.043$ vs Water–PQ+MB; unpaired t-test. Water [Saline control ($n=8$); PQ+MB ($n=12$)]; Allopurinol [Saline control ($n=8$); PQ+MB ($n=11$)].

combination with PQ+MB had no effect on TH− (negative) neuron number.

2.5. Allopurinol attenuates protein carbonyl levels in brains of PQ+MB-lesioned mice

Measurement of oxidative damage assessed by the protein carbonyl assay showed a non-significant increase after PQ+MB treatment to levels that were significantly reduced (27%) by allopurinol (Fig. 4).

3. Discussion

These findings demonstrate that we can pharmacologically lower levels of urate both in serum and brain (striatum) of mice by chronic oral administration of allopurinol. Our findings are confirmatory with others showing significant attenuation of striatal urate levels after allopurinol treatment (Desole et al., 1995; Enrico et al., 1997; Miele et al., 1995). Under these experimental conditions we showed PQ+MB treatment itself did not directly affect urate levels.

Motor function, as assessed using the pole test and beam traversal task provided a behavioral measure of pesticide toxicity. This was unaffected by allopurinol alone and while PQ+MB treatment worsened motor performance after at least 6 weeks of toxin exposure (12 injections), urate-lowering allopurinol treatment did not affect these deficits. Previously, it has been shown that under certain conditions of elevating urate levels (after injections of intraperitoneal (i.p.) administered urate) in rats exposed to 6-hydroxydopamine, behavioral outputs such as locomotion scores and forepaw adjusting step test scores can be improved (Wang et al., 2010). Differences in rodent species, toxin used, extent/direction of urate change, and even our approach of indirectly targeting urate levels by inhibiting the enzyme XOR in our model may all contribute to the lack of a hypothesized behavioral effect.

Striatal dopamine content was shown to be unaffected after either allopurinol or chronic pesticide exposure but was significantly reduced in mice exposed to the combination. Based on prior literature, our data are consistent with

allopurinol having no direct effect on striatal DA levels (Desole et al., 1995; Miele et al., 1995). The potentiated effect observed in the presence of a toxin may well be a result of allopurinol unmasking a PQ+MB-induced dopamine loss, possibly due to reduced endogenous antioxidant capacity resulting from lower striatal urate levels. In this setting PQ+MB may produce further increases in reactive oxygen species (ROS) and subsequent dopamine oxidation or dopaminergic nerve terminal injury.

In contrast to striatal dopamine levels, nigral dopaminergic cell counts were not reduced by allopurinol in the PQ+MB model of PD. Although such an exacerbation of neurotoxicity had been hypothesized based on the ability of allopurinol to lower levels of the putative antioxidant urate, we did not find a corresponding increase in oxidative damage markers in brain. Interestingly, brain levels of protein carbonyls were actually reduced by allopurinol in brains of mice treated with PQ+MB, suggesting a net antioxidant effect of allopurinol. Thus, at the level of nigral neuron survival, potentially deleterious urate-lowering effects of allopurinol may have been offset by antioxidant benefits. For example, in peripheral tissue, allopurinol or its metabolites can produce significant antioxidant effects on toxin-induced injury (Kitazawa et al., 1991; Knight et al., 2001), possibly PQ+MB via reduced XOR-driven H_2O_2 (as well as urate) generation. Why an antioxidant benefit of allopurinol would offset a detrimental effect of lower urate on nigral neuron numbers but not on striatal dopamine content is unclear, but may be related to the distinct anatomical and neurochemical nature of these of nigrostriatal neuron features. In any event, an alternative approach to testing urate reduction, such as may be achieved by increasing urate degradation (rather than by decreasing synthesis via allopurinol) could provide a simpler test of urate's role in models of PD. Finally, whether the synergistic toxicity of allopurinol and these pesticides on striatal dopamine levels (and the dissociation of allopurinol effects on nigral and striatal indices of dopaminergic neuron injury) were consistent across animal models of PD should be assessed in complementary, standard toxin (e.g., MPTP and 6-hydroxydopamine) and transgenic (e.g., α-synuclein) models of the disease.

4. Experimental procedures

4.1. Drug administration

Two-month-old male C57BL/6NCrl mice were obtained from Charles River Laboratories; Wilmington, MA and housed under a 12:12 h light:dark cycle. Food and water were provided ad libitum. All experiments were performed in accordance with Massachusetts General Hospital and NIH guidelines on the ethical use of animals, with adequate measures taken to minimize pain and discomfort. Prior to the start of the experiment, mice were either continued on water or placed on allopurinol dissolved in water for one month before exposure to intraperitoneal injections of saline or 10 mg/kg PQ (1,1′-dimethyl-4,4′-bipyridinium) dichloride hydrate followed immediately by 30 mg/kg MB (manganese bisethylenedithiocarbamate) in a volume of 10 ml/kg body

weight. Allopurinol was administered at a dosage of 150 mg/L and bottles replaced weekly. PQ and MB both obtained from Sigma were dissolved separately in saline on the day of administration and injected on the opposite sides (lower quadrants) of animal's abdomen. Mice were treated chronically (twice weekly for 7 weeks) in the following randomly assigned initial groups: Water [Saline control ($n=8$); PQ+MB ($n=18$)]; Allopurinol [Saline control ($n=8$); PQ+MB ($n=18$)]. Mortality rates of 33% and 39% in the toxin-treated mice on regular and allopurinol-treated water, respectively, resulted in final group numbers of 12 and 11. Body weights were obtained twice a week prior to injection during the course of the experiment. Drug treatments did not affect body weight (DNS). Mice were continued on allopurinol or water until their sacrifice one week after the last PQ+MB or Saline+Saline injection.

4.2. Behavioral testing

Behavioral testing involved mice being exposed to the pole test and the beam traversal task (Kachroo and Schwarzschild, 2012). Baseline values for individual mice on these tests were taken prior to, and during, allopurinol treatment but before toxin exposure. No behavioral differences were observed between either time-point. The average of both baselines was used to normalize subsequent assay values and thus reduce their variability. Mice were tested at 4 time-points referred to as Tests 1–4 which correspond to behavioral assessments at 2, 5, 6 and 7 weeks respectively, after the start of the toxin injections.

4.3. Tissue processing

One week after the last injection (week 8), mice were sacrificed by cervical dislocation, decapitated and trunk blood collected. The brains were removed and the rostral and caudal portions separated by an axial cut made across the whole brain at the tail end of the striatum. Both striata as well as a portion of cortex were removed and frozen at $-80\,°C$ until use. The remaining caudal brain portion was immediately fixed in 4% PFA for 3 days, placed in cryoprotectant and stored at $-80\,°C$ until use. The striatum was assayed for dopamine and urate by standard reverse phase high performance liquid chromatography with electrochemical detection as routinely performed in our laboratory (Chen et al., 2001; Ascherio et al., 2009). The striatum and cortex were used for purine and protein carbonyl assays, respectively. Fixed brains were cut on a Leica microtome into 30 μm-thick sections and stored for immunolabeling studies in a cryoprotectant consisting of 30% sucrose and 30% ethylene glycol in 0.1 M phosphate buffer. As previously described (Kachroo et al., 2010), sections were chromogenically stained for TH immunoreactivity (IR) followed by counterstaining with Nissl. Tyrosine hydroxylase-positive (TH+) and -negative (TH−) neurons were counted in the region of the SNpc.

4.4. Striatal and serum sample preparation for HPLC purine analysis

Brain samples were weighed and homogenized in 50 mM phosphoric acid, 0.1 mM EDTA, 50 μM methyl-DOPA (internal standard), and 1 μM DHBA (internal standard). Homogenates were centrifuged at 14,000 rpm for 15 min and the supernatant was transferred to a Costar SpinX (#8161) 0.22 μm CA filter tube and centrifuged at 14,000 rpm for 5 min. Samples were then stored at $-80\,°C$ until needed.

Whole blood was collected from the submandibular vein using Goldenrod animal lancets, and centrifuged at 14,000 rpm at 4 °C for 15 min. Serum was collected and de-proteinated by adding 0.4 M perchloric acid. The mixture was allowed to incubate on ice for 10 min and then centrifuged at 1400 rpm for 15 min. The supernatant was then collected and added to 0.2 M potassium phosphate. The resulting solution was added to a Costar SpinX (as above) and centrifuged at 14,000 rpm for 5 min. Samples were then stored at $-80\,°C$ until needed.

4.5. Purine determination in striatal and serum samples

A dual-pump gradient method was used to measure the concentrations of purines in the samples using a Varian Microsorb-MV reverse-phase column (150×4.6 mm, C_{18}, 5 μm pore size). Mobile phase A contained 0.52 mM sodium 1-pentanesulfonate and 0.20 M KH_2PO_4 monobasic at pH 3.5 using 85% phosphoric acid (HPLC-Grade, Fisher Scientific, Pittsburgh, PA). Mobile phase B had the same final concentrations as mobile phase A, except for the addition of 10% acetonitrile (v:v). All analyses were performed at a flow rate of 1 mL/min. The method ran at 0% B for 6 min and then linearly ramped to 70% B between 6 and 14 min. 70% B was maintained until 17.4 min, at which point, it returned to 0% B and was allowed to equilibrate until 20 min. The sample injection volume was 12 μL. Detection was performed by linking a UV–vis spectrophotometer upstream of two coulometric cells. The UV–vis detection was set to a wavelength of 254 nm. The first electrode was set to -100 mV and acted as conditioning cell. The analytical electrodes 1 and 2 were set at $+150$ and $+450$ mV, respectively. Data were collected using CoulArray Data Station 3.0 software (ESA Biosciences) with auto-range gain enabled. A standard curve was analyzed at the beginning of each run to determine the concentrations of purines in the biological samples. Methyl-DOPA and DHBA were used as internal standards to correct for minor variations between samples in the same run.

4.6. Protein carbonyl assay

Protein carbonyl levels were determined using the OxyBlot Protein Oxidation detection kit from Millipore. A 5 mg sample of cortex was removed from frozen brain tissue (kept frozen on dry ice) and added to a tube containing 40 μl of lysis buffer (RIPA buffer+50 mM DTT). The sample was hand homogenized, 5 μl of the resulting homogenate was added to 5 μl of SDS to a final concentration of 6% SDS. The samples were then derivatized by adding 10 μl of $1\times$ 2,4-dinitrophenylhydrazine (DNPH) and incubated at room temperature for 15 min. The derivatization was halted using 7.5 μl of Neutralization buffer (aqueous solution of Trometamol and glycerol). The resulting solution was run on a 4% stacking/10% resolving polyacrylamide gel at 90 V until the samples had cleared the stacking gel, and then at 110 V for 1 h. The gel was then transferred to a nitrocellulose membrane. The membrane was then probed with a rabbit anti-

DNP (1:150) primary antibody and goat anti-rabbit IgG (1:300) secondary antibody for 1 h each. The blots were then visualized using horseradish peroxidase.

4.7. Statistical analysis

Optical density measurement for protein carbonyl level assessment was performed using the ImageJ software. All values are expressed as mean±SEM. For behavioral tests repeated measures ANOVA (RMANOVA) with *post hoc* analysis was performed. For all other analyses unpaired *t*-tests were performed.

Conflict of interest

No conflict of interest.

Acknowledgments

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PROTECTION BY INOSINE IN A CELLULAR MODEL OF PARKINSON'S DISEASE

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Abstract—Inosine (hypoxanthine 9-beta-D-ribofuranoside), a purine nucleoside with multiple intracellular roles, also serves as an extracellular modulatory signal. On neurons, it can produce anti-inflammatory and trophic effects that confer protection against toxic influences *in vivo* and *in vitro*. The protective effects of inosine treatment might also be mediated by its metabolite urate. Urate in fact possesses potent antioxidant properties and has been reported to be protective in preclinical Parkinson's disease (PD) studies and to be an inverse risk factor for both the development and progression of PD. In this study we assessed whether inosine might protect rodent MES 23.5 dopaminergic cell line from oxidative stress in a cellular model of PD, and whether its effects could be attributed to urate. MES 23.5 cells cultured alone or in presence of enriched murine astroglial cultures MES 23.5–astrocytes co-cultures were pretreated with inosine (0.1–100 μM) for 24 h before addition of the oxidative stress inducer H_2O_2 (200 μM). Twenty-four hours later, cell viability was quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay or immunocytochemistry in pure and MES 23.5–astrocytes co-cultures, respectively. H_2O_2-toxic effect on dopaminergic cells was reduced when they were cultured with astrocytes, but not when they were cultured alone. Moreover, in MES 23.5–astrocytes co-cultures, indicators of free radical generation and oxidative damage, evaluated by nitrite (NO_2^-) release and protein carbonyl content, respectively, were attenuated. Conditioned medium experiments indicated that the protective effect of inosine relies on the release of a protective factor from inosine-stimulated astrocytes. Purine levels were measured in the cellular extract and conditioned medium using high-performance liquid chromatography (HPLC) method. Urate concentration was not significantly increased by inosine treatment however there was a significant increase in levels of other purine metabolites, such as adenosine, hypoxanthine and xanthine. In particular, in MES 23.5–astrocytes co-cultures, inosine medium content was reduced by 99% and hypoxanthine increased by 127-fold. Taken together these data raise the possibility that inosine

might have a protective effect in PD that is independent of any effects mediated through its metabolite urate. © 2014 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: MES 23.5 cells, astrocytes, urate, HPLC, cell viability, oxidative stress.

INTRODUCTION

Inosine is a purine shown to have trophic protective effects on neurons and astrocytes subjected to hypoxia or glucose-oxygen deprivation (Haun et al., 1996) and to induce axonal growth following neuronal insult *in vivo* and *in vitro* (Zurn and Do, 1988; Benowitz et al., 1998; Petrausch et al., 2000; Chen et al., 2002; Wu et al., 2003). Moreover, inosine showed anti-inflammatory effects in the central nervous system (CNS) and periphery (Jin et al., 1997; Hasko et al., 2000; Gomez and Sitkovsky, 2003; Shen et al., 2005; Rahimian et al., 2010). Some (Toncev, 2006; Markowitz et al., 2009) but not all (Gonsette et al., 2010) clinical studies have suggested a possible antioxidant protective effect of inosine in multiple sclerosis patients (Markowitz et al., 2009). In these trials inosine consistently elevated serum urate, which was proposed to mediate any protective effect of inosine (Markowitz et al., 2009; Spitsin et al., 2010).

Oxidative stress is thought to be a key pathophysiological mechanism in Parkinson's disease (PD) leading to cellular impairment and death (Ross and Smith, 2007). Urate – a major antioxidant circulating in the human body – has emerged as an inverse risk factor for PD. Clinical and population studies have found the urate level in serum or CSF to correlate with a reduced risk of developing PD in healthy individuals and with a reduced risk of clinical progression among PD patients (Weisskopf et al., 2007; Schwarzschild et al., 2008; Ascherio et al., 2009). Moreover, in cellular and animal models of PD, urate elevation has been shown to reduce oxidative stress and toxicant-induced loss of dopaminergic neurons (Wang et al., 2010; Cipriani et al., 2012a,b; Gong et al., 2012; Zhu et al., 2012; Chen et al., 2013). Although inosine can elevate urate concentration in the periphery in animals and humans, little is known about its effect on the urate level in the CNS (Ceballos et al., 1994; Scott et al., 2002; Rahimian et al., 2010; Spitsin et al., 2010). A cellular study indicated that inosine added to cortical astroglial (but not neuronal) cultures increases urate concentration in the medium (Ceballos et al., 1994).

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Abbreviations: CNS, central nervous system; DHBA, 3,4-dihydroxybenzylamine; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; HPLC, high-performance liquid chromatography; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PD, Parkinson's disease; SDS, sodium dodecyl sulfate.

In the present study we characterized a protective effect of inosine on oxidative stress-induced dopaminergic cell death in a cellular model of PD and investigated whether urate elevation might mediate the effect.

EXPERIMENTAL PROCEDURES

Animals

C57BL/6 mice were employed to obtain astroglial cultures. All experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals with approval from the animal subjects review board of the Massachusetts General Hospital.

MES 23.5 cell line

The rodent MES 23.5 dopaminergic cell line (Crawford et al., 1992) was obtained from Dr. Weidong Le at the Baylor College of Medicine (Houston, USA). MES 23.5 cells were cultured on polyornithine-coated T75 flasks (Corning Co, Corning, NY) in culture medium; Dulbecco modified Eagle medium (DMEM, Invitrogen/Gibco), added with Sato components (Sigma Immunochemicals), and supplemented with 2% newborn calf serum (Invitrogen), 1% fibroblast growth factor (Invitrogen), penicillin 100 U ml^{-1} and streptomycin 100 $\mu g\ mL^{-1}$ (Sigma), at 37 °C in a 95% air–5% carbon dioxide, humidified incubator. Culture medium was changed every 2 days. At confluence, MES 23.5 cells were either sub-cultured new T-75 flasks or used for experiments. For experiments, MES 23.5 cells were seeded at a density of 600 cells per mm^2 onto polyornithine-coated plates or flasks (according to the assay, see below) in culture medium. Twenty-four hours later, it was changed to DMEM serum-free medium. At this time, increasing concentrations of inosine (0–100 μM) were added to the cultures for 24 h and again during toxicant treatment. 200 μM H_2O_2 was added to the cultures for 24 h and then cells were used for assays.

Enriched astroglial cultures

Astroglial cultures were prepared from the brains of 1- or 2-day-old neonatal mice as previously described (Cipriani et al., 2012b). Briefly, cerebral cortices were digested with 0.25% trypsin for 15 min at 37° C. The suspension was pelleted and re-suspended in culture medium (DMEM, fetal bovine serum (FBS) 10%, penicillin 100 U ml^{-1} and streptomycin 100 $\mu g\ ml^{-1}$ to which 0.02% deoxyribonuclease I was added). Cells were plated at a density of 1800 cells per mm^2 on poly-L-lysine (100 $\mu g\ ml^{-1}$)/DMEM/F12-coated flasks and cultured at 37 °C in humidified 5% CO_2 and 95% air for 7–10 days until reaching confluence.

In order to remove non-astroglial cells, flasks were agitated at 200 rpm for 20 min in an orbital shaker and treated with 10 μM cytosine arabinoside (Ara-C) dissolved in cultured medium for 3 days. After the treatment, astrocytes were subjected to mild trypsinization (0.1% for 1 min) and then sub-plated (120 cells per mm^2) onto poly-L-lysine (100 $\mu g\ ml^{-1}$)/DMEM/F12-coated plates or flasks (according to the assay, see below) in DMEM plus 10% FBS for assays. Astroglial cultures comprised >95% astrocytes, <2% microglial cells and <1% oligodendrocytes; no neuronal cells were detected (Cipriani et al., 2012b).

MES 23.5–astrocytes co-cultures

MES 23.5 cells were cultured on a layer of enriched astroglial cultures prepared as described above. Briefly, astrocytes were allowed to grow for 48 h on poly-L-lysine (100 $\mu g\ ml^{-1}$)/DMEM/F12-coated plates or flasks (according to the assay, see below) in DMEM plus 10% FBS. Then, MES 23.5 cells were seeded on top at a concentration of 600 cells per mm^2 in MES 23.5 culture medium. An astrocyte:MES 23.5 cell ratio of 1:5 was chosen on the basis of our previous observations (Cipriani et al., 2012b), which indicated this proportion of astrocytes as sufficiently low to avoid a direct effect of astrocytes on dopaminergic cell survival. Twenty-four hours later, medium was changed to DMEM serum-free medium and subjected to treatments. Inosine was added to the cultures 24 h before and during 200 μM H_2O_2 treatment. In our previous study this H_2O_2 concentration was shown to have no effect on astrocyte viability (Cipriani et al., 2012b). At the end of treatment, MES 23.5 cells were easily detached from astrocytes and dissociated by gently pipetting up and down the medium before processing for biochemical assays.

Conditioned media experiments

Enriched-astrocyte cultures were grown on poly-L-lysine (100 $\mu g\ ml^{-1}$)/DMEM/F12-coated 6 well-plates in DMEM plus 10% FBS. Astrocytes were allowed to grow for three days and then the medium was changed to MES 23.5 culture medium in order to reproduce co-culture conditions. The day after, medium was changed to DMEM containing 100 μM inosine or vehicle. Twenty-four hours later, conditioned medium was collected and filtered through a 0.2 μM membrane to remove cellular debris. MES 23.5 cells were treated with increasing concentrations of conditioned medium 24 h before and during H_2O_2 treatment.

Drugs

Inosine was dissolved in DMEM as 20× concentrated stocks. H_2O_2 was dissolved in PBS (0.1 M, pH 7.4) as 100× concentrated stocks. Drugs were obtained from Sigma.

Cell viability and toxicity assessments

In MES 23.5 cultures, cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma). This assay is based on the conversion of the yellow tetrazolium salt MTT by mitochondrial dehydrogenase of live cells to the purple formazan (Hansen et al., 1989). Briefly, MES 23.5 cells were cultured in polyornithine-coated 96-well plates (600 cells per mm^2) and grown for at least 24 h. Then, the medium was changed to DMEM serum-free medium

for 24 h before H_2O_2 was added. In order to assess inosine protection, increasing concentrations of drug (0–100 µM) were loaded 24 h before and again during toxicant treatment. After washes, 100 µl of MTT solution (0.5 mg ml^{-1} in DMEM) was added for 3 h at 37 °C. Then, MES 23.5 cells were lysed with 10 µl/well of acidic isopropanol (0.01 M HCl in absolute isopropanol) to extract formazan that was measured spectrophotometrically at 490 nm with a Labsystems iEMS Analyzer microplate reader.

In MES 23.5–astrocytes co-cultures, surviving MES 23.5 cells were quantified by immunocytochemistry (Lotharius et al., 2005; Dumitriu et al., 2011; Cipriani et al., 2012b). MES 23.5 were grown on top of astrocytes in 96-well plates as described above. Increasing concentrations of drug (0–100 µM) were loaded 24 h before and again during toxicant treatment. After washing in PBS, cultures were fixed with 4% (wt/vol) paraformaldehyde for 1 h at room temperature. Then, cells were incubated with an Alexa 488-conjugated antibody specific for neuronal cells, Milli-Mark FluoroPan Neuronal Marker, (Millipore; 1:200, overnight at 4 °C). Fluorescence was read at 535 nm by using a microplate reader.

High-performance liquid chromatography (HPLC)

To determine purine content in cells and medium samples, MES 23.5, MES 23.5–astrocytes co-cultures or enriched astroglial cultures were prepared as described above and cultured in T75 flasks. Purine content was determined using our previously described HPLC-based analytical methods (Burdett et al., 2013). Briefly, cell medium was collected and added with 30% vol/vol of a buffer containing 150 mM phosphoric acid, 0.2 mM EDTA, and 1 µM 3,4-dihydroxybenzylamine (DHBA; used as internal standard). Cells were collected after washing in ice-cold PBS and purines were extracted in the same buffer used for medium. Samples were then filtered through a 0.2-µm Nylon microcentrifuge filter (Spin-X, Corning) at 4 °C. Samples were maintained at 4 °C and injected using an ESA Biosciences (Chelmsford, MA) autosampler, and chromatographed by a multi-channel electrochemical/UV HPLC system with effluent from the above column passing through a UV–VIS detector (ESA model 528) set at 254 nm and then over a series of electrodes set at -100 mV, $+250$ mV and $+450$ mV. To generate a gradient two mobile phases were used. Mobile phase A consisted of 0.2 M potassium phosphate and 0.5 mM sodium 1-pentanesulfonate; mobile phase B consisted of the same plus 10% (vol/vol) acetonitrile. Mobile phase B increased linearly from 0% to 70% between 6th and 14th min of the run.

Nitrite (NO_2^-) release

MES 23.5–astrocytes co-cultures were grown on a 96-well plate as described above. After treatments, nitrite release (NO_2^-), an indicator of free radical generation, was quantified in cell medium by the Griess assay. An azo dye is produced in the presence of nitrite by the Griess reaction and colorimetrically detected. Briefly, 100 µl of supernatant collected from treated cultures were added to 100 µl of Griess reagent (Sigma) and absorbance was read at 540 nm with a microplate reader. Blanks were prepared by adding medium containing toxicants and/or protectants to the Griess solution.

Protein carbonyl protein assay

MES 23.5–astrocytes co-cultures were grown in 6-well plates as described above. After treatments, cultures were washed with ice-cold PBS and oxidized proteins were detected in MES 23.5 cells, using the Oxyblot assay kit (Chemicon). MES 23.5 cells were detached from astrocytes in ice-cold PBS, spun to form a pellet at 4 °C and resuspended in ice-cold RIPA buffer containing 50 mM DTT. Cells were allowed to lyse on ice for 15′. For the assay, 20 µg of protein were derivatized in 10 µL of 2,4-dinitrophenylhydrazine (DNPH). After derivatization samples were subjected to sodium dodecyl sulfate (SDS)–polyacrylamide gel (10% [wt/vol] acrylamide, 0.1% [wt/vol] SDS) and transferred electrophoretically onto 0.2 µ nitrocellulose membranes. Membranes were loaded with an antibody specific to dinitrophenylhydrazone moiety of the proteins and reaction visualized by chemiluminescence.

Protein detection

After treatment, cells were washed in ice-cold PBS, collected and resuspended in ice-cold Ripa buffer. Cells were incubated on ice for 15′, followed by sonication for complete lysis. Proteins were quantified in 4 µl of each sample using Bio-Rad Protein Assay reagent (Biorad Laboratories) and measured at 600 nm with a microplate reader.

Statistical analysis

Statistical analysis was performed by GraphPad Prism version 4.00 (GraphPad Software Inc.). Unpaired Student's t-test was used when two group samples were compared. ANOVA analysis followed by Newman–Keuls was used when more than two group samples were compared. Values were expressed as mean ± SEM. Differences with a $P < 0.05$ were considered significant and indicated in figures by symbols explained in legends.

RESULTS

Astrocytes mediated protective effect of inosine on dopaminergic cells

Previously we showed that urate protected a dopaminergic cell line (MES 23.5) against oxidative stress when cells were cultured with astrocytes (Cipriani et al., 2012b). To assess whether inosine protected the dopaminergic cell line in a similar way we tested inosine on MES 23.5 cells cultured alone or with cortical astrocytes (MES 23.5–astrocytes co-cultures) treated with 200 µM H_2O_2.

Inosine on its own had no effect on MES 23.5 viability (one-way ANOVA, $P > 0.05$) (Fig. 1A), and showed only a trend toward modest protection with increasing
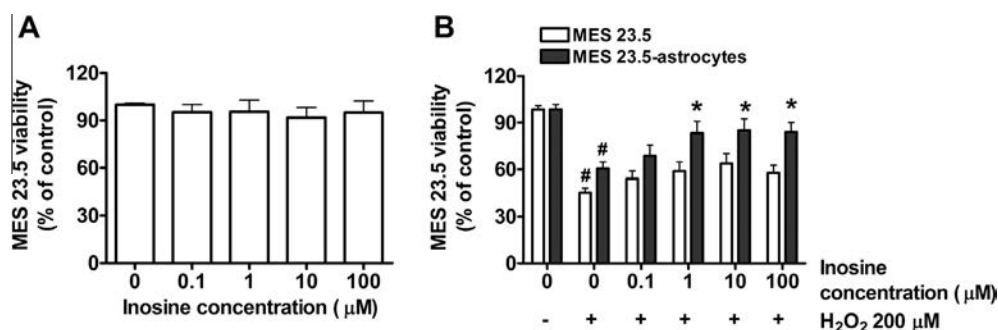
Fig. 1. Astrocytes potentiated the protective effect of inosine on 200 μM H_2O_2-treated MES 23.5 cells. (A) Viability of MES 23.5 cells treated for 24 h with increasing concentrations of inosine (0–100 μM). (B) Effect of inosine treatment (0–100 μM) at 24 h of toxic treatment with 200 μM H_2O_2 on viability of MES 23.5 cells cultured alone (white bars) or in the presence of astrocytes (gray bars). Cultures were treated with inosine 24 h before and during toxic treatment. Data represent mean ± SEM of values from four experiments, each of which yielded a mean of triplicate determinations for each condition. One-way ANOVA analysis ($P < 0.001$) followed by Newman–Keuls multiple comparison test ($^{\#}P < 0.01$ vs respective '0 inosine/ $-H_2O_2$' value; $^{*}P < 0.05$ vs '0 inosine/$+H_2O_2$' value).

concentrations from 0.1 to 100 μM against H_2O_2 toxicity (one-way ANOVA, $P > 0.05$) in pure MES 23.5 cultures. However, in the presence of a relatively low density of astrocytes (plated at a density of 120 cells per mm²), MES 23.5 cell viability significantly increased in comparison to inosine-untreated cells ($P < 0.05$; Fig. 1B).

Inosine decreased toxicant-induced oxidative stress

To determine whether protection was associated with reduced oxidative stress and protein damage, we measured the effect of inosine on oxidative stress markers in H_2O_2-treated co-cultures of MES 23.5 cells and astrocytes. At 24 h, inosine decreased the level of NO_2^- (nitrite), an indicator of free radical generation, from 2-fold to 1.4-fold of the control value in cell medium ($P = 0.00139$, Fig. 2A). Moreover, at 3 h inosine decreased protein oxidation, measured as protein carbonyl content in MES 23.5 cells (after removal from astrocytes), from 4.6- to 2.7-fold of control value ($P = 0.002$) (Fig. 2B).

Protection mediated by astrocytes does not require their physical contact with dopaminergic cells

We previously observed that astrocytes mediate urate's protective effect through the release of protective factor(s). To assess if astrocytes mediated inosine's protective effect in the same fashion, MES 23.5 cells were treated with increasing percentages of medium collected from untreated or inosine-treated astrocytes. Medium from untreated astrocytes did not show a statistically significant effect on H_2O_2-treated MES 23.5 viability at any given concentration ($P > 0.05$). On the other hand, conditioned medium from astrocytes treated for 24 h with 100 μM inosine improved MES 23.5 viability in a concentration-dependent manner ($P < 0.001$). This observation was confirmed by a two-way ANOVA analysis that showed significant effect of conditioned medium ($F_{1,151} = 46.28$, $P < 0.0001$) and conditioned medium percentage ($F_{1,151} = 7.31$, $P < 0.0001$) and significant interaction between these two factors ($F_{1,151} = 3.59$, $P = 0.0079$; Fig. 3).
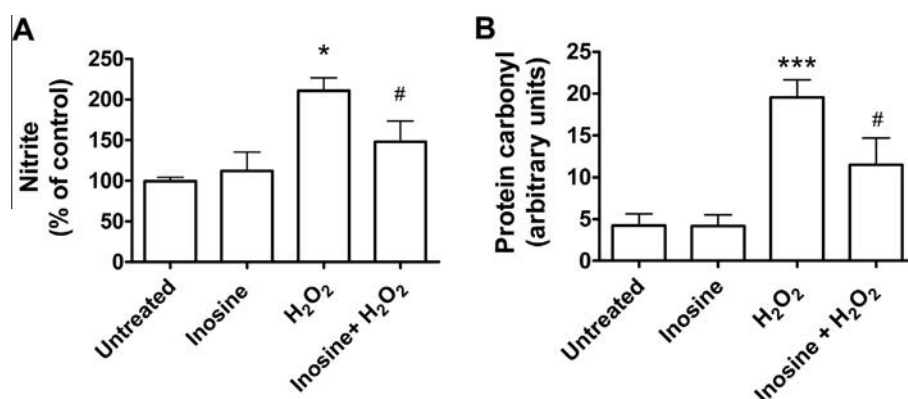

Fig. 2. Inosine reduced oxidative stress in MES 23.5 cells cultured with astrocytes. (A) Effect of inosine treatment (0–100 μM) on 200 μM H_2O_2 induced NO_2^- release in the medium of MES 23.5–astrocytes co-cultures at 24 h of toxic treatment. Cultures were treated with inosine 24 h before and during toxic treatment. Data represent mean ± SEM of three triplicate experiments. (B) Effect of inosine treatment (0–100 μM) on 200 μM H_2O_2 induced protein carbonylation in MES 23.5 cells cultured with astrocytes at 3 h of toxic treatment. Cultures were treated with inosine 24 h before and during toxic treatment. Data represent mean ± SEM of six replicates over three independent experiments. One-way ANOVA: $^{*}P < 0.05$ and $^{***}P < 0.001$ vs untreated and inosine (alone) values; $^{\#}P < 0.05$ vs H_2O_2 (alone) value.
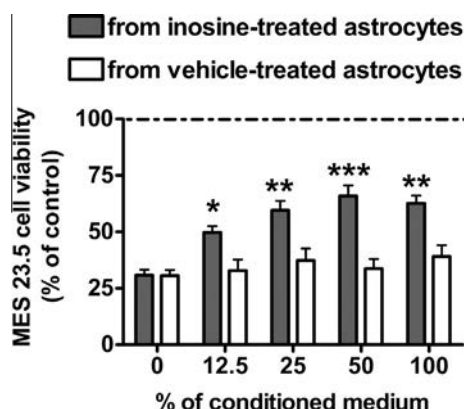
Fig. 3. Inosine-conditioned medium from astrocytes increased viability of H_2O_2-treated MES 23.5 cells. Effect of increasing concentration of cell medium collected from control (white bars) or 100 µM of inosine-treated astrocytes (gray bars) on 200 µM H_2O_2-induced cell death in MES 23.5 pure cultures. Cultures were treated with conditioned medium 24 h before and during toxic treatment. Data represent mean ± SEM of thirteen independent experiments. Two-way ANOVA analysis ($P = 0.0003$) followed by the Newman–Keuls multiple comparison test ($*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ vs respective control value).

Inosine treatment did not affect urate concentration

Extracellular inosine breakdown has been reported in astroglial cultures (Ceballos et al., 1994). To determine whether inosine degradation occurred in our cultures, purine metabolites of inosine were measured in the medium of MES 23.5–astroglial co-cultures treated with inosine. For this experiment we selected two time points: 0, when inosine was added to the cultures, and 24 h, when the cultures would be treated with toxicant. Over 24 h inosine concentration, reflecting both endogenous plus exogenous contributions, was reduced by 99% ($P < 0.0001$); over the same time period hypoxanthine and xanthine increased by 127-fold ($P < 0.0001$) and 1.5-fold ($P < 0.0001$), in comparison to time zero, respectively (Table 1). Thus, the hypoxanthine increment was 1.6-fold greater than the amount of inosine added.

Moreover, adenosine, an inosine 'precursor', increased by 4-fold ($P = 0.0001$, Table 1) over the 24 h. By contrast, urate content was not changed in the medium over the same time period ($P = 0.46$, Table 1),

indicating that extracellular urate unlikely mediated inosine's effects.

In our previous studies we found evidence that urate's protective effect on dopaminergic cells was correlated with its increase within astrocytes (Cipriani et al., 2012b). To assess whether inosine treatment increased intracellular urate in astroglial cells its concentration was measured in inosine-treated enriched astroglial cultures at time 0 and 24 h of treatment. Although adenosine increased 2-fold, intracellular concentrations of urate and other purines were not changed at 24 h in comparison to time 0 (Table 2) and vehicle-treated cells (data not shown). Similarly, no effect was seen on extracellular urate, where inosine induced an approximately 5-fold increase in hypoxanthine concentration ($P < 0.01$, Table 3). Thus despite the expression of functional xanthine oxidase, the enzyme that converts hypoxanthine to xanthine and in turn to urate in cortical astrocytes (Ceballos et al., 1994), we did not find evidence of the conversion of inosine to urate.

Purine increase induced by inosine in mixed-cultures might play a role in inosine protective effect. To assess whether this effect was selective for mixed-cultures, inosine metabolite concentration was also measured in the medium of MES 23.5 cultures after inosine treatment. Similarly to mixed cultures, in MES 23.5 cultures inosine concentration decreased to about 30% ($P < 0.0001$) and 3% ($P < 0.0001$) of control at 6 and 24 h, respectively. Hypoxanthine increased over the time up to 4-fold ($P < 0.0001$) at 24 h in comparison to time zero and xanthine by 1.8-fold in comparison to 6 h (Table 4). Moreover, adenosine increased about 9-fold ($P < 0.0001$) in comparison to time zero. Urate concentration did not change at any tested time (Table 4). These data exclude a direct effect of inosine metabolites on MES 23.5 cells since no protective effect was found in these experimental conditions.

DISCUSSION

We report that inosine prevented oxidative stress-induced cell death in dopaminergic MES 23.5 cells cultured with astrocytes. This effect appeared to be independent of increased intracellular urate, an inosine metabolite and established antioxidant.

Table 1. Extracellular purine content at time zero (0) and 24 h in 100 µM inosine-treated MES 23.5–astrocytes co-cultures

Analites	Concentration (µM)	
	0	24 h
Adenosine	0.16 ± 0.01	0.82 ± 0.04***
Inosine	104 ± 8	0.74 ± 0.09***
Hypoxanthine	1.25 ± 0.26	160 ± 28***
Xanthine	0.34 ± 0.03	0.85 ± 0.05***
Urate	0.89 ± 0.04	0.83 ± 0.05

Purine content of co-cultures cell medium was analyzed by high-performance liquid chromatography. Data are expressed as µM. Significance was determined by Student's t test: ***$P < 0.001$ vs 0 time point value. Data are presented as mean ± SEM of eight experiments.

Table 2. Intracellular purine content at time zero (0) and 24 h in 100 µM inosine-treated enriched astroglial cultures

Analites	Concentration (nmol/g of protein)	
	0	24 h
Adenosine	130 ± 48	390 ± 137*
Inosine	607 ± 230	477 ± 204
Hypoxanthine	320 ± 77	400 ± 199
Xanthine	3 ± 1	6 ± 3
Urate	38 ± 5	26 ± 2

Purines were extracted from enriched astroglial cultures by cell trituration in extracting buffer (see methods) and measured by high-performance liquid chromatography. Data are expressed as nmol/g of protein. Significance was determined by Student's t test: *$P = 0.012$. Data are presented as mean ± SEM of four experiments.

Table 3. Extracellular purine content at time zero (0) and 24 h in 100 µM inosine-treated enriched astroglial cultures

Analites	Concentration (µM)	
	0	24 h
Adenosine	1.7 ± 0.1	1.6 ± 0.4
Inosine	105 ± 1	9.0 ± 0.1[***]
Hypoxanthine	22.6 ± 0.1	127 ± 21[**]
Xanthine	8.5 ± 0.1	13 ± 2
Urate	20.3 ± 0.1	24 ± 4

Purine content of astrocyte medium was measured by high-performance liquid chromatography. Data are expressed as µM. Significance was determined by Student's t test: [**]$P < 0.01$ and [***]$P < 0.001$ vs 0 time point value. Data are presented as mean ± SEM of four experiments.

Table 4. Extracellular purine content in 100 µM inosine-treated MES 23.5 cells over 24 h of treatment

Analites	Concentration (µM)		
	0	6 h	24 h
Adenosine	0.28 ± 0.09	2.28 ± 0.08	2.8 ± 0.4[**]
Inosine	89 ± 20	30 ± 2[**]	2.6 ± 0.1[***]
Hypoxanthine	149 ± 10	680 ± 43[***]	780 ± 81[***]
Xanthine	N.D.	0.10 ± 0.04	0.28 ± 0.07[*]
Urate	N.D.	N.D.	N.D.

Purine content of astrocyte medium was measured by high-performance liquid chromatography.
Data are expressed as µM. Student's t test, $n = 8$, [*]$P < 0.05$ vs 6 h value. One-way ANOVA followed by Newman–Keuls test: [**]$P < 0.01$ and [***]$P < 0.001$ vs 0 time point value. Data are presented as mean ± SEM of eight experiments.

As a close structural homolog of adenosine, inosine may confer protection by direct mechanisms, activating multiple subtypes of adenosine receptors that are known to modulate cell death. Several studies have implicated A_1, A_{2A} or A_3 receptors as mediators of inosine effects in the setting of inflammatory or ischemic injury (Jin et al., 1997; Gomez and Sitkovsky, 2003; Shen et al., 2005; Rahimian et al., 2010). For example, inosine was found to reduce ischemic brain injury in rats likely via an adenosine A_3 receptor-dependent pathway (Shen et al., 2005).

In vitro studies showed inosine to be protective in models of hypoxia (Litsky et al., 1999) and glucose–oxygen deprivation (Haun et al., 1996) where it mediated adenosine protective effects. Inosine has been shown to protect neurons with a neurotrophic effect, promoting axonal regeneration *in vivo* and *in vitro* (Zurn and Do, 1988; Chen et al., 2002; Wu et al., 2003) and inducing the expression of axonal growth-associated genes (Benowitz et al., 1998; Petrausch et al., 2000). This neuroprotective effect can be exerted with a receptor-independent mechanism, for example, activating the cytoplasmic protein kinase Mst3b as shown in the setting of stroke or traumatic brain injury in rodents (Zai et al., 2011). *In vitro* and *in vivo* studies showed inosine to have anti-inflammatory effects in inflammatory or ischemic injury (Jin et al., 1997; Hasko et al., 2000; Gomez and Sitkovsky, 2003; Shen et al., 2005; Rahimian et al., 2010). Moreover, a clinical study raised the possibility that

inosine may have antioxidant properties improving structural and neurological impairment in multiple sclerosis patients (Markowitz et al., 2009).

A previous study reported that inosine protection against chemical hypoxia was dependent on the presence of astrocytes in cultures (Litsky et al., 1999). Similarly, we show that inosine's protective effect on dopaminergic cells was mediated by astrocytes, suggesting a mechanism more complex than a direct protective effect exerted by inosine. Moreover, the rapid inosine degradation occurring in cultures would suggest more of an indirect effect of inosine, which would be consistent with stimulated production and release of an astrocytic protective factor(s) (Imamura et al., 2008).

The rapid elimination of exogenous inosine and increase in its precursor and metabolites are also consistent with the possibility that a purine related to inosine mediates its protective effect. Treatment with inosine at a high concentration relative to endogenous levels increased the concentration of its precursor adenosine in co-cultures, suggesting either conversion of inosine into adenosine (Murray, 1971) or feedback inhibition of adenosine deaminase (Meyskens and Williams, 1971) leading to reduced degradation of endogenous adenosine. Extracellular adenosine in turn may act on its own receptors to enhance survival of dopaminergic neurons in cultures (Michel et al., 1999) or it can be taken up by neurons (Hertz and Matz, 1989).

Alternatively, increased metabolism of inosine may have mediated its protective effect. Inosine breakdown protected cells subjected to glucose deprivation or hypoxia-reoxygenation preserving cellular ATP content (Jurkowitz et al., 1998; Shin et al., 2002; Módis et al., 2009; Szoleczky et al., 2012). Intracellular inosine (and adenosine by way of inosine) was shown to be transformed to hypoxanthine and ribose 1-phosphate by purine nucleotides phosphorylase (Jurkowitz et al., 1998). In turn, ribose 1-phosphate was converted to an intermediate that can enter the anaerobic glycolytic pathway providing the ATP necessary to maintain cell integrity (Jurkowitz et al., 1998). Inhibition of the enzyme purine nucleoside phosphorylase notably prevented the neuroprotective effect of inosine in glial cells and mixed astrocyte-neuronal cultures (Jurkowitz et al., 1998; Litsky et al., 1999). Moreover, this pathway can represent the primary energy source for erythrocytes lacking functional glucose transporters (Young et al., 1985). In our study we found that the hypoxanthine increment was about 24-times higher in MES 23.5-astrocytes co-cultures than in MES 23.5 cells alone after inosine treatment. Purine nucleoside phosphorylase is highly expressed in astrocytes (Ceballos et al., 1994); thus the presence of astrocytes in cultures might provide conditions sufficient for enhanced ATP production during the toxic insult. This raises the possibility that the anaerobic glycolytic pathway might contribute to the protective effect of inosine on dopaminergic cells during oxidative stress. A role for this pathway and the associated production of hypoxanthine by increased purine nucleoside phosphorylase activity in astrocytes may also account for the observed hypoxanthine increase in molar excess of

exogenous inosine introduced. Regardless of whether altered cellular energy metabolism induced by inosine breakdown or a specific metabolite of inosine is protective, these scenarios support the hypothesis that inosine treatment might induce release of factor(s) from astrocytes to protect dopaminergic cells.

Inosine has been shown to be converted to urate in cultures (Ceballos et al., 1994) and to elevate urate serum level in rodents and humans (Ceballos et al., 1994; Scott et al., 2002; Rahimian et al., 2010; Spitsin et al., 2010). Although we observed higher extracellular concentrations of inosine's metabolites, such as hypoxanthine, we did not find increased urate levels in media or in astrocytes. It is unlikely that an earlier increase in urate was missed due to its being metabolized to allantoin since we have already shown that cortical astrocytes and MES 23.5 cells do not express urate oxidase, the enzyme that converts urate to allantoin (Cipriani et al., 2012b). Together these observations argue against a role for urate as the mediator of inosine's protective effects in this cellular model of oxidative stress in PD. However, purine metabolism is of course different in intact humans versus murine culture models and the present findings of a urate-independent protective effect in culture do not preclude the protective effect of urate, which can be substantially elevated in people treated with inosine (The Parkinson Study Group SURE-PD Investigators et al., 2014).

In PD the degeneration of dopaminergic neurons is thought to be induced by accumulation of oxidative damage that leads to mitochondrial impairment and protein aggregation. The finding that inosine prevents oxidant-induced dopaminergic cell loss may be of substantial epidemiological and therapeutic significance for PD. A phase II clinical trial of inosine in early PD showed that inosine was safe, tolerable and effective in raising CSF and serum urate levels (The Parkinson Study Group SURE-PD Investigators et al., 2014). Our results suggest that if CNS inosine itself was elevated in the CNS of treated individuals it could produce a neuroprotective effect independent of urate.

CONCLUSIONS

Inosine had antioxidant and protective effects on dopaminergic cells with a mechanism that does not require increased urate concentration. This finding further supports inosine as a candidate for PD therapy.

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PLOS ONE

Chronic Treatment with Novel Small Molecule Hsp90 Inhibitors Rescues Striatal Dopamine Levels but Not α-Synuclein-Induced Neuronal Cell Loss

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Abstract

Hsp90 inhibitors such as geldanamycin potently induce Hsp70 and reduce cytotoxicity due to α-synuclein expression, although their use has been limited due to toxicity, brain permeability, and drug design. We recently described the effects of a novel class of potent, small molecule Hsp90 inhibitors in cells overexpressing α-synuclein. Screening yielded several candidate compounds that significantly reduced α-synuclein oligomer formation and cytotoxicity associated with Hsp70 induction. In this study we examined whether chronic treatment with candidate Hsp90 inhibitors could protect against α-synuclein toxicity in a rat model of parkinsonism. Rats were injected unilaterally in the substantia nigra with AAV8 expressing human α-synuclein and then treated with drug for approximately 8 weeks by oral gavage. Chronic treatment with SNX-0723 or the more potent, SNX-9114 failed to reduce dopaminergic toxicity in the substantia nigra compared to vehicle. However, SNX-9114 significantly increased striatal dopamine content suggesting a positive neuromodulatory effect on striatal terminals. Treatment was generally well tolerated, but higher dose SNX-0723 (6–10 mg/kg) resulted in systemic toxicity, weight loss, and early death. Although still limited by potential toxicity, Hsp90 inhibitors tested herein demonstrate oral efficacy and possible beneficial effects on dopamine production in a vertebrate model of parkinsonism that warrant further study.

Introduction

Protein aggregates such as beta amyloid in Alzheimer's disease, tau deposits in frontotemporal dementia, and Lewy bodies in Parkinson disease (PD) are a common pathological feature in neurodegenerative disorders. Molecular chaperones, such as heat shock proteins, co-localize with aggregates in neurodegenerative disease and play a critical role in protein processing and homeostasis [1,2]. Heat shock proteins (Hsp) such as Hsp70 direct misfolded and potentially toxic proteins for degradation via the proteasome or autophagy-lysosomal system [3–5]. Furthermore, induction of Hsp70 is protective in models of neurodegenerative disorders, such as Huntington's disease, spinocerebellar ataxias,

and tauopathy disorders (i.e., Alzheimer's disease) [6–8]. We and others have demonstrated that Hsp70 can enhance the degradation of misfolded α-synuclein, reduce oligomer formation, and mediate toxicity due to α-synuclein overexpression [9–11]. Moreover, direct pharmacological upregulation of Hsp70 with geldanamycin, an Hsp90 inhibitor, results in decreased cytotoxicity from α-synuclein [12]. Thus targeting molecular chaperones, such as Hsp70 or Hsp90, has reasonable therapeutic potential not only for parkinsonism, but also for related neurodegenerative disorders.

A number of small molecule inhibitors of Hsp90 have been tested in models of PD and other neurodegenerative disorders [13,14]. Hsp90 negatively regulates Hsp70 expression by blocking

activation of the transcription factor HSF-1; thus inhibitors result in Hsp70 induction [15]. Geldanamycin is a naturally occurring benzoquinone that blocks Hsp90 interaction with HSF-1 resulting in enhanced Hsp70 expression [16]. However, its utility is limited by hepatotoxicity and poor brain permeability. In contrast, the analogues 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) and 17-dimethylaminoethylamino-17-demethoxy-geldanamycin (17-DMAG) have greater potency, reduced toxicity, and cross the blood brain barrier more efficiently [6,17]. Preliminary testing also showed neuroprotection in models of polyglutamine disorders. However, despite promising effects in clinical trials for cancer, these compounds have been pursued only in a limited fashion due to hepatotoxicity, poor oral bioavailability, and formulation issues [18,19].

Recently, a novel class of Hsp90 inhibitors with structure different from that of geldanamycin and derivatives was discovered among a screen for drugs that bind the ATP pocket of Hsp90. SNX-2112 (4-[6,6-dimethyl-4-oxo-3-(trifluoromethyl)-4,5,6,7-tet-rahydro-1H-indazol-1-yl]-2-[(trans-4-hydroxycyclohexyl)amino]-benzamide; PF-04924473) was the initial drug described and exhibited potent Hsp90 inhibition, anti-tumor activity, blood-brain permeability, and oral bioavailability [20,21]. We recently tested compounds from the same class in a PD cell model [22]. Several of these novel Hsp90 inhibitors, in particular SNX-0723 (PF-04924868), significantly reduced α-synuclein oligomer formation and cytotoxicity concomitant with Hsp70 induction. SNX-0723 also exhibited favorable pharmacokinetic properties and induced Hsp70 in rat brain [22]. Based on these findings we next wanted to test the effect of these novel Hsp90 inhibitors in a rat model of parkinsonism. We and others have demonstrated that AAV expression–utilizing a variety of viral serotypes: 1, 2, 5, 6, and 8–of α-synuclein results in progressive, dopaminergic nigro-striatal neurodegeneration over the course of several weeks [23–25]. This model allowed us to test whether chronic oral administration of novel Hsp90 inhibitors in rats could protect against progressive α-synuclein-induced nigrostriatal toxicity.

Methods

Viral Production

Construction of rAAV vectors used to express human wild-type α-synuclein was as previously described (AAV-CBA-Syn-WPRE construct) [26]. Recombinant AAV2/8 virus was generated by the Harvard Gene core (Harvard Gene Therapy Initiative, Harvard Medical School) via tripartite transfection of the *cis*-transgene, packaging (*rep* and *cap*) genes, and helper plasmid into HEK 293A cells. Viral particles were purified by iodixanol density gradient, isolated, and titered by dot blot hybridization. Final titer for rAAV expressing human α-synuclein was 5.6×10^{12} gc/mL.

Stereotaxic Surgery and Drug Treatment

Animal protocols and procedures were approved by the MGH Subcommittee on Research Animal Care (IACUC #2005N000156) and followed recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. All surgery was performed under ketamine/xylazine anesthesia, and all efforts were made to minimize suffering. Male Sprague Dawley rats (300–350 g) were anesthetized, skull exposed, and then unilaterally injected in the substantia nigra (SN) with rAAV2/8 expressing human α-synuclein as previously described [23]. Each rat was injected with 2 μL of rAAV2/8 (1.12×10^{10} viral genomes) at 0.4 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. The scalp was sutured and animals were monitored until fully recovered.

Four days following recovery from surgery, rats began receiving drug or vehicle (0.5% methylcellulose) by oral gavage on a biweekly basis. Figure 1 illustrates the experimental paradigm and structures for each compound. Drug groups included SNX-0723 (PF-04924868) at 10 mg/kg and SNX-9114 (PF-04944733) at 1.5 mg/kg and 3 mg/kg. All rats were weighed routinely prior to surgery, and then at each treatment session for the duration of the experiment. Rats treated with 10 mg/kg SNX-0723 showed toxicity characterized by weight loss and failure to thrive, thus mid-treatment the dose was reduced to 6 mg/kg dose (see results).

Tissue Preparation and Immunohistochemistry

Eight weeks post-injection, rats were deeply anesthetized and transcardially perfused with cold 0.01M phosphate buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in PBS. Brains from a subset of animals were harvested fresh, without fixation, and the cortex, striatum, and midbrain quickly dissected on ice, snap-frozen in isopentane, and kept at $-80°C$ for use in biochemical analyses. Perfused brains were postfixed 24 hours, then cryoprotected in 30% sucrose/PBS, and serially sectioned at 40 μm with a sliding microtome. For immunohistochemistry, free-floating sections were rinsed with PBS, then treated with endogenous peroxidase inhibitor (10% methanol and 3% H_2O_2), permeablized with 0.3% Triton X-100 in PBS, and blocked in 5% normal goat serum. Coronal sections through the striatum and SN 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 rinsing, immunostaining was visualized with biotinylated secondary, followed by avidin-biotin (Vectastain Elite Kit), and 3,3′-diaminobenzidine reaction. Immunostained sections were washed and mounted on Superfrost slides, and then counterstained with 0.05% cresyl violet per standard protocols and coverslipped (Permount, Sigma Chemicals).

Microscopy and Stereology

Immunostained sections were viewed using an Olympus BX51 microscope, and photomicrographs were taken with Olympus DP70 digital camera and adjusted for suitable contrast and brightness. Cases in which the AAV injection was improperly placed in the SN (missed target) and poor expression of α-synuclein in the nigrostriatal system were excluded from analyses. Nigrostriatal cell loss was assessed using unbiased stereology according to the optical fractionator principle [27] as previously described [23]. The examiner was blinded to treatment group. Cell counts included the injected side compared to the uninjected contralateral SN as control. At least 8 sections (240 μm apart) though the SN for each case were analyzed and counted using the Olympus CAST Stereology System. Sampling frequency was sufficient for a coefficient of error of less than 0.1.

Immunoblotting

Striatal and midbrain tissues were separately suspended in 8×volume/wet weight tissue of lysis buffer (50 mM Tris-HCL, pH 7.4; 175 mM NaCl; 5 mM EDTA, pH 8.0; and protease inhibitor, Roche Inc.) and homogenized on ice for 10–15 seconds with Teflon pestle. A 100 μL aliquot of this tissue suspension was removed for HPLC and treated with 100 mM H_3PO_4 plus 100 μM methyldopa (internal standard for HPLC recovery). Each sample was centrifuged for 15 minutes at 4°C, filtered (0.22 μm Spin-X filter, Corning, NY), and then 1% Triton X-100 added to the non-HPLC lysate. Lysates were then centrifuged for 60 min at
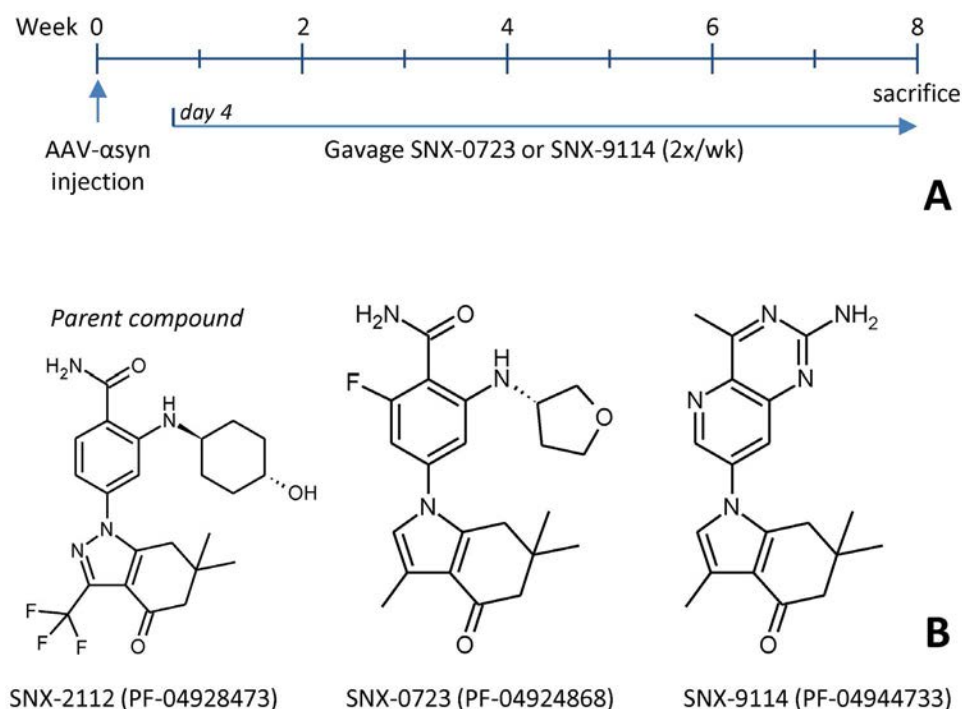
Figure 1. Study paradigm and structure of compounds. A) Illustrates the study paradigm and timecourse for drug testing in animals. **B**) Shows the structures of parent and the two derivative SNX compounds used in experiments.
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4°C to collect the triton-X 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. Samples were separated on a 4–12% Bis-Tris NuPage pre-cast gel (Invitrogen) with MES buffer, transferred to PVDF, and immunoblotted for Hsp70 (rabbit anti-Hsp70, Stressgen), tyrosine hydroxylase (TH; mouse TH-2 antibody, Sigma), or α-synuclein (mouse Syn1, BD Transduction Laboratories, or Syn (LB509) antibody, Zymed). All blots were immunostained for GAPDH or actin as loading control. Immunoblotted α-synuclein, TH and GAPDH were detected with secondary antibody conjugated to HRP and reacted with ECL (GE Healthcare), per protocol. Films were digitally scanned and analyzed with ImageJ software (NIH). TH and α-synuclein content for each sample was normalized to loading control.

Hsp70 ELISA

Quantitative analysis of Hsp70 levels in rat cortical (or striatal) tissues after treatment with Hsp90 inhibitors was performed using ELISA (Stressgen, Ann Arbor, MI, USA) according to the manufacturer's instructions and similar to that detailed by Danzer et al. [10]. Hsp70 concentrations in tissues were determined by generating a standard curve with calibrated Hsp70 protein standard and then interpolating absorbance readings using fitting software (Graph Pad 5.0).

Dopamine Content

Striatal tissues were thawed, weighed, homogenized, and mixed in lysis buffer with methyldopa added as an internal control as described above. Dopamine (DA) and 3,4-dihydroxyphenlyacetic acid (DOPAC) were measured by HPLC with electrochemical detection and normalized to protein content per sample [28].

Statistics

All data are expressed as group mean ± SEM. Stereological estimates of nigral TH cell survival were analyzed using one-way ANOVA with Tukey's multiple comparison post-hoc (Prism GraphPad 5.0, San Diego, CA). Dopamine and DOPAC content were analyzed with repeated measures ANOVA and Bonferroni multiple comparisons posthoc and Spearman's correlation. Alpha was 0.05 for all tests.

Results

Preliminary testing of SNX-0723 in rats at doses 10 mg/kg or higher showed lasting induction of Hsp70 in brain at least 24 hours post oral gavage (Figure 2A). The newer compound SNX-9114 likewise demonstrated excellent brain permeability and even greater potency than SNX-0723 in terms of Hsp70 induction. Limited dose-response testing also suggested more prolonged Hsp70 induction, 72 hours or greater, in brain for both Hsp90 inhibitors. Based on these findings we compared the effects of chronic oral treatment of rats with SNX-0723 at 10 mg/kg versus SNX-9114 at 1.5 and 3 mg/kg for 7–8 weeks post injection of AAV-α-synuclein. Western blot analysis of striatal extracts collected 3–4 days post final treatment confirmed a sustained 2-fold increase in Hsp70 induction in SNX-9114 treatment groups compared to vehicle (Figure 2B, C).

Tolerability and Toxicity

Although chronic treatment with SNX-9114 was generally well tolerated, SNX-0723 at 10 mg/kg resulted in toxicity manifest by diarrhea, weight loss, failure to thrive, and early death in 7 of 21 animals. As a result, the dose of SNX-0723 was reduced mid-treatment to 6 mg/kg for all remaining rats in this group. Dose reduction was effective in reducing toxicity, reversing weight loss and mortality. However, rats did not gain weight at the same rate
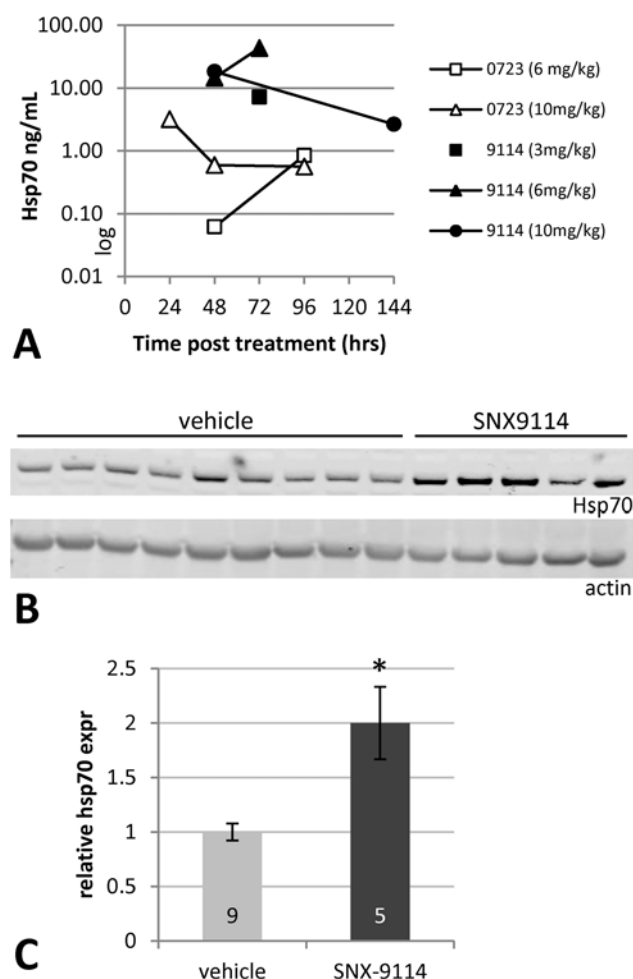
Figure 2. Hsp70 induction in brain. A) Graph of hsp70 ELISA data from cortical tissue lysates after treatment of rats with novel small molecule Hsp90 inhibitors. Tissue was harvested at 1–6 days post treatment and shows sustained hsp70 induction at ≥72 hrs for both SNX-0723 and SNX-9114. B) Western blot of striatal tissue homogenates from rats injected with WT α-synuclein and treated with SNX9114 (n = 5) or vehicle (n = 9), immunoblotted for hsp70 and actin as loading control. C) Densitometry shows significant (p = 0.037) striatal Hsp70 induction following treatment with SNX-9114.
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as vehicle control animals (Figure 3). Similarly, although rats treated with 3 mg/kg SNX-9114 did not show overt signs of toxicity, weight gains were less than that of control. Halving the SNX-9114 dose to 1.5 mg/kg in a separate treatment group made little difference in weight gain. No overt behavioral changes were observed in either of the groups or treatment regimens.

Effects of Hsp90 Inhibitors on Nigrostriatal Toxicity

Chronic treatment with SNX-0723 or with the more potent SNX-9114 did not rescue nigral dopaminergic neurons from α-synuclein dependent toxicity. Figure 4A-C shows the distribution of TH-immunoreactive cell loss at comparable levels of the SN for both drug and vehicle treatment after viral injection. Stereological counts revealed mean TH cell loss (relative to the contralateral unlesioned SN) of 21.1%±3.7 for vehicle, 21.6%±5.0 for 6–10 mg/kg SNX-0723, and 17.0%±3.2 and 24.1%±4.7for 1.5 and 3 mg/kg doses of SNX-9114, respectively (Figure 4E). Although the lower dose of SNX-9114 appeared to have slightly

less TH cell loss, one-way analysis of variance revealed no significant differences among treatment groups ($F[3,31] = 0.42$, $p = 0.39$). Similarly, analysis of striatal TH terminal density as shown in representative cases (Figure 5) demonstrated no differences between drug and vehicle control groups. Likewise, among different treatment groups there was also no apparent change in α-synuclein-positive inclusion-like structures in nigrostriatal terminals or cell bodies.

Hsp90 Inhibitor Effects on Dopamine Terminals

We performed biochemical analysis of striatal DA and its metabolite, DOPAC, to gauge drug effects on nigrostriatal terminal plasticity. Striatal DA and DOPAC measurements ipsilateral (ip) to rAAV α-synuclein injection were normalized to the contralateral (ct) uninjected/unlesioned side within each animal and shown as ratio of ip/ct (Figure 6A). Repeated measures ANOVA demonstrated a significant main effect for drug ($F[3,25] = 7.05$, $p = 0.001$) and interaction with DA and DOPAC measures ($F[3,25] = 3.31$, $p = 0.036$). Vehicle treated animals, as expected, showed (~50%) reduction in striatal DA ipsilateral to rAAV α-synuclein injection with mean DA ratio of 0.51 ± 0.10 relative to that in the contralateral striatum. SNX-0723 at the 6–10 mg/kg dose did not significantly alter striatal DA levels (0.62 ± 0.10) and was similar to vehicle control. However, treatment with SNX-9114 resulted in significant increase and trend toward normalization of striatal DA and DOPAC levels compared to vehicle. DA content for the 1.5 mg/kg dose was 1.04 ± 0.13 ($p = 0.061$) and the 3 mg/kg dose 1.45 ± 0.32 ($p = 0.003$). DOPAC was also significantly increased for SNX-9114 1.5 mg/kg dose, 1.36 ± 0.15 ($p = 0.005$), and likewise showed a non-significant trend for normalization at 3 mg/kg dose, 1.12 ± 0.22 ($p = 0.28$). We also examined an index of DA turnover to DOPAC (DOPAC/DA ratio), which negatively correlated with DA changes, $r_s = -0.67$, $p<0.01$ (Figure 6B). Decreases in DA for control and SNX-0723 at 6–10 mg/kg corresponded to increase in DOPAC/DA ratio (1.57 ± 0.2 and 1.34 ± 0.16, respectively), or turnover. By contrast, in the case of the 3 mg/kg dose of SNX-9114 relative increase in striatal DA resulted in non-significant decrease in DOPAC/DA ratio (0.81 ± 0.09).

Discussion

Modulation of molecular chaperones with small molecule Hsp90 inhibitors has gained recent attention as potential novel therapeutics for parkinsonism and other neurodegenerative disorders that manifest proteinopathy [13,29,30]. We recently reported that novel small molecule Hsp90 inhibitors in a neuroglioma cell model of parkinsonism can reduce formation of toxic dimer/oligomeric species of α-synuclein and prevent cytotoxicity [22]. In the current study, we examined whether chronic treatment with candidate, small molecule Hsp90 inhibitors could protect against α-synuclein-induced nigrostriatal toxicity in a targeted viral model of parkinsonism in the rat. Chronic treatment twice weekly was best tolerated with SNX-9114, but neither SNX-0723 nor SNX-9114 protected against loss of dopaminergic nigrostriatal neurons in our model. Several possibilities may explain these results including length of treatment, onset of therapy, and inter-animal variability. Longer incubation with AAV-synuclein (12 vs 8 weeks, personal observation) can result in greater dopamine cell loss and, combined with chronic Hsp90 inhibitor therapy, might have increased our ability to detect potential differences between vehicle and drug groups. Although we started treatment early–4 days post viral injection when viral transgene expression is only beginning–pretreatment before AAV
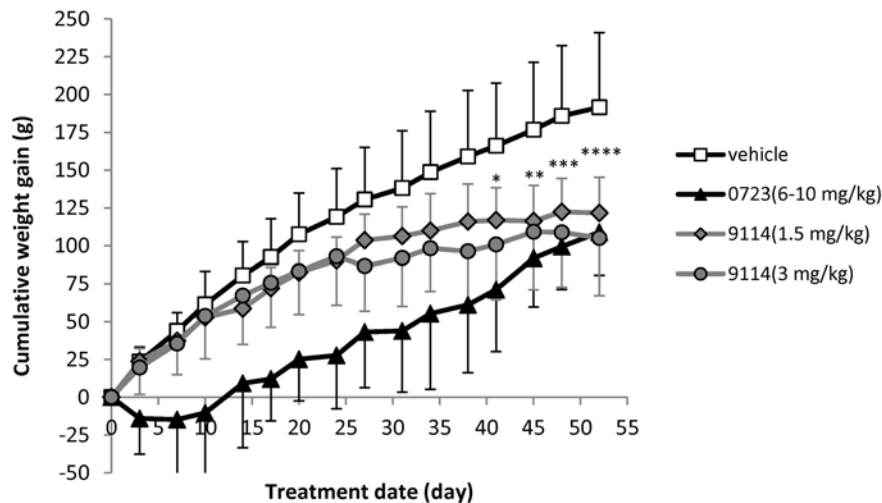
Figure 3. Graph of cumulative weight gain for treatment groups. SNX-0723 caused the most toxicity, weight loss, and failure to thrive at the higher dose of 10 mg/kg. All treatment groups gained weight at lower doses, including SNX-9114, but not at the rate of vehicle control (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 for comparison of vehicle and 9114 at 1.5 mg/kg; 2-way ANOVA with Bonferroni correction). (n = 14, 14, 12, 10, and 14 for vehicle, 0723 [3mg/kg], 0723 [6–10 mg/kg], 9114 [1.5 mg/kg] and 9114 [3 mg/kg], respectively).
doi:10.1371/journal.pone.0086048.g003
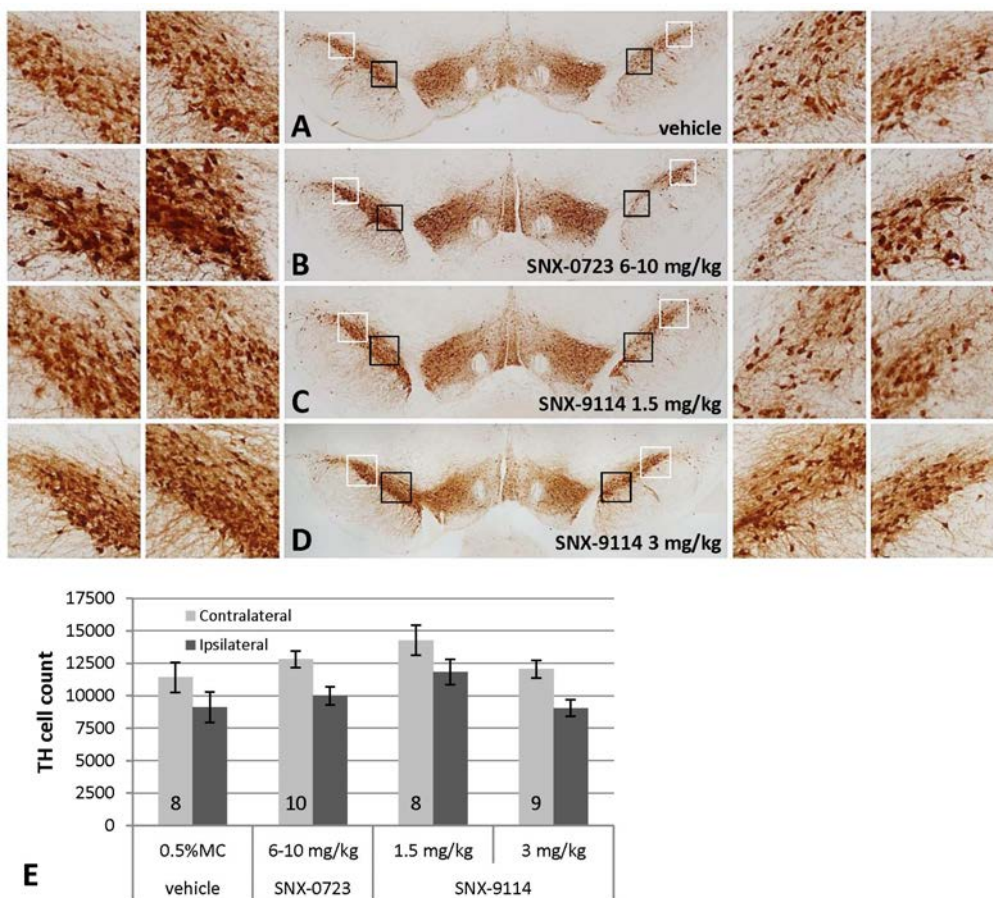
Figure 4. Comparison of higher dose SNX-0723 and SNX-9114 effects on nigrostriatal toxicity. A–D) Photos show low power images of injected SN (right) and contralateral uninjected side (left) for each drug treatment group. Black (medial) and white (lateral) squares indicate regions of interest for higher magnification photos shown. There is modest cell loss on the side of the lesion for all treatments. **E**) Graph of stereological counts (mean ±SEM) of TH-positive cells in the SN ipsilateral and contralateral to AAV-α-synuclein lesion. Numbers at base of bars indicate N for each group. Analysis of variance revealed no significant differences among treatment groups.
doi:10.1371/journal.pone.0086048.g004
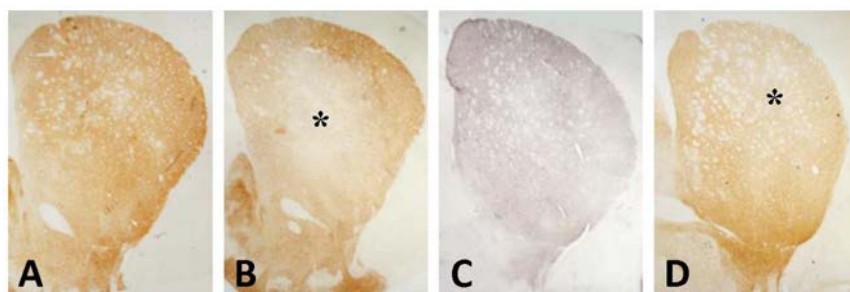
Figure 5. Illustration of drug effects on nigrostriatal terminal density for SNX-9114, 3 mg/kg dose. The representative photos show the distribution of TH+ terminals in the striatum contralateral and ipsilateral to AAV-α-synuclein injection in panels **A** and **B,** respectively. **C)** α-Synuclein-positive nigrostriatal terminals ipsilateral to AAV injection in same case. **D)** Photo of TH+ terminals in striatum from animal treated with lower dose SNX-9114, 1.5 mg/kg. *Marks region of TH terminal loss due to α-synuclein toxicity.
doi:10.1371/journal.pone.0086048.g005

Figure 6. Striatal DA and DOPAC content and turnover. A) Graph shows the mean (±SEM) DA or DOPAC content in the striatum ipsilateral (ip) to AAV-α-synuclein injection normalized to the contralateral (ct) uninjected side (ratio ip/ct). Repeated measures ANOVA (DA and DOPAC) showed a main effect for 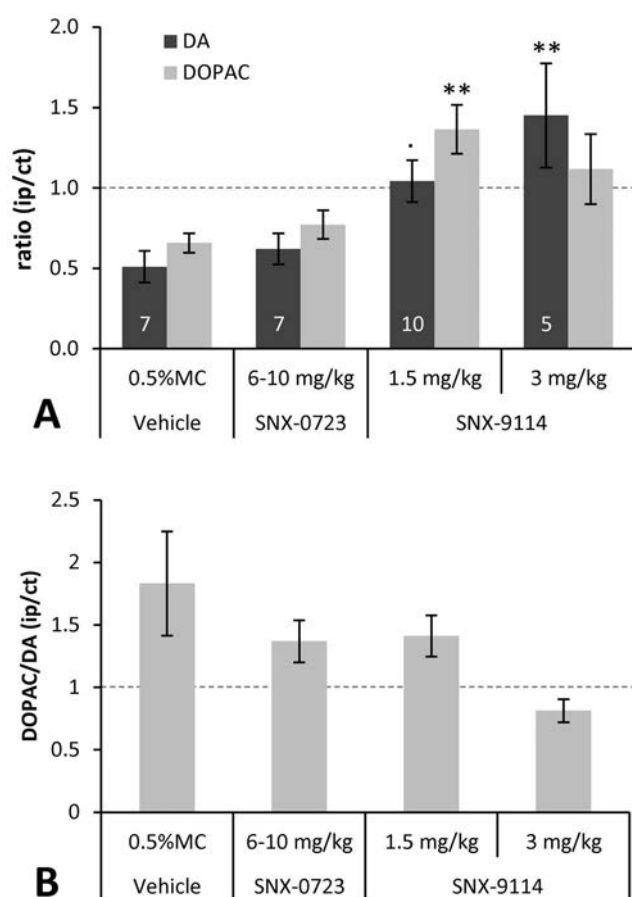drug (F[3,25] = 7.05, p = 0.001) and interaction with DA metabolites (F[3,25] = 3.31, p = 0.036). DA levels were significantly increased for SNX-9114 at both 1.5 (p = 0.061) and 3 (p = 0.003) mg/kg doses compared to vehicle, whereas no change for SNX-0723. DOPAC levels increased significantly only for SNX-9114 at 1.5 mg/kg (p = 0.005), but appeared also to trend toward normal for the higher dose. N for each group is noted at base of each bar (7, 7, 10, 5, respectively). **B)** Graph of DA turnover (DOPAC/DA ratio, normalized to contralateral control) for each case shows an inverse correlation between DA level and rate of turnover. ·p = <0.1, **p<0.01.
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injection may be required in our model to prevent dopamine cell loss in the rat SN as seen in prior cell studies [22]. Variability in this model and treatment paradigm likewise could have contributed to lack of findings, indicating need for greater animal numbers to the increase the power of our observations.

Although Hsp70 expression or induction (i.e., via Hsp 90 inhibition) has been shown to reduce α-synuclein dimer/oligomers and cytotoxicity in cell models [10,12,31,32], few studies have examined the effects of increased Hsp70 on α-synuclein toxicity in animal models. In *Drosophila* Hsp70 expression has been shown to reduce dopaminergic neuronal loss associated with α-synuclein [11]. Crossing Hsp70 expressing mice with transgenic mice that express human wild-type α-synuclein (line D), we subsequently demonstrated that Hsp70 specifically reduces "toxic" high-molecular weight α-synuclein species [9]. In contrast, Shimsheck et al. (2010) examined transgenic mice co-expressing both human A53T mutant α-synuclein and Hsp70(HspA1A) under the control of the Thy1 promoter and found that mice overexpressing Hsp70 actually performed worse on behavioral tests than single transgenic α-synuclein(A53T) mice [33]. Moreover, Hsp70 overexpression did not cause change in α-synuclein expression, oligomers, phosphorylation, or localization in brain. These findings are difficult to explain, but possibilities include inadequate level of Hsp70 expression, non-functional Hsp70, or lack of other co-chaperones such as Hsp40 or Hsp90 which enhance Hsp70 ATPase activity [34]. Differences in interaction between Hsp70 and wild-type vs A53T α-synuclein may also contribute but remain unclear. Besides Hsp70 other heat shock proteins may be (more) effective, such as Hsp104 which when tested in a rat model of α-synuclein overexpression reduced dopaminergic cell loss and phosphorylated α-synuclein-containing inclusions [35]. In vitro Hsp27 expression has also been shown to have more potent effect than Hsp70 on toxicity associated with mutant and wild-type α-synuclein [36]. Recent studies by Daturpalli et al. (2013) suggest also that Hsp90 itself interacts with oligomeric α-synuclein and can inhibit fibril formation and α-synuclein toxicity in SHSY5Y cells [37]. Together these data indicate need for further study of heat shock protein effects on α-synuclein in both cell and animal models.

Despite the lack of apparent rescue of nigrostriatal dopamine cells, we observed a significant drug effect on striatal DA content and metabolism. In animals treated with SNX-9114 striatal DA and DOPAC levels increased and "normalized," suggesting a possible effect on the remaining nigrostriatal terminals and neurochemical plasticity. These preliminary findings are potentially significant as restoration of dopamine content in the striatum improves behavioral deficits in PD models and overall function in

PD patients [38,39]. Potential mechanisms for the observed increase in striatal DA include increased TH activity or L-DOPA (L-dihydroxyphenylalanine) supply, decrease in monoamine oxidase B activity, or increased terminal DA reuptake. Compensatory mechanisms for nigrostriatal injury are well-established and residual striatal terminals can compensate for nearly 80% loss of DA innervation [38,40]. Recent data, however, suggests that α-synuclein expression negatively regulates TH activity and can affect dopaminergic neurotransmission [41]. Results in vehicle control animals are consistent with these findings and show reduced striatal DA without evidence of compensation despite relative small nigrostriatal lesion (~21% TH cell loss). However, the cause of increase or normalization of DA levels with SNX-9114 is less clear. Although we did not measure TH activity, striatal levels of TH for treated and control animals appeared similarly reduced due to AAV-α-synuclein lesion and there was no evidence of TH terminal sprouting as seen previously in other partial lesion models [40,42]. While Hsp70 induction has been shown to protect dopaminergic neurons against toxic insult, including α-synuclein, very little is known about the potential effects on DA production (i.e. TH activity) or metabolism [43,44]. Further studies are needed to evaluate possible neuromodulatory effects of small molecular Hsp90 inhibitors and Hsp70 induction on dopaminergic neurons.

A major limitation of Hsp90 inhibitor therapy unfortunately has been toxicity, which was also found for the drugs used in this study [13]. Modifications to geldanamycin leading to development of the analogues 17-AGG and 17-DMAG were initially purported to reduce toxicity, mainly hepatic, and increase potency as well as brain penetration [6,17]. Clinical trials of these compounds primarily for cancer therapy have shown some promise, but significant concerns about hepatotoxicity and delivery issues remain, limiting their use in particular for non-oncology indications [18,19]. Recent efforts have focused on developing novel small molecule Hsp90 inhibitors, such as those studied herein which potently inhibit Hsp90, cross the blood-brain barrier, and are orally bioavailable [20,21]. Our initial studies in rodents demonstrate that candidate drugs, administered orally, were brain permeable at concentrations used and produced lasting induction of Hsp70 in brain tissue. However, SNX-0723 given chronically at 10 mg/kg caused animals to loose significant weight, fail to thrive, and die, forcing decrease in the dose to 6 mg/kg which was better tolerated. Although the more potent SNX-9114 did not cause overt toxicity at either dose used, rats still did not gain weight at rates equivalent to vehicle treated animals. While SNX-9114 induced Hsp70 in brains, it too had an insignificant neuroprotective effect on synucleinopathy. It is tempting to speculate whether Hsp70 induction in brain had a causal relationship to weight loss/failure-to-thrive in animals, but based on prior studies it is more likely that our candidate drugs caused peripheral toxicity (i.e., hepatic, gastrointestinal) [29,45]. No studies to our knowledge so far have linked Hsp70 (or Hsp90) to weight homeostasis or metabolism. Further studies are needed to elucidate the source of toxicity for future trials.

To date clinical trials for Hsp90 inhibitors have primarily been limited to cancer therapy, based on their selectivity for tumor cells, modulation of Hsp90 function, and binding of client proteins [29,46]. Kamal *et al.* have suggested that the geldanamycin derivative, 17-AAG, preferentially binds Hsp90 when it is part of a multi-chaperone complex, including co-chaperones and client protein [47,48]. Although it is unclear if novel small molecule Hsp90 inhibitors such as those used here function similarly, such selectivity may provide similar advantage for use in neurodegenerative disorders particularly due to the probable need for long-term, chronic therapy, relative to that in cancer. Our findings, however, indicate that Hsp70 induction in brain was widespread rather than limited to tissues affected by viral α-synuclein expression. Though potentially concerning, such effects in brain may actually be advantageous. Heat shock protein induction (i.e., stress response) by Hsp90 inhibition has been shown to have purported neuroprotective effects in a variety of neurodegenerative models including Huntington's disease [7], spinocerebellar ataxias [6], tauopathies [8], and parkinsonism [10] in which pathology spreads. Neuroprotective effects of Hsp70 induction in particular include reduction in aggregate ("toxic" oligomer) formation, cellular toxicity, and apoptosis [9,10,49]. Thus, targeting Hsp90 and augmenting the cellular response to stressors may still be a reasonable therapeutic approach for neurodegenerative diseases.

This study represents a first attempt to examine the ability of novel small molecule Hsp90 inhibitors to protect against α-synuclein dependent nigrostriatal toxicity in mammalian model of PD. Compared to vehicle neither compound tested protected against nigral TH-cell loss; however, our results suggest possible nigrostriatal terminal effects with normalization of DA content and turnover in striatum. These results are significant as restoration of DA in the brain is an aim of current therapeutics in Parkinson disease. Although the mechanism of nigrostriatal dopamine restoration remains unclear, these findings suggest that Hsp90 inhibition may represent a potential novel therapeutic approach to Parkinson disease and related disorders. Further study of these novel small molecule Hsp90 inhibitors is warranted and must also address toxicity concerns for future trials in neurodegenerative disease.

Acknowledgments

Author Contributions

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REVIEW

Treatment of Parkinson's Disease: What's in the Non-dopaminergic Pipeline?

Albert Y. Hung · Michael A. Schwarzschild

Abstract Dopamine depletion resulting from degeneration of nigrostriatal dopaminergic neurons is the primary neurochemical basis of the motor symptoms of Parkinson's disease (PD). While dopaminergic replacement strategies are effective in ameliorating these symptoms early in the disease process, more advanced stages of PD are associated with the development of treatment-related motor complications and dopamine-resistant symptoms. Other neurotransmitter and neuromodulator systems are expressed in the basal ganglia and contribute to the extrapyramidal refinement of motor function. Furthermore, neuropathological studies suggest that they are also affected by the neurodegenerative process. These non-dopaminergic systems provide potential targets for treatment of motor fluctuations, levodopa-induced dyskinesias, and difficulty with gait and balance. This review summarizes recent advances in the clinical development of novel pharmacological approaches for treatment of PD motor symptoms. Although the non-dopaminergic pipeline has been slow to yield new drugs, further development will likely result in improved treatments for PD symptoms that are induced by or resistant to dopamine replacement.

Keywords Parkinson's disease · Non-dopaminergic · Dyskinesias · Motor fluctuations · Glutamate · Adenosine

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder that is characterized clinically by the classical motor symptoms of bradykinesia, rigidity, and resting tremor. These symptoms are primarily caused by the selective loss of dopaminergic neurons in the substantia nigra pars compacta, which results in decreased levels of dopamine in the striatum. Dopamine replacement strategies have been the mainstay of treatment for motor symptoms of PD, and nearly 50 years since its introduction, levodopa (the precursor of dopamine) remains the most effective treatment. However, despite its beneficial effects on motor function, dopaminergic therapy has significant limitations, making development of other therapeutic approaches targeting non-dopaminergic pathways a priority [1]. First, neither levodopa nor dopamine agonists have been demonstrated to slow the progression of nigrostriatal cell loss. Second, while initially successful in ameliorating motor symptoms, long-term treatment with levodopa is complicated by the onset of motor fluctuations (with alternating periods of mobility and relative immobility) and involuntary dyskinesias. Last, symptoms that develop at later stages of PD, both motor (e.g., postural instability and freezing of gait) and non-motor, are frequently not responsive to dopaminergic treatments. These symptoms are likely to be caused by the degeneration of neurons in other parts of the nervous system as a result of the same disease process that affects the nigrostriatal system [2].

In this review, we discuss potential non-dopaminergic approaches to treatment of PD symptoms. Multiple neurotransmitters are recognized to play a role in modulating the basal ganglia and other neural circuits thought to be involved in PD. We will focus primarily on neurotransmitter targets in which there have been therapeutic advances in targeting motor symptoms. Agents targeting non-dopaminergic pathways are also being actively explored for treatment of non-motor symptoms.

Neurotransmitter Diversity in the Basal Ganglia and Motor Control

To understand the potential use of pharmacologic agents targeting non-dopaminergic pathways, it is helpful to briefly

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review the role of these neurotransmitter systems in regulating motor function [3]. In the classic model of basal ganglia organization (Fig. 1), the cerebral cortex sends excitatory glutamatergic inputs to the striatum. Dopamine, via the nigrostriatal pathway, modulates these inputs, either through an excitatory effect on a subpopulation of striatal neurons that contain gamma-aminobutyric acid (GABA) and substance P (direct pathway), or through an inhibitory effect on a separate subpopulation of neurons that co-express GABA and enkephalin (indirect pathway). The effects on the direct and indirect pathways are mediated by dopamine binding to D1 and D2 receptors, respectively, both of which are highly expressed in the striatum (Fig. 2). In the direct pathway, striatal neurons send inhibitory GABAergic inputs directly to the output

nuclei of the basal ganglia, the globus pallidus pars interna (GPi) and substantia nigra pars reticulata (SNr), which then send GABAergic fibers to ventral thalamic nuclei. In contrast, axons from striatofugal neurons in the indirect pathway form GABAergic synapses with cells in the globus pallidus pars externa, which then send GABAergic projections to the subthalamic nucleus (STN). The STN then uses glutamate to modulate basal ganglia output from the GPi/SNr. This classic model suggests that dopamine regulates basal ganglia activity by balancing opposing effects on the direct and indirect pathways. Loss of striatal dopamine in PD disrupts this balance, producing a hypokinetic (parkinsonian) state. In contrast, subsequent treatment with dopaminergic agents predisposes to hyperkinetic (dyskinesia) responses. While this model is
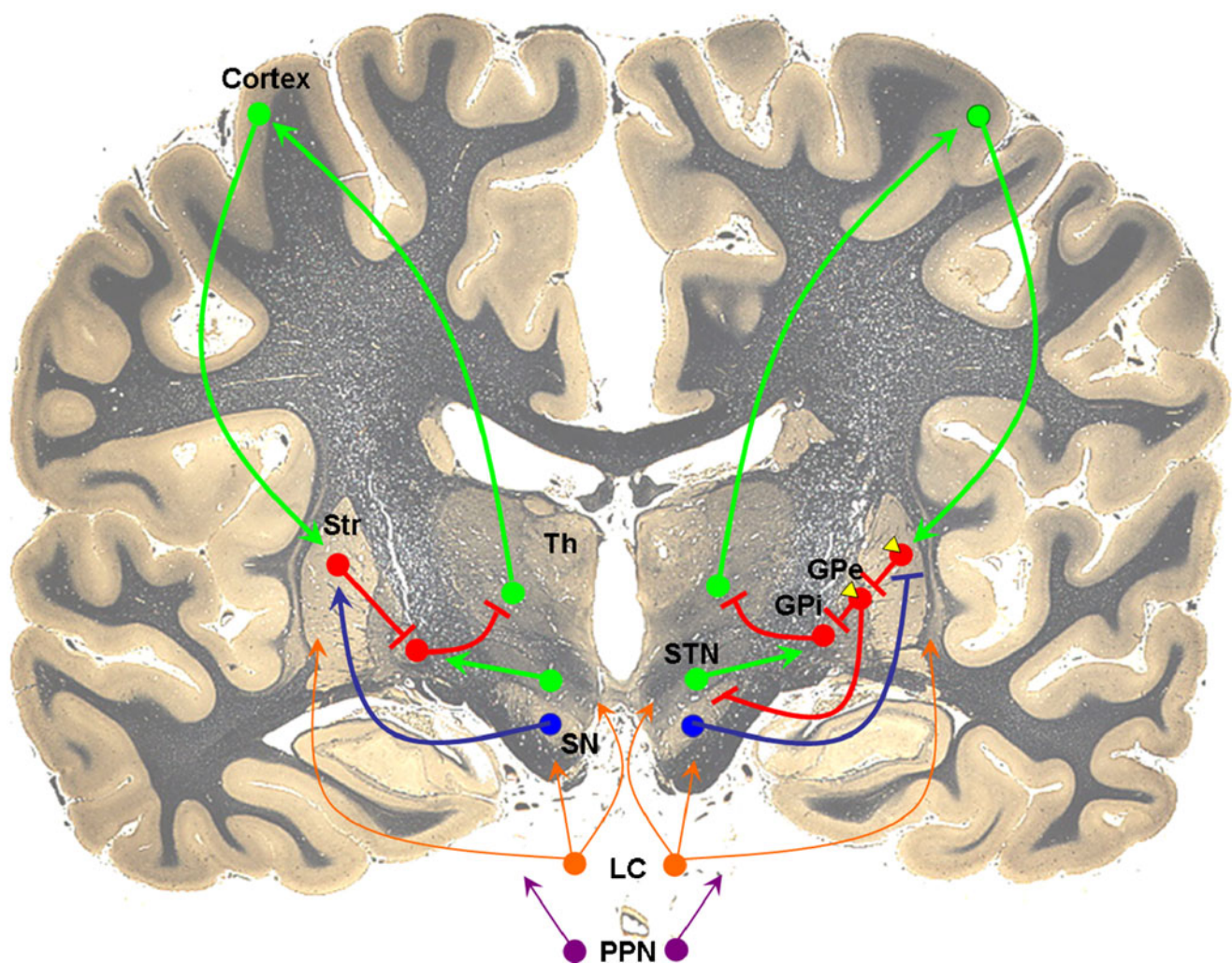
Fig. 1 Neurotransmitter systems involved in basal ganglia circuitry. Excitatory glutamatergic efferents (green) from cortex project to gamma-aminobutyric acid (GABA)ergic (red) striatal neurons. In the direct pathway (left), striatal neurons receive excitatory dopaminergic inputs (blue) from substantia nigra and project directly to globus pallidus interna (GPi). In the indirect pathway (right), dopamine inhibits striatal GABAergic output to the globus pallidus externa (GPe), which then projects to GPi. Adenosine A_{2A} receptors (yellow) are localized to dopamine D2 receptor-containing cells in the indirect pathway. Noradrenergic and cholinergic efferents from the locus coeruleus (orange) and pedunculopontine nucleus (purple), respectively, project widely to multiple brain regions, including cortex and basal ganglia. The coronal brain image is adapted with permission from http://www.brains.rad.msu.edu and http://brainmuseum.org, supported by the US National Science Foundation

Fig. 2 Expression patterns of neurotransmitter systems in the rodent brain. Dopamine D1 and D2 receptors and adenosine A_{2A} receptors are localized and highly expressed in the striatum, while glutamic acid decarboxylase [GAD, present in gamma-aminobutryic acid (GABA)ergic neurons], N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid (AMPA), and metabotropic glutamate receptor (mGlu5) subunits, and serotonin (5-HT) receptor subtypes are not concentrated in specific brain regions. In situ hybridization images are obtained from the Allen brain atlas (www.brain-map.org)
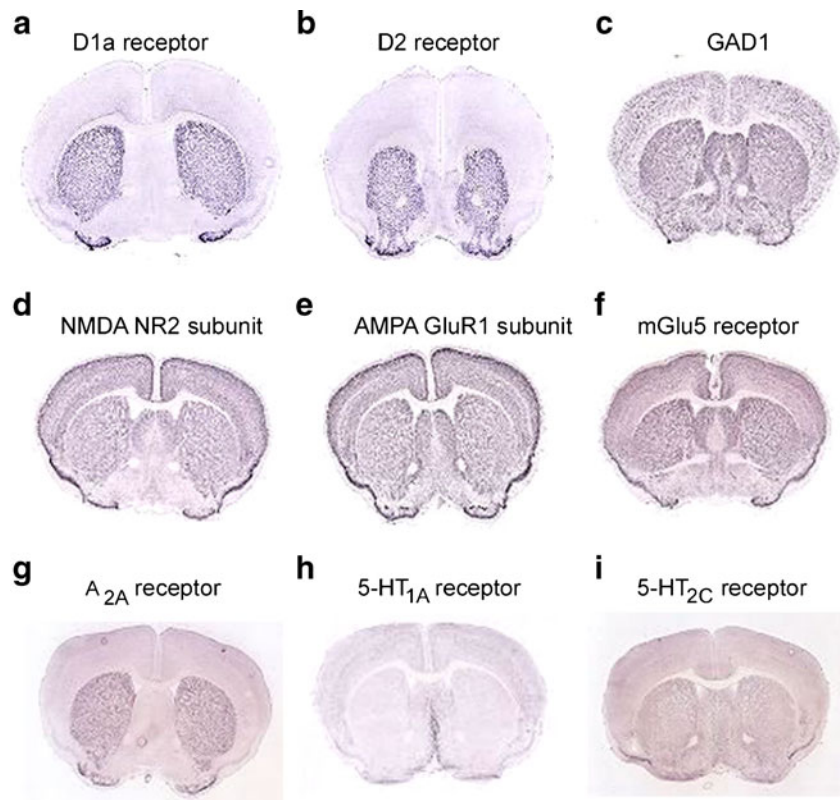
useful in accounting for some of the phenomenology associated with PD, basal ganglia circuitry is likely to be more complicated. For example, a recent rodent study suggests that both direct and indirect pathways are concurrently activated during initiation of action [4].

Glutamate receptors are expressed at high levels in the striatum. However, unlike dopamine receptors (which are highly enriched in the basal ganglia), they are present at high density throughout the brain (Fig. 2). GABA, which is synthesized from glutamate by the enzyme glutamic acid decarboxylase (GAD), is also expressed widely in the central nervous system (CNS). Given their primary role in basal ganglia circuitry, these neurotransmitter systems are potentially attractive targets to treat parkinsonian symptoms. However, their lack of regional specificity raises the potential challenge of side effects from actions on other brain regions.

Other neurotransmitters have also been implicated in the regulation of basal ganglia function. Adenosine is a purine nucleoside that acts to modulate synaptic function in the CNS. Its action is mediated by 4 G-protein-coupled receptor subtypes: A_1, A_{2A}, A_{2B}, and A_3. Of these, the A_{2A} receptor has received considerable attention as a potential treatment target because, like dopamine receptors, its expression is highly enriched in the striatum (Fig. 2) [5, 6]. Alterations in the serotonergic system have also been recognized in PD [7]. Of the 14 subtypes of serotonin (5-HT) receptors [8], multiple subtypes, including 5-HT$_{1A}$ and 5-HT$_{2C}$ receptors, are present

in striatal neurons (Fig. 2). Serotonergic inputs from the raphe nuclei form widespread connections throughout the brain, including the substantia nigra, striatum, globus pallidus, STN, thalamus, and cortex.

Neuropathological studies have suggested that neurodegeneration in PD is not restricted to dopaminergic neurons and the basal ganglia. According to the Braak staging system [2], inclusion bodies containing α-synuclein are found in caudal brainstem nuclei (stage 1) prior to involvement of the substantia nigra (stage 3). At stage 2, 5-HT-producing raphe nuclei neurons are affected, as are projection neurons in the locus coeruleus that produce noradrenaline. At later stages (through stage 6), acetylcholine (ACh)-producing neurons in the pedunculopontine tegmental nucleus and neocortex also undergo degeneration. The diversity of affected neurotransmitter systems yields a number of symptoms that may only respond to adjunct non-dopaminergic therapies.

Symptomatic Treatment and Motor Fluctuations

The presence of multiple neurotransmitters modulating the basal ganglia circuitry that coordinates movement suggests that non-dopaminergic strategies may be helpful in treating motor symptoms. These approaches offer potential advantages, including providing antiparkinsonian benefits either as monotherapy or in combination with dopamine replacement,

allowing reduction in dose of dopaminergic agents to ameliorate treatment-related side effects, or directly reducing motor fluctuations and/or dyskinesias associated with chronic levodopa use.

Adenosine

Adenosine A_{2A} receptors are localized to dendrites, cell bodies, and axon terminals of GABAergic striatopallidal neurons of the indirect pathway, in close association with dopamine D_2 receptors [9–11]. By binding to A_{2A} receptors, adenosine activates striatopallidal neurons, opposing the inhibitory effects mediated by D2 receptor binding [12, 13]. These findings suggest that blockade of A_{2A} receptors should inhibit the excessive activity of the indirect pathway that results from dopamine depletion. Indeed, in rodent and non-human primate models of PD, A_{2A} antagonists consistently reversed parkinsonian deficits without development of tolerance to prolonged treatment [14]. The preclinical data have motivated multiple clinical trials investigating whether these agents are effective in treating PD symptoms (Table 1).

Among A_{2A} antagonists that have been investigated clinically, istradefylline (KW-6002) has been studied most extensively. In a phase II randomized clinical trial, istradefylline did not improve motor function when used as monotherapy [15]. However, multiple phase II clinical trials in levodopa-treated PD patients with motor fluctuations demonstrated a significant reduction in off time [16–20]. Several of these trials demonstrated an increase in on time with dyskinesias, although they were not troublesome and did not impair mobility [17–19]. A long-term, open-label study showed persistent improvement in off time over a 52-week treatment period, suggesting a sustained symptomatic benefit [21]. However, despite the initial optimism based on the early studies, subsequent phase III clinical trials have yielded conflicting results. Two studies demonstrated a significant reduction in daily off time with an increased incidence of dyskinesias [22, 23], but istradefylline did not affect off time in another trial [24]. In the latter study, motor function in the on state was improved compared with placebo, and a large placebo response may account for the negative effect on off time [24]. Although the US Food and Drug Administration issued a not approvable letter for istradefylline based on available data in 2008 [25], the drug was later approved for use in Japan as adjunctive treatment for PD [26], and phase III clinical development recently resumed in North America [27].

More recently, preladenant, a second-generation A_{2A} antagonist with higher affinity and greater selectivity, had been moving through the therapeutic pipeline. In a phase II, dose-finding, 12-week randomized, placebo-controlled trial, preladenant at a dose of 5 mg and 10 mg twice daily was well-tolerated and reduced off time without increasing on time with troublesome dyskinesias [28]. In a 36-week open-label extension study, the drug similarly provided a reduction in off time, but with an increased incidence of dyskinesias (33 % *vs* 9 % in the randomized study) [29]. Three separate phase III randomized, controlled clinical trials have been ongoing, 2 assessing preladenant when added to levodopa in patient with moderate-to-severe PD, and another as monotherapy in early PD. Results have not been presented or published, but a press release from the manufacturer [30] indicated that initial review did not show evidence of efficacy; as a result, extension studies were discontinued and there are no plans to pursue regulatory filings.

A phase IIb randomized clinical trial investigating the safety and efficacy of the A_{2A} antagonist tozadenant (SYN115) to treat end-of-dose wearing off in 420 patients with moderate-to-severe PD patients has been completed, and a preliminary communication reported good tolerability and significant reduction in off time [31]. A previous smaller clinical study of tozadenant in PD patients provided functional magnetic resonance imaging evidence that the drug enters the CNS and engages its putative target of striatopallidal adenosine A_{2A} receptors to reduce the inhibitory influence of the indirect pathway on motor function [32].

Lastly, it is worth noting that the non-specific adenosine receptor antagonist caffeine, likely acting by blocking striatal A_{2A} receptors [33], has recently demonstrated evidence of significant antiparkinsonian actions in a randomized clinical trial. Although the study by Postuma et al. [34] was designed primarily to investigate potential alerting effects, they observed a reduction in Unified Parkinson's Disease Rating Scale score comparable to that with more specific A_{2A} antagonists and are now pursuing a long-term phase III study to investigate potential disease-modifying benefits, as well as to possibly confirm short-term motor benefits. Convergent epidemiological and laboratory animal data also support the neuroprotective potential of A_{2A} antagonists, including caffeine, in PD [35]. Similarly, clinical, pathological, imaging, and laboratory findings have suggested these agents may help prevent the development of dyskinesias in PD [36–39].

GABA: Glutamic Acid Decarboxylase Gene Therapy

In PD, loss of dopaminergic neurons in the nigrostriatal pathway and reduction of striatal dopamine levels results in disinhibition of the subthalamic nucleus that causes parkinsonian symptoms. The enzyme GAD converts glutamate into GABA, the major inhibitory neurotransmitter in the brain. GAD gene transfer using an adeno-associated virus (AAV) has been explored as an approach

Table 1 Non-dopaminergic therapies for motor symptoms of Parkinson's disease: Results from clinical trials

Mechanism	Drug	Phase	Use	n	Dose	Duration	Primary Outcome	Result	Ref
SYMPTOMATIC TREATMENT AND MOTOR FLUCTUATIONS									
Adenosine									
A$_{2A}$ antagonist	Istradefylline (KW-6002)	II	Mono	176	40 mg/day	12 weeks	Change from baseline in UPDRS motor score	Negative	[15]
			Adjunct	15	40 or 80 mg/day	6 weeks	Duration of L-dopa effect	Positive	[16]
			Adjunct	83	5/10/20 or 10/20/40 mg/day	12 weeks	Reduction in off time	Positive	[17]
			Adjunct	395	20 and 60 mg/day	12 weeks	Reduction in off time	Positive	[18]
			Adjunct	196	40 mg/day	12 weeks	Reduction in off time	Positive	[19]
			Adjunct	363	20 or 40 mg/day	12 weeks	Reduction in off time	Positive	[20]
		III	Adjunct	231	20 mg/day	12 weeks	Reduction in off time	Positive	[22]
			Adjunct	373	20 or 40 mg/day	12 weeks	Reduction in off time	Positive	[23]
			Adjunct	584	10, 20, or 40 mg/day	12 weeks	Reduction in off time	Negative	[24]
	Preladenant	II	Adjunct	253	1, 2, 5, or 10 twice daily	12 weeks	Reduction in off time	Positive (for 5, 10 mg doses)	[28]
	Tozadenant	II	Adjunct	420	60, 120, 180, or 240 mg twice daily	12 weeks	Reduction in off time	Positive (for 120, 180 mg doses)	[31]
GABA									
GAD gene therapy	AAV2–GAD	II	Adjunct	45	Bilateral AAV2–GAD delivery	6 months	Change from baseline in off state UPDRS motor score	Positive	[42]
Serotonin									
5-HT$_{1A}$ agonist	Pardoprunox	II	Mono	139	9-45 mg/day	3 weeks	Change from baseline in UPDRS motor score	Positive	[49]
		III	Mono	468	6, 12, or 12-42 mg/day	24 weeks	Change from baseline in UPDRS motor score	Positive (high dropout rate)	[50]
			Mono	334	12-42 mg/day (vs pramipexole)	24 weeks	Change from baseline in UPDRS motor score	Positive (high dropout rate)	[50]
		III	Adjunct	295	12-42 mg/day	12 weeks	Reduction in off time	Positive (high dropout rate)	[51]
LEVODOPA-INDUCED DYSKINESIAS									
Glutamate									
NMDA receptor antagonist	Traxoprodil (CP-101,606)	II	Adjunct	12	Low, high-dose infusion	Single dose	Change in Dyskinesia Rating Scale score	Positive (dose-related side effects)	[71]
	Memantine	II	Adjunct	12	30 mg/day	2 week treatment	Change in dyskinesia score after single levodopa challenge	Negative	[72]
AMPA receptor antagonist	Perampanel	III	Adjunct	763	2 or 4 mg/day	30 weeks	Reduction in off time and severity of dyskinesias (UPDRS IV)	Negative	[82]
			Adjunct	751	2 or 4 mg/day	20 weeks	Reduction in off time and severity of dyskinesias (UPDRS IV)	Negative	[82]
mGluR5 negative allosteric modulator	Mavoglurant (AFQ056)	II	Adjunct	31	50–300 mg/day	16 days	Change in LFADLDS	Positive	[95]
			Adjunct	28	50–300 mg/day	16 days	Change in mAIMS score	Positive	[95]
			Adjunct	197	20, 50, 100, 150, or 200 mg/day	13 weeks	Change in mAIMS score	Positive (for 200 mg dose)	[96]
	Dipraglurant (ADX48261)	II	Adjunct	76	Dose titration to 300 mg/day	4 weeks	Change in mAIMS score	Positive (on Days 1, 14)	[99]

Table 1 (continued)

Mechanism	Drug	Phase	Use	n	Dose	Duration	Primary Outcome	Result	Ref
Noradrenaline									
α2 Adrenergic receptor antagonist	Fipamezole	II	Adjunct	179	90, 180, or 270 mg/day	4 weeks	Change in levodopa-induced dyskinesia scale	Negative	[109]
Serotonin									
5-HT$_{1A}$ receptor agonist	Sarizotan	II	Adjunct	398	2, 4, or 10 mg/day	12 weeks	Change in diary-based *on* time without dyskinesias	Negative	[110]
GAIT AND BALANCE									
Cholinesterase inhibitor	Donepezil	IV	Adjunct	23	5–10 mg/day	6 weeks	Reduction in fall frequency	Positive	[119]
Noradrenergic reuptake inhibitor	Methylphenidate	IV	Adjunct (+ STN DBS)	69	1 mg/kg/day	90 days	Change in number of steps in stand-walk-sit test	Positive	[125]
			Adjunct	23	Up to 80 mg/day	12 weeks; 3-week washout	Change in gait composite score	Negative	[126]
NMDA receptor antagonist	Memantine	IV	Adjunct	25	20 mg/day	90 days	Change in stride length	Negative	[127]

GABA = gamma-aminobutyric acid; GAD = glutamic acid decarboxylase; 5-HT = serotonin; NMDA = *N*-methyl-D-aspartate; AMPA = alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid; mGlu= metabotropic glutamate; AAV = adeno-associated virus; STN = subthalamic nucleus; DBS = deep brain stimulation; UPDRS = Unified Parkinson Disease Rating Scale; LFADLDS = Lang-Fahn Activities of Daily Living Dyskinesia Scale; mAIMS, modified Abnormal Involuntary Movement Scale

to convert STN neurons from being excitatory to inhibitory [40]. In an initial phase I, open-label study, 12 patients with advanced PD were followed for 12 months after unilateral injection of AAV–GAD into the STN. Improvements in contralateral on and off motor function were observed 3 months after the injection and persisted for 12 months [41]. In a phase II double-blind, randomized trial comparing bilateral delivery of AAV2–GAD to sham surgery, patients receiving active gene therapy demonstrated a significant improvement in motor Unified Parkinson's Disease Rating Scale score in the off state, but not the on state, at 6 months [42]. Despite these initial promising proof-of-principle findings for non-dopaminergic modulation of STN neurotransmission and for gene therapy in PD, the long-term follow-up study has been terminated owing to financial reasons [43] and there are no plans for phase III studies.

Serotonin

In PD, serotonergic neurons in the raphe nuclei degenerate, leading to a reduction in 5-HT levels [7]. Loss of 5-HT is thought to contribute to both motor and non-motor symptoms. In preclinical models, several 5-HT$_{1A}$ receptor agonists have shown efficacy in improving motor activity and reducing dyskinesias. However, interpretation of these results is complicated in that these agents can also interact with other receptors. In levodopa-treated parkinsonian rats, the partial 5-HT$_{1A}$ receptor agonist piclozotan improved motor function [44]. A randomized pilot study using piclozotan in a small number of PD patients on levodopa was also reported to show improvements in off and on time without dyskinesias [45, 46]. However, results have been published only in abstract form and additional trials have not been registered.

Pardoprunox (SLV308) is a full 5-HT$_{1A}$ agonist that also has partial dopamine D2/D3 agonist properties. As monotherapy in animal models it reduced parkinsonian symptoms and induced only mild dyskinesias [47, 48]. In a double-blind study of pardoprunox in early PD, treatment resulted in improvement in motor function and activities of daily living [49]. Two large, randomized, phase III dose-finding trials also showed significant improvement in motor function, although dropout rates were high owing to treatment-emergent adverse events (e.g., nausea, somnolence, and dizziness) at higher doses [50]. As adjunctive therapy to levodopa, pardoprunox reduced off time and improved on time without troublesome dyskinesias in a phase III study [51]. However, a high dropout rate was similarly noted with the selected dose range, and the most recent registered clinical trial of pardoprunox was terminated "due to strategic considerations" [52].

Levodopa-induced Dyskinesias

Repeated administration of levodopa results in the development of motor complications, including involuntary dyskinesias. Age of PD onset, disease severity, and high levodopa dose are risk factors for the development of levodopa-induced dyskinesias (LID). Based on a literature review, the rate of development of dyskinesias has been reported to be about 35–40 % by 4–6 years of treatment, and nearly 90 % within a decade [53]. The mean time to onset of dyskinesias in a recent community-based study was 6.6 years [54]. LID can be clinically expressed in a variety of ways, occurring when levodopa effects are maximal (peak-dose dyskinesias, generally choreiform, but may be dystonic), with rising or falling levels of medication (diphasic dyskinesias), or at low levels of levodopa (off-period dystonia) [55, 56].

Evidence from postmortem and pharmacological preclinical studies supports a role for multiple non-dopaminergic systems in the development of LID [57–59]. These studies have led to exploratory trials investigating drugs targeting other neurotransmitters as adjunctive therapy with the goal of decreasing LID without compromising motor function.

Glutamate

Glutamate is the most abundant excitatory neurotransmitter in the brain and is directly involved in activating basal ganglia motor circuits. Loss of nigrostriatal dopamine input is believed to induce changes in synaptic connectivity in the striatum [60]. Repeated exposure to dopaminergic drugs, particularly in a hypodopaminergic parkinsonian state, results in maladaptive plasticity in glutamatergic synapses that contributes to the expression of dyskinesias [57, 61, 62].

Glutamate signaling in the CNS is mediated by a variety of receptors, including ionotropic receptors (those that directly conduct ion flow in response to glutamate binding) and metabotropic receptors (those whose actions are mediated via intracellular signaling pathways). Among ionotropic receptors, N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid (AMPA) receptors have been most extensively studied for a possible role in LID [59, 63].

Changes in NMDA receptor levels, phosphorylation state, and cellular distribution have been identified in the dyskinetic state in animal models [59]. Highlighting the complexity of antidyskinetic strategies targeting these receptors, preclinical studies using NMDA antagonists directed at specific receptor subunits in nonhuman primate models have yielded conflicting results. In one study, a negative allosteric modulator (Co-101,244/PD-174,494) acting on NR2B receptors decreased LID, while antagonists with increased specificity for NR1A/NR2A receptors exacerbated dyskinesias [64]. In contrast, another NR2B-specific antagonist (traxoprodil, CP-101,606) increased severity of dyskinesias in levodopa-treated animals [65].

As clinical support for a role for NMDA receptor modulation as an approach to treat LID, the nonselective NMDA antagonist amantadine is currently the only accepted treatment for dyskinesia in PD [66]. It has been recommended by the American Academy of Neurology for the treatment of dyskinesias (level C evidence) [67], and has also been suggested to be efficacious in treatment of LID in an evidence-based review by the Movement Disorders Society [68]. In a recent double-blind, randomized, placebo-controlled cross-over study of 36 patients with PD and dyskinesias, 64 % of patients showed improvement in LID [69]. Treatment with amantadine can also be limited by neuropsychiatric and other side effects. Remacemide, another nonselective NMDA antagonist, did not show a significant benefit in reducing dyskinesias in a randomized, controlled trial [70]. Hence, there is a need to develop better NMDA receptor antagonists with antidyskinetic properties.

To date, several small pilot studies have been conducted investigating other NMDA receptor blockers in PD. Traxoprodil, an antagonist selective for NR2B subunits, reduced the maximum severity of acute LID by approximately 30 % in response to a 2-h levodopa infusion in 12 PD patients with motor fluctuations and dyskinesias, but did not improve parkinsonism and caused dose-related neuropsychiatric side effects [71]. However, memantine, an NMDA receptor antagonist approved for treatment of dementia, did not improve dyskinesias in a small cross-over study [72], although there are case studies reporting a positive response [73–75]. An early small, double-blind, cross-over study suggested that the NMDA antagonist dextromethorphan may be effective in reducing LID [76]. AVP-923, a combination agent combining dextromethorphan and quinidine that has been approved for treatment of pseudobulbar affect [77], is currently being studied to assess its efficacy in reducing dyskinesias in a small phase IIa study [78]. Interestingly, recent preclinical data suggests that the potential antidyskinetic effect may be mediated by indirect 5-HT$_{1A}$ agonism rather than through an NMDA antagonist effect [79].

The role of AMPA receptors in the development of LID has received less attention, although the AMPA antagonists talampanel (LY-300,164) [80] and topiramate [81] reduced LID in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned primates. To date, 2 phase III human clinical trials using the AMPA antagonist perampanel as potential treatment for PD motor fluctuations have been published, but the drug did not show benefit either in reducing dyskinesias or "off" time [82]. Two small phase II studies investigating talampanel as an antidyskinetic agent have been completed, but results have not been published [83, 84].

Currently, metabotropic glutamate (mGlu) receptors are receiving significant attention as potential therapeutic targets [85, 86]. In particular, mGlu5 receptors are highly expressed

in the striatum and globus pallidus. Expression of mGlu5 is upregulated in MPTP-lesioned primates treated with levodopa, and this increase is associated with the development of LID [87]. Administration of mGlu5 antagonists has been shown to attenuate abnormal involuntary movements in rodent models [88, 89] and LIDs in primates [90–93].

Negative allosteric modulators (NAM) of G-protein-coupled receptors target binding sites distinct from the active site and inhibit the response to endogenous ligand. Drugs targeting allosteric sites may provide greater receptor selectivity and potentially decrease adverse side effects [94]. Clinically, the selective mGlu5 NAM mavoglurant (AFQ056) was demonstrated to show a significant antidyskinetic effect in 2 small phase II randomized clinical trials [95]. Findings from a larger dose-finding study of 197 patients with PD and dyskinesias provided further evidence of anti-dyskinetic benefit without worsening of parkinsonism [96]. A phase II study exploring the efficacy and safety of a modified release form was also recently completed [97]. Another mGlu5 NAM, dipraglurant (ADX48261), has similarly been under investigation as a putative antidyskinetic agent [98]. Although not yet published, preliminary results presented in abstract form suggest a significant reduction in peak dose LIDs without affecting levodopa efficacy [99]. Together, these results suggest that mGlu5 antagonists offer promise for the treatment of LID.

Noradrenaline

Noradrenaline exerts its action by binding to G-protein-coupled adrenergic receptors, which are expressed in the striatum, STN, and substantia nigra [100]. Of particular interest are $\alpha 2$ adrenergic receptors, which may act to modulate GABA [101, 102] and dopamine release [103]. In pharmacological studies using primate models, $\alpha 2$ antagonists have been shown to reduce LID [104, 105], possibly through preferential effects on the direct pathway [57]. These preclinical findings have motivated clinical trials exploring these agents as potential antidyskinetic therapies.

Pilot studies using idazoxan yielded conflicting results [106, 107], and this drug is no longer in clinical development for PD. Currently, the selective $\alpha_{2A/2C}$ receptor antagonist fipamezole is being studied for LID. An initial small pilot study demonstrated good tolerability and suppression of LID without exacerbating parkinsonian symptoms [108]. In a phase II study conducted in the USA and India, fipamezole failed to show a statistically significant reduction in dyskinesias [109]. However, separate outcome analysis of the US patients did show a benefit at the highest dose used; it has been proposed that this differential result may be owing to heterogeneity between the US and Indian study populations. An additional clinical trial may be helpful to determine whether fipamezole is indeed useful for treatment of LID.

Serotonin

Serotonin has also been implicated to play a role in LID, and 5-HT$_{1A}$ receptor agonists and 5-HT$_{2A}$ receptor antagonists have been explored as promising antidyskinetic agents. In a large phase IIb study, sarizotan, a full 5-HT$_{1A}$ agonist with additional affinity for dopamine D3/D4 receptors, did not show benefit in increasing on time without dyskinesias, and resulted in increased off time at higher doses [110]. Eltoprazine, a mixed 5-HT$_{1A/1B}$ receptor agonist, is effective in suppressing LID in animal models [111]. A small, human, randomized clinical trial has been completed in Sweden, but results have not yet been published [112]. It has been proposed that drugs aimed at reducing LID by modulating serotonergic function may need to demonstrate anatomic selectivity, as well as receptor selectivity [113].

Gait and Balance

Postural instability and gait difficulty are cardinal features of idiopathic PD, but typically do not cause prominent functional disability until later stages of disease. In particular, patients at more advanced stages of PD may become unable to initiate locomotion and develop freezing of gait (FOG) [114]. FOG is often associated with gait imbalance and can result in falls [115]. In some cases, FOG may respond to dopaminergic therapy at earlier stages [116]; however, PD-associated gait disorders may become progressively resistant to dopamine replacement or can be unresponsive from the start. Neurodegeneration in non-dopaminergic brainstem structures may contribute directly to this lack of response [117]. Cholinergic neurons in the pedunculopontine tegmental nucleus (PPN) and the prefrontal and frontal cortex are thought to be involved in gait control. Noradrenaline-producing cells in the locus coeruleus are also severely affected in PD. As a result of striatal dopamine depletion, excessive glutamatergic activity at projections from STN to PPN may also contribute to locomotor dysfunction. Interest in the role of PPN in PD gait disorders has been supported by the finding that low-frequency deep brain stimulation may reduce falls and FOG, either alone or in combination with high-frequency STN stimulation [118].

Strategies to increase ACh transmission have been used to target gait and balance symptoms unrelated to FOG. A small, randomized, placebo-controlled, crossover study in PD patients with falls showed that the centrally-acting cholinesterase inhibitor donepezil reduced falls by approximately half [119]. A single-center study in the UK is similarly exploring the effects of rivastigmine on gait and balance [120]. In the striatum, nicotinic ACh receptors are located presynaptically, and include subtypes $\alpha 4\beta 2$, $\alpha 6\beta 2$, and $\alpha 7$ receptors. A single-site study investigating the use of varenicline, a partial

$\alpha4\beta2$ and full $\alpha7$ agonist used as an aid for smoking cessation, to improve balance is ongoing [121].

Methylphenidate is an amphetamine-like stimulant that inhibits presynaptic noradrenaline and dopamine transporters. Three small pilot studies using different dosing protocols demonstrated improvement in various gait measures, including gait speed and freezing [122–124]. Two subsequent randomized studies have been completed and reported conflicting results. In a study of 69 PD patients treated with STN–DBS, methylphenidate treatment improved the number of steps in the stand-walk-sit test; the treated group experienced significantly more adverse events [125]. However, another trial of 23 patients did not show any improvement in a gait composite score of stride length and velocity [126].

A recent study of 25 patients explored the use of the NMDA receptor antagonist memantine as treatment for axial symptoms of PD. Although the treated group showed improvement in axial motor symptoms and dyskinesias, no improvement was noted in stride length [127]. Similarly, a randomized, double-blind, placebo-controlled crossover trial of the non-specific NMDA antagonist amantadine failed to show benefit against FOG resistant to dopaminergic therapy [128].

Conclusions

Dopamine deficiency due to degeneration of the nigrostriatal pathway is the primary cause of motor symptoms in PD. Nevertheless, multiple other neurotransmitter systems play an important role in modulating basal ganglia function and motor control. Targeting these systems, in particular, offers potential approaches to treating motor complications of dopamine replacement and symptoms that are resistant to dopaminergic therapy. At first glance, candidate non-dopaminergic agents would appear to be the proverbial "low-hanging" fruit in the PD pipeline. Receptors for neurotransmitters, including adenosine, GABA, serotonin, glutamate, and noradrenaline, have been well characterized biochemically with extensive knowledge of their neuroanatomic distribution and intracellular signaling pathways. Moreover, preclinical studies in rodent and nonhuman primate models have demonstrated effectiveness in reducing parkinsonian symptoms.

Based on the promise of the animal studies and early phase clinical studies, a number of randomized clinical trials directed at a variety of neurotransmitter targets have been completed [129]. Unfortunately, no compound specifically targeting non-dopaminergic pathways has yet received broad regulatory approval of an indication for use in the therapeutic armamentarium for PD. Drawing on previous studies, a potential hurdle may be finding agents that show high receptor specificity and also target specific brain regions. For example, the presence of multiple glutamate and serotonin receptor subtypes offers the potential for designing drugs that act on one, or a narrow, subset of receptors. However, the widespread distribution of these receptors throughout the CNS poses the challenge of finding doses that do not cause limiting side effects through action at undesired neuroanatomic sites.

Adenosine A_{2A} receptors have been an attractive potential target, as they are highly enriched in the striatum. Phase III studies investigating 2 A_{2A} antagonists, istradefylline and preladenant, have been conducted, and while phase II studies have consistently shown benefit in reducing motor fluctuations, the large phase III studies have yielded conflicting results, slowing progression through the therapeutic pipeline. These discrepancies raise questions about the design of clinical trials addressing motor fluctuations. Determination of on/off fluctuations relies on patient diaries, which may be subject to variability despite appropriate training. Also, while there are multiple dyskinesia rating scales [130], the clinical variability in the types of dyskinesias and their timing offers challenges in quantifying response to treatment. There is similarly a need to define the most appropriate outcome measures for trials focusing on PD gait symptoms. The recent approval of istradefylline in Japan [26] will hopefully provide additional experience about the effect of A_{2A} antagonists as adjunctive therapy. Additional phase III studies with mGlu5 receptor antagonists will be necessary to confirm the initial promising results from phase I/II studies.

Given the complexity of the pharmacology of dopamine-induced and dopamine-refractory PD symptoms, it may be necessary to target multiple non-dopaminergic systems in order to optimize clinical response while minimizing side effects from any particular pathway. This presents obvious obstacles to clinical trial design. However, despite the challenges thus far, ongoing development of these strategies remains a critical and hopeful pursuit toward improved treatment of PD.

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Original Paper

Postmortem Brain Levels of Urate and Precursors in Parkinson's Disease and Related Disorders

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Abstract
Background: Increasing evidence suggests that urate may play an important role in neurodegenerative disease. In Parkinson's disease (PD) higher, but still normal, levels of blood and cerebrospinal fluid urate have been associated with a lower rate of disease progression. ***Objective:*** We explored the hypothesis that lower levels of urate and its purine precursors in brain may be associated with PD and related neurodegenerative disorders, including Alzheimer's disease (AD) and Lewy body dementia (DLB). ***Methods:*** Human postmortem brain tissues were obtained from PD, AD, and DLB patients and non-neurodegenerative disease controls. We measured urate and other purine pathway analytes in the frontal and temporal cortex, striatum, and cerebellum, using high-performance liquid chromatography with electrochemical and ultraviolet detection. ***Results:*** Age was well-matched among groups. Mean postmortem interval for samples was 16.3 ± 9.9 h. Urate levels in cortical and striatal tissue trended lower in PD and AD compared to controls in males only. These findings correlated with increased urate in male versus female control tissues. By contrast, in DLB urate levels were significantly elevated relative to PD and AD. Measurement of urate precursors suggested a decrease in xanthine in PD compared to AD in females only, and relative increases in inosine and adenosine in DLB and AD samples among males. Xanthine and hypoxanthine were more concentrated in striatal tissue than in other brain regions. ***Conclusions:*** Though limited in sample size, these findings lend support to the inverse association between urate levels and PD, as well as possibly AD. The finding of increased urate in DLB brain tissue is novel and warrants further study.

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Introduction

Increasing clinical, epidemiological, and laboratory evidence suggests that urate (or uric acid) may play a role in neurodegenerative disease, and Parkinson's disease (PD) in particular. Urate is a natural antioxidant, found abundantly in blood and human brain tissue due to mutations of the *urate oxidase (UOx)* gene during primate evolution [1]. In humans, urate is thus the enzymatic end product of purine metabolism (fig. 1) and circulates at high plasma concentrations. Urate's antioxidant capacity
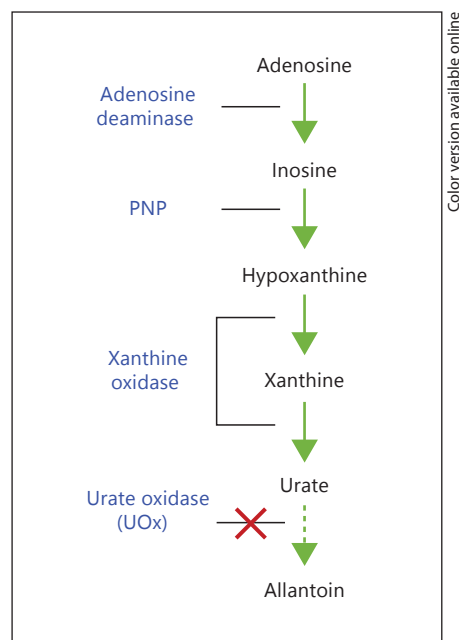
Fig. 1. Metabolism of purines in humans. Loss of urate oxidase (UOx) function results in elevated urate. PNP = Purine nucleoside phosphorylase.

is comparable to that of ascorbate [2, 3] and suggests that the loss of urate oxidase activity in our primate ancestors may have provided additional antioxidant health benefits [4]. Urate also inhibits free-radical formation and forms complexes with iron (particularly Fe^{3+}), crucial to limiting oxidative damage [2]. As oxidative stress is thought to contribute to loss of nigrostriatal dopamine neurons in PD and the pathophysiology of other neurodegenerative disorders, levels of urate and its metabolites may help determine disease susceptibility and predict rate of progression [5, 6].

In the early 1990s, Church and Ward [7] provided initial, postmortem evidence that nigrostriatal levels of urate (but not ascorbate) as well as dopamine are reduced in PD. Epidemiological and clinical studies subsequently linked lower urate levels to a greater risk of PD and a faster rate of its progression [8–10]. Lower urate levels have likewise been reported for patients with mild cognitive impairment and Alzheimer's disease (AD) [11–13]. Similarly, higher urate has been associated with reduced progression in Huntington's disease [14] and multiple system atrophy [15]. Furthermore, recent data suggest a potential link between higher plasma urate levels and reduced progression of cognitive decline and adjusted risk of dementia [16, 17]. However, other studies have linked higher urate to an increased risk of dementia, though these generally have not been adjusted for cerebral ischemia, which is frequently comorbid with both dementia and elevated urate levels and may mediate the association between urate and cognitive dysfunction [18].

In this study, we explored the hypothesis that lower brain urate levels may be associated with PD and related neurodegenerative disorders, including AD and dementia with Lewy bodies (DLB). A secondary aim was to determine whether levels of urate and its precursors vary among brain regions and correlate with affected areas in each disease. We analyzed human postmortem brain tissue obtained from the Massachusetts General Alzheimer Disease Research Center (ADRC)/Harvard NeuroDiscovery Center neuropathology core from PD, AD, and DLB patients and age-matched non-neurodegenerative disease controls. Urate pathway analytes were measured in multiple brain regions, including the frontal and temporal cortex, striatum, and cerebellum using high-performance liquid chromatography (HPLC) with electrochemical and ultraviolet (UV) detection.

Methods

Standard Protocol Approvals and Patient Consents
Postmortem tissue collection and protocols were approved by the Partners/Massachusetts General Hospital Institutional Review Board. Prior written, informed consent was obtained from brain donor participants or from their family members or authorized representatives.

Tissue Selection
Brain samples were obtained from the Massachusetts General ADRC/Harvard NeuroDiscovery Center neuropathology core B repository based on tissue availability and confirmed neuropathological diagnosis. Relevant clinical information such as age, gender, race, comorbidities, and clinical diagnosis was acquired from the brain bank database. Criteria used for neuropathological diagnosis included those established by the London Brain Bank for PD [19, 20], the DLB Consortium [21], and the National Institute on Aging and Reagan Institute Working group for AD [22]. Fresh frozen tissues (~100–200 mg, stored at –80°C) from PD, DLB, AD, and age-matched, non-neurodegenerative disease control brains were collected and included samples from the frontal and temporal cortices, striatum (caudate and putamen), and cerebellum. There was insufficient midbrain tissue for adequate sampling. Cases with combined AD and Lewy body pathology, and those with extensive cerebrovascular disease considered likely to cause dementia, were excluded. To help ensure stability of urate and metabolites, the average postmortem interval (PMI) for cases was kept as short as possible (generally <24 h) and matched across the disease and control groups (table 1). Five to 10 samples for each disease state, gender, and specified brain region were examined.

Table 1. Group statistics

	Control	PD	DLB	AD	p
Total, n	13	17	13	19	–
M/F	8/5	8/9	7/6	12/7	–
Mean age (SD), years	78.3 (11.3)[a]	79.2 (7.5)[a]	75.6 (9.8)[a]	82.2 (7.6)[a]	0.342
Mean PMI (SD), h	22.7 (14.7)[a]	16.0 (9.1)[a,b]	15.5 (7.9)[a,b]	12.0 (7.5)[b]	0.035*

Kruskal-Wallis test of disease vs. age or PMI (* p < 0.05). Values in the same row for age and PMI not sharing the same superscript (a, b) are significantly different (p < 0.05) for two-sided test of column means (Dunn's multiple comparisons test). Mean PMI for control was greater than for AD (p = 0.023).

Table 2. Regression data for age and PMI versus urate and precursors

	Regression data		Age		PMI	
	β	t	p	β	t	p
Urate	0.114	1.528	0.128	0.042	0.560	0.576
Xanthine	−0.172	−2.323	0.021*	0.243	3.349	0.001*
Hypoxanthine	−0.053	−0.713	0.477	0.137	1.845	0.067
Inosine	−0.026	−0.344	0.731	−0.135	−1.816	0.071
Adenosine	0.039	0.519	0.604	−0.235	−3.227	0.001*

Xanthine levels correlated with both age and PMI (* p < 0.05), whereas they do not predict urate or other precursor levels (PMI vs. adenosine appears significant, but values are at the limit of detection and violate homoscedasticity assumptions).

Urate and Precursor Measurements

We compared urate pathway metabolites in human postmortem tissue across select brain regions and neurodegenerative diseases, including PD, DLB, AD, and controls. Tissue samples were homogenized on ice in 50 mM phosphoric acid solution (containing 0.1 mM EDTA) with 50 μM methyldopa and 1 μM 3,4-dihydroxybenzylamine as internal standards, and then centrifuged at 16,000 *g* for 15 min. The supernatant was filtered through a 0.22-μm Spin-X (Costar) cellulose acetate filter [23]. Purines in the brain homogenate filtrates were separated over a reverse-phase HPLC column, and then measured in the effluent by serial UV and electrochemical detectors. Specifically, adenosine, inosine and hypoxanthine were quantified based on UV absorbance at 254 nm, whereas urate and xanthine were quantified based on oxidation at a coulometric detector set at 150 and 450 mV, respectively [24]. For calibration, standard concentrations of each purine were also measured. All data were collected using a CoulArray Data Station with 3.0 software (ESA Biosciences, Chelmsford, Mass., USA) with autorange gain enabled. Measurements for each sample were normalized to the methyldopa standard peak and wet weight of tissue analyzed (expressed as ng of analyte per wet weight of brain tissue (wwt) in figures).

Statistical Analysis

All statistical analyses were performed in SPSS 20.0 (IBM Corp.). For comparison of group statistics, we performed analysis of variance (Kruskal-Wallis, α = 0.05) with post hoc Dunn's mul-

tiple comparison tests. Linear regression analysis was done on urate and precursor measurements for all areas (average) versus PMI and age. Normalized data for urate and precursors were analyzed by multivariate general linear model (MANCOVA) with disease, brain region, and gender as independent variables and with age and PMI as covariates. Post hoc pairwise comparisons were performed with Bonferroni correction. Data in graphs are expressed as means ± SEM.

Results

A total of 62 cases was collected and included 17 PD, 13 DLB, 19 AD, and 13 age-matched non-neurodegenerative disease controls. Controls died of various causes, including cardiovascular disease, pneumonia, cancer, and gastrointestinal bleed. For each group, basic demographic and specimen features are displayed in table 1. Disease and control groups were generally well matched for age and PMI. There was no significant difference in age among groups with mean (±SD) age for all groups being 79.2 ± 9.0 years. Overall mean PMI was 16.1 ± 10.4 h and differed among groups (H = 8.6, d.f. = 3, p = 0.035)

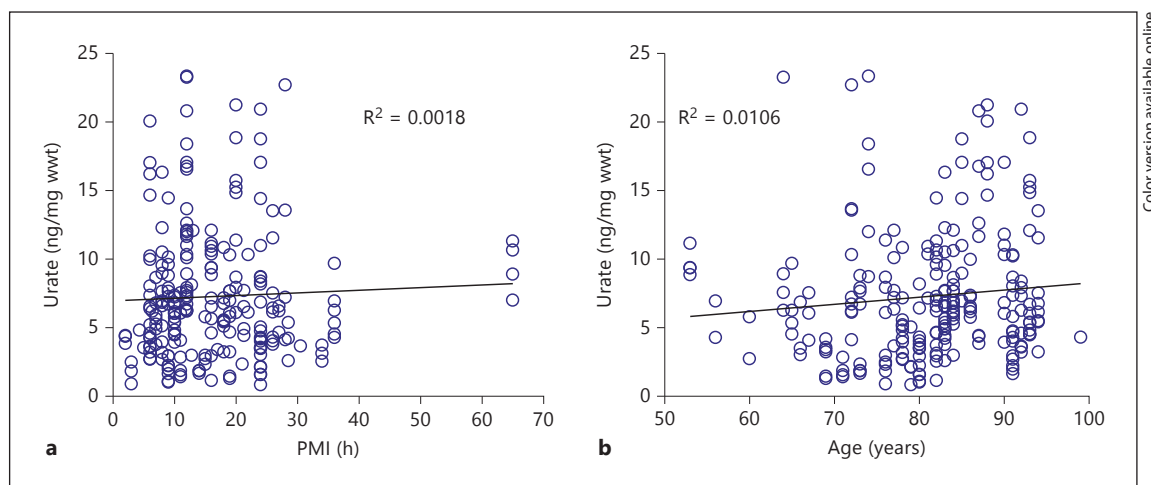Postmortem Brain Levels of Urate in
Parkinson's Disease

Fig. 2. Regression analysis of PMI versus urate levels ($\beta = 0.042$, $p = 0.576$) (**a**), and age versus urate ($\beta = 0.114$, $p = 0.128$) (**b**). No significant relationships were found.

with PMI for AD (12 ± 7.5 h) being significantly lower than for controls (22.7 ± 12.4 h). Given uncertainty over the stability of urate and enzymatic precursors in postmortem tissue, we performed regression analysis of brain urate and precursor levels versus PMI and age (table 2). Although we found no significant correlation with urate levels (fig. 2a), PMI appeared to predict higher levels of xanthine. There was also a trend for higher levels of hypoxanthine, whereas lower levels of both inosine and adenosine were suggested. The cause of these trends is unclear, but may indicate postmortem, active enzymatic or non-enzymatic metabolism of purines early in the pathway. Regression analysis revealed a non-significant trend toward increased urate levels with age but is consistent with that found in serum urate levels (fig. 2b) [25, 26]. Among precursors, only xanthine showed a significant correlation and decline in levels with age.

Urate and Precursor Levels in PD and Gender Differences

Two-way analysis of primary measurements of urate in postmortem brain samples was restricted to disease-relevant regions, including cortical and striatal samples only, and showed main effects for both disease ($F[3, 136] = 7.44$, $p < 0.0005$) and gender ($F[1, 138] = 6.38$, $p = 0.013$), as well as a significant interaction between disease and gender ($F[3, 136] = 4.10$, $p = 0.008$). In control tissue, urate levels in males were significantly higher (8.21 ±1.0 ng/mg wwt, $p = 0.014$) than in females (4.06 ± 1.44 ng/mg wwt), consistent with levels reported in serum [27,

28]. Urate levels in PD trended lower than in control tissue ($p = 0.096$) among males, whereas there was no difference among females (fig. 3). Interestingly, urate in DLB among males was significantly elevated compared to PD ($p < 0.0005$) and AD ($p < 0.0005$). Analysis of xanthine levels also showed main effects for disease ($F[3, 136] = 3.53$, $p = 0.017$) but not gender, though there was a significant interaction between disease and gender ($F[3, 136] = 7.75$, $p < 0.0005$). Pairwise comparisons showed that xanthine levels in PD were significantly lower ($p = 0.019$) in females (22.6 ± 3.7 ng/mg wwt) than in males (32.9 ± 2.3 ng/mg wwt), whereas in controls and DLB, levels were higher in females (42.0 ± 4.1 ng/mg wwt, $p = 0.002$ and 43.1 ± 2.9 ng/mg wwt, $p < 0.000$, respectively). Additionally, among females, xanthine was reduced in PD compared to DLB ($p < 0.0005$) and to control tissues ($p = 0.004$). No significant differences in xanthine levels were found in males. There were no interactions or main effects detected for hypoxanthine levels either. However, interaction between disease and gender effects on inosine levels trended toward significance ($F[3, 136] = 2.21$, $p = 0.09$). Post hoc analysis revealed that in PD, females have significantly elevated brain levels of inosine ($p = 0.012$) compared to males (149.6 ± 20.1 vs. 89.1 ± 12.6 ng/mg wwt, respectively). Among males, inosine levels in AD were also increased (137.8 ± 10.8 ng/mg wwt, $p = 0.025$) relative to PD. For adenosine, there were no significant main effects due to the high variability of adenosine readouts, although pairwise comparisons suggested differences in levels for DLB between
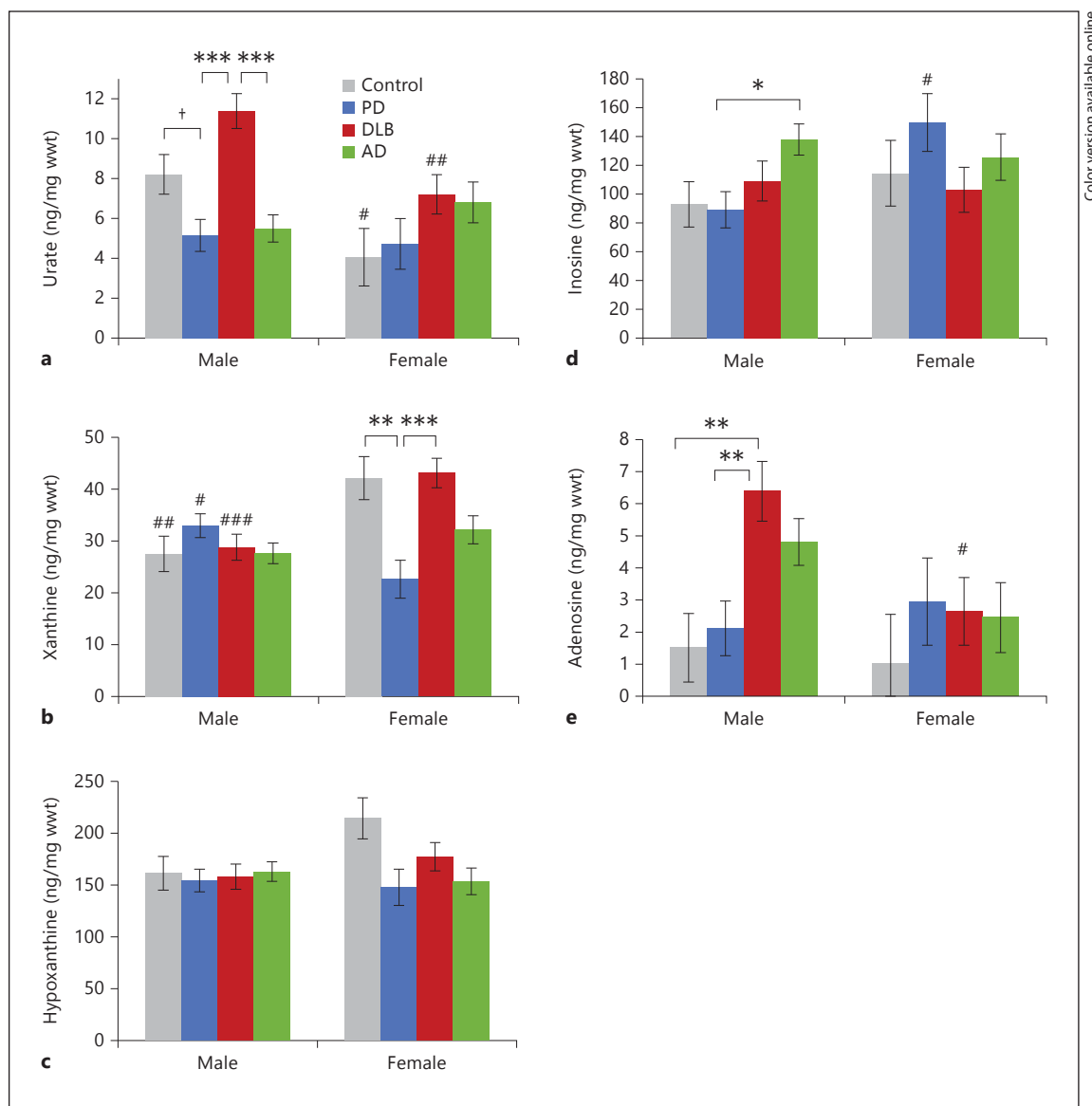
McFarland/Burdett/Desjardins/Frosch/
Schwarzschild

Fig. 3. Disease versus gender differences in brain purine levels for non-neurodegenerative disease control, PD, DLB, and AD tissue. Urate levels (**a**) in controls are significantly lower in female versus male tissue. Urate in PD males appears reduced and is more comparable to that in female control tissue, but represents a trend only ($p = 0.096$). In contrast, urate in DLB is significantly elevated compared to PD and AD tissue ($p < 0.0005$). Among precursors, xanthine (**b**) in PD is decreased in females versus males, whereas in-creased in DLB females. Xanthine in DLB females is also significantly elevated compared to PD. Inosine (**d**) levels are increased in AD compared to PD tissues. In PD tissues, inosine is also significantly elevated in female versus male samples. Adenosine (**e**) is increased in DLB relative to control and PD tissue among males. [†] $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Gender comparison: [#] $p < 0.05$, [##] $p < 0.01$, [###] $p < 0.001$.

Fig. 4. Region versus disease group differences in brain purine levels. Graphs of both disease versus region (left) and region versus disease (right) are shown for clarity. For urate (**a, b**), there is a clear trend toward lower urate in PD and AD, but significant elevation in DLB ($p < 0.0005$). Xanthine (**c, d**) shows no relation to disease, but is increased in the striatum. Similarly, hypoxanthine (**e, f**) levels are greater in the striatum and cerebellum compared to fron-tal and temporal cortices ($p < 0.0005$). There is no disease effect. For inosine (**g, h**), no regional or disease effects are seen, though AD levels are greater than levels in PD in frontal tissues. Adenosine (**i, j**) levels are increased in DLB compared to PD ($p = 0.015$) and control ($p = 0.006$). Significant interactions (post hoc) between region and disease for urate and precursors are also shown. [†] $p < 0.1$, * $p < 0.05$, ** $p < 0.01$. CBL = Cerebellum.
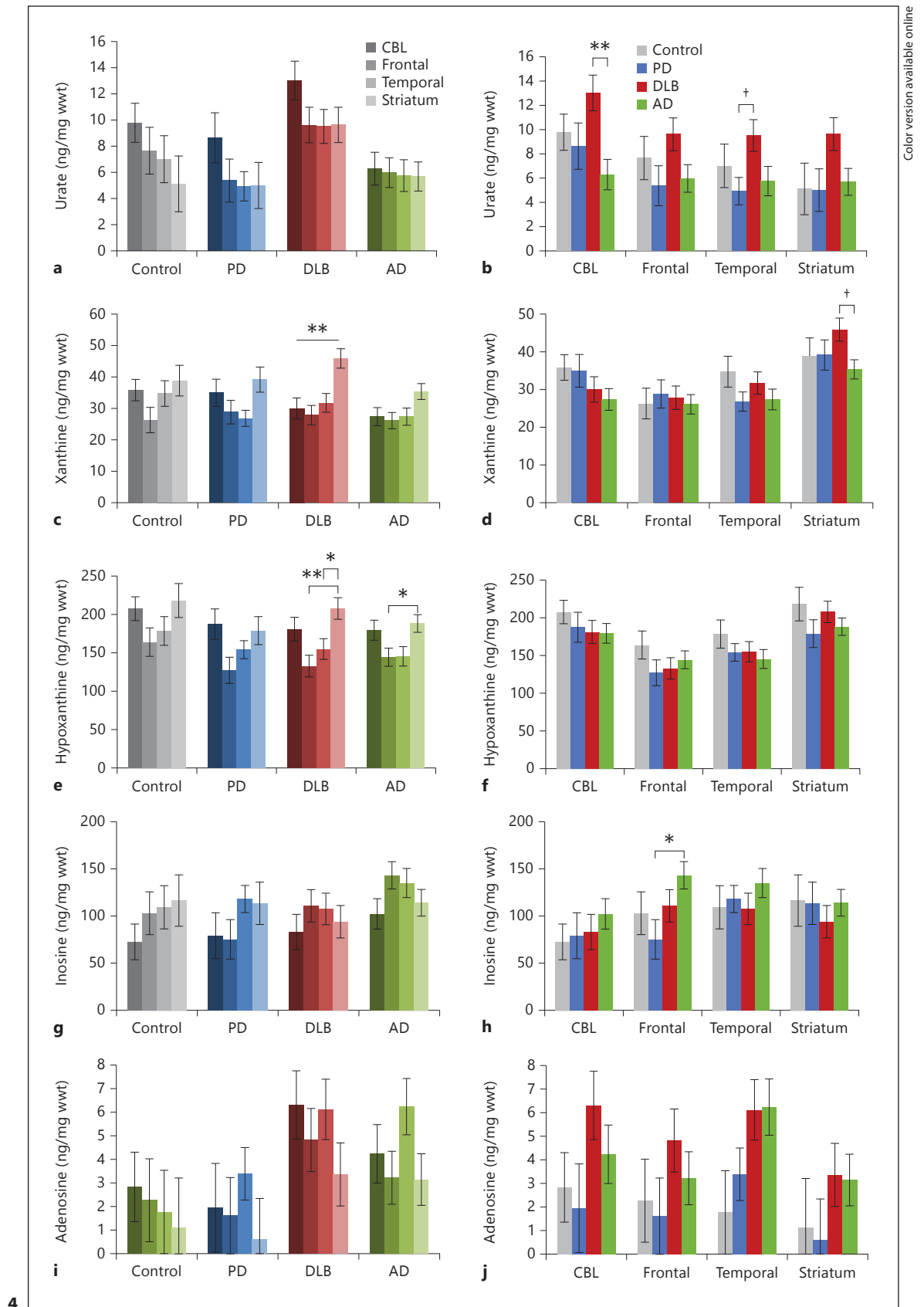
male and female (p = 0.01) tissues, as well as between male DLB and control (p = 0.005) and PD levels (p = 0.006).

Urate/Precursor Levels by Region and Disease

Analyses of urate and precursor levels in postmortem brain samples by region and disease include both genders pooled (fig. 4). We also performed separate male and female analyses which showed similar results, though there were limited female PD samples for all brain regions (data not shown). For urate, there were main effects for region (F[3, 162] = 3.09, p = 0.029) and disease (F[3, 162] = 8.83, p < 0.0005; fig. 4a, b) on postmortem brain levels. Simple effects analysis indicated that cerebellar urate levels were higher than levels in the striatum (p = 0.041). Among disease groups, urate was elevated in DLB brains versus control, AD, and PD brains (p = 0.029, <0.0005, and <0.0005, respectively). Post hoc analyses similarly showed significant increase in urate in the cerebellum for DLB compared to AD (p = 0.004). Though urate in PD and AD appeared lower than in control tissues, differences did not reach significance. By contrast, brain xanthine levels did not differ among disease groups but trended higher in the striatum compared to other tissues (F[3, 162] = 9.15, p < 0.0005). Likewise, there was no association between hypoxanthine levels and disease, but significant regional differences in hypoxanthine (F[3, 162] = 11.66, p < 0.0005) were noted for the striatum compared to the frontal and temporal cortices (p < 0.0005), as well as the cerebellum compared to the frontal (p < 0.0005) and temporal (p = 0.024) cortices. Inosine levels displayed a disease effect (F[4, 162] = 2.71, p = 0.047) with relative increase in AD versus control tissues (p = 0.20), but no significant regional associations. Although adenosine values were low and variable, we detected a main effect for disease (F[3, 162] = 5.32, p = 0.002) with DLB levels being higher than those in PD (p = 0.015) and control subjects (p = 0.006).

Discussion

Urate, the end product of enzymatic purine metabolism in humans, has emerged as a potential biomarker for PD with serum and CSF levels correlating inversely with risk and progression rates [9, 28]. Recent studies also indicate a potential link between urate, cognitive decline, and AD [11, 12, 16, 17]. In this study, we analyzed postmortem brain levels of urate and its purine precursors among multiple select brain regions and neurodegenerative diseases, including PD, DLB, and AD, to test the hypothesis that lower levels correlate with disease. In males, but not females, there was a clear trend toward lower urate levels in PD versus control brains, though levels did not quite reach significance (p = 0.096). This finding correlated with significantly higher urate levels in control tissue in males versus females, mirroring similar gender differences reported in serum [29]. Our findings are generally in agreement with Church and Ward [7], who also found lower levels of urate in PD substantia nigra and striatum compared to control. These findings appear to support the notion that in males, PD brains may, as a result of lower urate levels, have a decreased antioxidant capacity and greater risk for oxidative damage and dopaminergic cell loss [2, 5], consistent with epidemiological data suggesting an inverse correlation with risk and disease progression rate [9, 10].

Conversely, a lower brain urate concentration in PD patients may be secondary to their putatively higher levels of oxidative stress and reactive oxygen species (ROS) [5]. Urate is consumed as it exerts its antioxidant action, which entails the non-enzymatic oxidation of urate by ROS resulting in irreversible conversion to allantoin. Thus, it would be informative to determine whether lower levels of urate in PD are associated with higher levels of allantoin, with a higher allantoin:urate ratio potentially serving as an index of oxidative stress [30] and potentially a more robust prognostic biomarker of PD compared to urate alone. Because urate's purine ring structure is disrupted upon its conversion to allantoin, the electrochemical or UV methods employed here were not adequate on their own to measure the analyte in brain tissue, but may be coupled with an allantoin derivatization step toward this end in future studies.

Whereas brain urate levels trended lower for PD, unexpectedly we found that urate appeared significantly elevated in DLB brains in all brain regions examined. Ours is the first study to our knowledge to demonstrate this finding in postmortem brain. Similar to PD, differences in urate in DLB reached significance only in males. Greater numbers are needed to increase the power of analysis, however. Although dementia in some studies has been linked to higher urate levels, this association has been attributed at least in part to urate's covariance with vascular risk factors [31–33]. Indeed, other studies have shown the opposite association [11, 12], with *lower* urate levels linked to a reduced risk of de-

mentia after adjusting for potential cardiovascular confounds. Thus, the higher urate levels we observed in postmortem DLB brain could reflect associated vascular risk factors, which have been shown to be a determinant of DLB diagnosis [34]. Vascular confounds, however, would not readily explain why urate was elevated in DLB but not in AD, which is similarly thought to be more likely among those with vascular risk factors. Information on whether the DLB subjects differed from other groups based on vascular risk factors was not available in the present study, but would be helpful to factor into future studies investigating the role of urate in neurodegenerative diseases to which vascular disease may contribute.

The findings in DLB also contrast those of a recent study of urate levels in Lewy body disorders with or without dementia, which reported lower levels of cerebrospinal fluid (CSF) urate in dementing Lewy body disorders (including DLB) compared to non-demented PD patients [35]. However, this study also reported differences between serum and CSF urate relationships to neurodegenerative diseases, suggesting that brain too may have a distinct association with urate. Thus, the role of brain urate in DLB in particular remains unclear and warrants further study.

Gender differences seen in this study add to increasing data that the urate-PD link is stronger in men than in women. For instance, men with gout have decreased risk of PD, whereas data for women were not significant, although interestingly use of anti-gout treatment was associated with reduced PD risk in both groups [36]. Serum levels also negatively correlated with risk of PD, disease duration, and daily levodopa dose in men, but again not in women [37], though a trend toward reduced risk in women has been observed in at least one epidemiological study [38]. Studies on disease progression likewise show an inverse correlation between serum or CSF urate and rate of clinical decline that is significant in men but not in women [10, 28]. More recently, a similar inverse association between serum urate and presence of dopaminergic deficit on [I^{125}]β-CIT SPECT was seen in men but did not quite reach statistical significance (p = 0.051) in women [39]. In our study, lower brain urate levels in females correlated with those found in serum and CSF, and were not different among control and PD tissue, which may help to explain the lack of association in women. Precursor levels, however, differed only for inosine, which was significantly elevated in woman and not men, a finding not previously reported. Together, these studies support the possibility that factors other

than urate may play a more primary role for Parkinsonism in women, and that perhaps their oxidative burden is not as high as in men, who require or produce more urate.

In addition to gender, we explored regional differences in urate and metabolite levels as differences might be expected to be more prominent in areas affected by disease, such as the nigrostriatal system in PD. Previous reporting of postmortem brain urate in PD also reported a reduced urate concentration compared to that in control subjects but was based on a small sampling (n = 4) and was limited to nigral and striatal tissues [7]. In combination with these results, the present findings suggest a more generalized reduction in urate in PD brain rather than one specific to the nigrostriatal system.

Among urate's immediate precursors, xanthine and hypoxanthine, we observed few disease-specific alterations with the exception of a relative decrease in xanthine levels for PD compared to AD in females. Precursor levels, however, did appear to vary significantly by region in some disease as well as control brains, with striatal levels generally being the highest. Though consistent with a regionally specific decrease in striatal xanthine oxidase function, there was no accompanying decrease in striatal urate, and the significance and reproducibility of these differences remain to be determined.

This study has several limitations including the small sample size, lack of relevant midbrain tissue, and limited corresponding clinical information. Tissue samples were primarily obtained from one source, the Massachusetts General ADRC, and thus limited in scope and regions available. We further limited tissue samples, particularly in choosing controls, to those without concurrent severe cerebrovascular pathology and reported dementia that could confound urate measurements. Cardiovascular disease was reported in some cases (likely an underestimate given limited records), but other comorbidities, such as alcoholism, diabetes, and obesity, as well as medications were not detailed. Although cardiovascular risk is associated with elevated urate levels [40], it remains unclear whether this translates to higher levels in brain.

In conclusion, this study provides further support for a role of urate in PD by strengthening the direct evidence that urate levels in degenerating brain tissue of male PD patients, as well as in their CSF and blood, are lower than in control subjects. Although we did not examine oxidative stress in brain tissues, previous studies suggest that oxidation of dopamine and other markers of neuronal integrity are increased in the PD nigrostriatal system and

that urate may play an important antioxidant role [7, 41]. Testing in animal models of Parkinsonism may help further clarify the role of urate. Based on findings herein, the importance of urate and its metabolites in other neurodegenerative disorders remains unclear and warrants future investigation.

Acknowledgements

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RESEARCH ARTICLE

Mendelian Randomization of Serum Urate and Parkinson Disease Progression

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Objective: Higher serum urate concentrations predict more favorable prognosis in individuals with Parkinson disease (PD). The purpose of this study was to test the causality of this association using a Mendelian randomization approach.

Methods: The study was conducted among participants in DATATOP and PRECEPT, 2 randomized trials among patients with early PD. The 808 patients with available DNA were genotyped for 3 *SLC2A9* single nucleotide polymorphisms (SNPs) that identify an allele associated with lower urate concentrations, and for selected SNPs in other genes encoding urate transporters that have modest or no effect on serum urate levels. An *SLC2A9* score was created based on the total number of minor alleles at the 3 *SLC2A9* loci. Primary outcome was disability requiring dopaminergic treatment.

Results: Serum urate concentrations were 0.69mg/dl lower among individuals with $\geq$4 *SLC2A9* minor alleles as compared to those with $\leq$2 ($p = 0.0002$). The hazard ratio (HR) for progression to disability requiring dopaminergic treatment increased with increasing *SLC2A9* score (HR = 1.16, 95% confidence interval [CI] = 1.00–1.35, $p = 0.056$). In a comparative analysis, the HR was 1.27 (95% CI = 1.00–1.61, $p = 0.0497$) for a 0.5mg/dl genetically conferred decrease in serum urate, and 1.05 (95% CI = 1.01–1.10, $p = 0.0133$) for a 0.5mg/dl decrease in measured serum urate. No associations were found between polymorphisms in other genes associated with urate that do not affect serum urate and PD progression.

Interpretation: This Mendelian randomization analysis adds to the evidence of a causal protective effect of high urate levels.

Previous longitudinal investigations have shown that individuals with higher serum urate levels[1–3] or a diet that increases serum urate[4] have a lower risk of developing Parkinson disease (PD). Furthermore, in individuals with early PD, higher urate predicts milder clinical and radiographic progression.[5,6] Urate is a potent antioxidant,[7] and several lines of evidence support a role for oxidative stress in the neurodegenerative process of PD,[8] but whether the inverse association between serum urate and PD progression reflects a neuroprotective effect remains uncertain due to the possibility of unmeasured confounders. Because urate levels are in part heritable (the estimate of between-person variation due to inherited genetic factors ranges from 25 to 70%[9]), we sought to use a Mendelian randomization design[10] to investigate whether genetic polymorphisms that predict serum urate levels predict the rate of clinical progression among individuals with early PD. Although several genes are

associated with serum urate, and a multiple genes score has been used in a previous study of PD risk,[11] we selected as an instrumental variable for this investigation only the gene for solute carrier family 2 (facilitated glucose transporter), member 9 (*SLC2A9*, also known as *GLUT9*),[12] which explains most of the genetically specified variability in serum urate.[13–19] By using a single gene with a strong effect on serum urate, but no known direct effects in the central nervous system, we minimized the possibility of violating the assumption that there are no genetic effects on PD progression other than those mediated by urate levels.[10] Other genes encoding urate transporters that are known to have modest or no effects on serum urate, but could nevertheless modulate its biological effects, were included in exploratory analyses.

Subjects and Methods

Study Population

The source population for this study includes participants in 2 randomized clinical trials of PD: the Parkinson Research Examination of CEP-1347 (PRECEPT) and the Deprenyl and Tocopherol Antioxidative Therapy of Parkinsonism (DATATOP) trials. The details of these studies and their participants are described elsewhere.[20,21] We have previously reported an inverse association between serum urate and rate of disease progression in 804 individuals enrolled in PRECEPT and 774 enrolled in DATATOP.[5,6] The population for this study comprises the subset of these individuals from whom DNA was also available. In DATATOP (2-year study with enrollment from September 1987 to November 1988), DNA was collected at the end of the extended follow-up in 1995. DNA was not collected during PRECEPT (a 2-year trial with enrollment from April 2002 to April 2004), but DNA collection began during a follow-up investigation, known as POSTCEPT, in which all the surviving individuals previously enrolled in the original trial at participating sites were invited to participate. Overall, DNA was available for 808 individuals, of whom 63 were excluded from the Mendelian randomization analyses due to lack of serum urate levels or failure in genotyping of *SLC2A9*; furthermore, we excluded 10 patients who reported use of allopurinol at baseline, leaving 735 patients (390 in DATATOP and 345 in PRECEPT). Exploratory analyses of other genes included between 759 and 783 patients, because we excluded only those patients missing the specific single nucleotide polymorphism (SNP) of interest.

SNPs and Genotyping

Numerous SNPs in *SLC2A9* (a urate transporter[22]) have been identified in several genome-wide association studies (GWAS) as the strongest genetic predictors of serum urate levels and gout.[13–19] Because these SNPs are in high linkage disequilibrium (LD),[15] and a single causal variant has not been identified, we selected 3 of the top SNPs for the present study.

Specifically, the following SNPs in *SLC2A9* were genotyped: rs6855911, an intronic SNP with minor allele frequency (MAF) of 0.31 (G allele); rs7442295 (intronic, MAF = 0.21 for G allele); and rs16890979 (missense mutation, MAF = 0.22 for T allele; using HapMap data from Utah residents with ancestry from northern and western Europe, abbreviated CEU[23]), for which each minor allele has been associated with a 0.30 to 0.43mg/dl decrease in serum urate in individuals of European descent.[13,18] Because these 3 SNPs are in strong LD (pairwise r^2 range = 0.68–0.76 from Haploview[24] with HapMap CEU data), we used information from these 3 SNPs to create an *SLC2A9* score with values equal to 0 ($\leq$2 minor alleles; ie, preponderance of wild-type alleles), 1 (3 minor alleles and 3 wild-type alleles), and 2 ($\geq$4 minor alleles; ie, preponderance of minor alleles).

Other genes of interest because of their role in the transport of urate include solute carrier family 22, member 12 (*URAT1/SLC22A12*), which encodes a urate–anion exchanger,[25] adenosine triphosphate (ATP)-binding cassette subfamily G, member 2 (*ABCG2*), and solute carrier family 19 (sodium phosphate), member 3 (*SLC17A3*). All genotyping was performed through the Harvard Partners Center for Genetics and Genomics at the Harvard Partners Genotyping Facility using the OpenAssay SNP Genotyping System (BioTrove, Woburn, MA). Concordance rates for blinded duplicate quality control samples were 100%. Test of Hardy–Weinberg equilibrium revealed no significant deviations (all $p > 0.05$).

Serum Urate and Clinical Outcomes

Serum urate was measured in PRECEPT and DATATOP participants at baseline prior to treatment assignment, as previously reported.[5,6] The outcome evaluated in this study for both DATATOP and PRECEPT participants was the accumulation of disability sufficient to require dopaminergic therapy (this was also the primary outcome of the original studies).[20,21] The mean duration of follow-up until endpoint or study termination was 13.6 months in DATATOP and 13.3 months in PRECEPT.

Statistical Analysis

Initial analyses were conducted separately in DATATOP and PRECEPT. Because all tests of heterogeneity between studies were not significant ($p > 0.05$), data from the 2 trials were pooled, and all models were adjusted for study group and treatment. Differences in serum urate according to genotype were assessed using generalized linear models. Primary analyses to assess the relation between genetic variants and PD progression assumed additive models (per unit increase in score for *SLC2A9*, per allele associations for other genes); secondary analyses used separate indicators for each genetic score category or genotype. Cox proportional hazards models were used to estimate hazard ratios (HRs) and 95% confidence intervals (CIs) for reaching the primary endpoint according to number of minor alleles or genotype. Analyses were adjusted for study, treatment, gender, age, and use of thiazide diuretics at baseline. We assessed potential effect modification by gender and, in
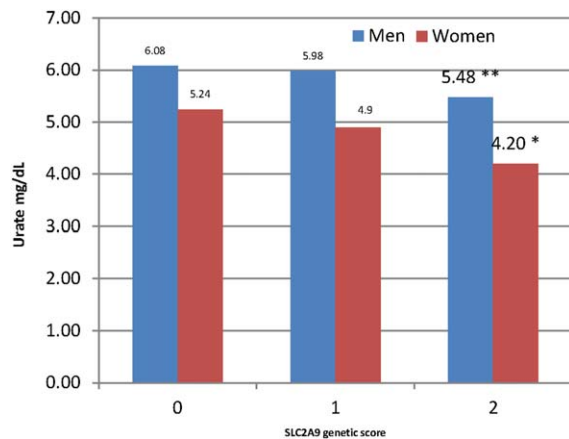
FIGURE 1: Serum urate by *SLC2A9* score. *$p<0.001$, **$p<0.01$ for comparison with score = 0 (≤2 minor alleles).

DATATOP, by randomization to α-tocopherol supplementation, which was included in some of the treatment arms in DATA-TOP. These interactions were assessed by including in the regression models an interaction term that was the cross product of the number of minor alleles of each individual SNP by gender (male/female) or α-tocopherol (yes/no). The association between genetically determined serum urate and PD progression was estimated by 2-stage regression; first, we fitted a generalized linear regression model with serum urate as the dependent variable and the *SLC2A9* score and potential confounders (study, gender, age, and use of thiazide diuretics) as independent variables, then the predicted urate level from the first stage regression was used as a continuous independent variable to determine its association with PD progression in a Cox proportional hazard model, adjusting for potential confounders. Sensitivity analyses were conducted estimating the genetically predicted urate level in a generalized linear model with separate indicators for each *SLC2A* SNP.

Results

As expected, serum urate concentrations decrease with increasing number of minor *SLC2A9* alleles, with a
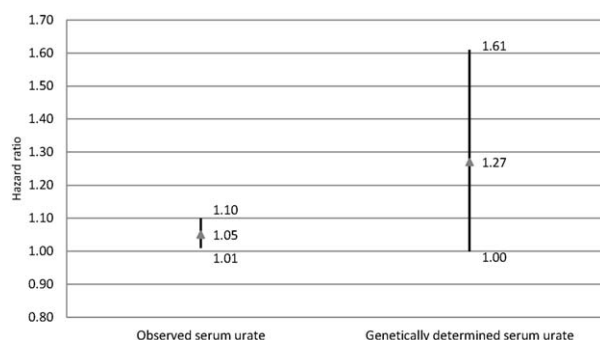

FIGURE 2: Hazard ratios and 95% confidence intervals for initiation of dopaminergic therapy for a 0.5mg/dl observed decrease in serum urate or for genetically conferred decrease in serum urate.

stronger association in women than in men (Fig 1). In an additive model, the rate of progression to a level of disability requiring dopaminergic treatment increased with the number of the minor alleles associated with lower serum uric acid (UA; HR = 1.16 for each point increase in genetic score, 95% CI = 1.00–1.35, $p = 0.056$). As compared with individuals with ≤2 minor *SLC2A9* alleles, the HR was 1.12 (95% CI = 0.89–1.41) for individuals with 3 minor alleles, and 1.39 (95% CI = 0.98–1.96) for individuals with ≥4 minor alleles. The HR for a genetically determined lower serum urate was higher (HR for 0.5mg/dl lower urate = 1.27) than the corresponding HR for directly measured 0.5mg/dl lower serum urate (HR = 1.05; Fig 2). Results were not materially changed if each *SLC2A9* SNP was used as an independent predictor of serum urate; in this analysis, the HR for 0.5mg/dl genetically predicted lower urate was 1.24 (95% CI = 0.99–1.54).

There was no significant effect modification by either gender or α-tocopherol supplementation (in DATATOP only) on the association between *SLC2A9* score and initiation of dopaminergic therapy (all p for interaction > 0.05). Additionally, there was no evidence of interaction between *SLC2A* score and serum urate.

Overall, polymorphisms in genes other than *SLC2A9* were not significantly associated with serum UA (Table 1) or subsequent initiation of dopaminergic therapy adjusting for age, gender, and treatment (Table 2).

Discussion

In this investigation, we found that among individuals with early PD, SNPs in *SLC2A9* predicted differences in serum urate that are similar to those previously reported in the general population.[13–19] Furthermore, the rate of progression to a level of disability requiring dopaminergic treatment was faster among those patients carrying the *SLC2A9* genotypes associated with lower serum UA. Although the statistical significance was marginal according to conventional levels, these novel results suggest that among participants in DATATOP and PRECEPT the previously reported better prognosis of early PD patients with higher urate levels[5,6] is due to a protective effect of urate itself rather than to confounding by unknown factors.

A limitation of this study is that DNA was collected only several years after the trial completion and was only available for a subset of participants in DATA-TOP and PRECEPT, so that patients with more rapidly progressive disease may be underrepresented. It is unlikely, however, that this selection would result in a spurious inverse association between the *SLC2A9* SNPs and the rate of PD progression during the trials.

TABLE 1. Serum Urate according to Urate Transport–Related Genotype

SNP	Serum Urate, Mean ± SD	p^a
URAT1-rs11231825, n = 764		
TT	5.5 ± 1.4	0.18
TC	5.3 ± 1.4	
CC	5.3 ± 1.4	
URAT1-rs11602903, n = 780		
AA	5.5 ± 1.3	0.56
AT	5.3 ± 1.4	
TT	5.3 ± 1.4	
URAT1-rs3825016, n = 780		
CC	5.6 ± 1.3	0.36
CT	5.3 ± 1.4	
TT	5.3 ± 1.4	
URAT1-rs3825018, n = 759		
AA	5.3 ± 1.4	0.68
AG	5.4 ± 1.4	
GG	5.4 ± 1.4	
URAT1-rs475688, n = 770		
CC	5.3 ± 1.5	0.95
CT	5.3 ± 1.3	
TT	5.4 ± 1.3	
URAT1-rs476037, n = 763		
AA	6.1 ± 1.0	0.35
AG	5.4 ± 1.4	
GG	5.3 ± 1.4	
URAT1-rs7932775, n = 783		
CC	5.7 ± 1.5	0.86
CT	5.2 ± 1.3	
TT	5.3 ± 1.4	
URAT1-rs893006, n = 761		
AA	5.3 ± 1.4	0.46
AC	5.3 ± 1.4	
CC	5.6 ± 1.4	
ABCG2-rs2231142, n = 779		
GG	5.3 ± 1.4	0.07
GT	5.6 ± 1.5	
TT	4.9 ± 1.1	

TABLE 1: Continued

SNP	Serum Urate, Mean ± SD	p^a
SLC17A3-rs1165205, n = 773		
AA	5.3 ± 1.4	0.49
AT	5.3 ± 1.4	
TT	5.4 ± 1.4	

[a]Probability value for trend test, adjusted for study.
SD = standard deviation; SNP = single nucleotide polymorphism.

Furthermore, as in the previous studies including all trial participants, baseline serum urate was inversely related to time to initiation of dopaminergic therapy.

A Mendelian randomization approach has been used to investigate the causality of the association between serum urate and PD risk in 3 previous studies. In the first, conducted among individuals with PD in Italy, Croatia, and Germany, a *SLC2A9* SNP predicting lower serum urate was associated with a younger age at onset of PD.[26] In the second, a case–control study in Spain, individuals in the highest tertile of a genetic score predicting lower serum urate were found to have a 50% higher risk of PD.[11] In the third study, only 1 of 12 genotyped SNPs in *SLC2A9* was associated with a significantly increased PD risk in women, and none in men.[27] In this last study, however, serum urate levels were not available, and it is thus possible that the association between the genotyped SNPs and serum urate in the study population was weaker than expected. An important limitation of these studies is that even for those SNPs with the most robust associations with serum urate, the expected effects on PD risk are small, and power to detect an association is thus modest. Considering these limitations, overall the results of these studies support the hypothesis that higher urate levels reduce PD risk.

The association between genetically decreased serum UA levels and PD prognosis was somewhat stronger than the comparable association for measured circulating UA, suggesting that the latter may have been attenuated by unmeasured confounding. The lower measurement error and long-term stability of genetically determined changes in serum urate may have contributed to this difference, but it is also possible that the association between serum urate and PD progression is attenuated by unmeasured confounders and thus underestimates the true effect of urate on PD progression. Serum urate is associated with obesity and insulin resistance, which in some investigations have been

TABLE 2. Hazard Ratio for Initiating Dopaminergic Therapy according to Urate Transport–Related Genotype				
SNP	Genotypes	Risk Allele	Genotype Frequencies	HR (95% CI)[a]
URAT1-rs11231825	TT/TC/CC	C	89/313/375	1.10 (0.95–1.27)
URAT1-rs11602903	AA/AT/TT	T	81/351/362	1.07 (0.92–1.24)
URAT1-rs3825016	CC/CT/TT	T	78/365/351	1.10 (0.95–1.28)
URAT1-rs3825018	AA/AG/GG	G	355/334/84	0.91 (0.78–1.05)
URAT1-rs475688	CC/CT/TT	T	404/319/61	0.94 (0.81–1.10)
URAT1-rs476037	AA/AG/GG	G	9/145/623	1.03 (0.83–1.29)
URAT1-rs7932775	CC/CT/TT	T	34/258/505	1.05 (0.89–1.25)
URAT1-rs893006	AA/AC/CC	C	358/332/85	0.92 (0.80–1.07)
ABCG2-rs2231142	GG/GT/TT	T	614/169/10	1.04 (0.84–1.29)
SLC17A3-rs1165205	AA/AT/TT	T	221/380/186	0.89 (0.78–1.03)

[a]HR for increasing number of risk alleles, adjusted for gender and age, stratified by a 4-level treatment by study variable.
CI = confidence interval; HR = hazard ratio; SNP = single nucleotide polymorphism.

associated with an increased risk of PD,[28,29] and could be a marker of dysfunctional energy metabolism.[30] In vitro, urate production is stimulated by compounds that lower ATP, including inhibitors of mitochondrial respiration,[30] which have been implicated in the pathogenesis of PD.[31] The observed association between serum urate and PD progression could thus reflect in part the protective effect of urate on neurodegeneration, and in part the adverse effects of the upstream metabolic dysregulation that results in elevated serum urate. Although we did not find a significant interaction between *SLC2A9* genotype and α-tocopherol supplementation or gender, the power for these analyses was modest, and effect modification by these factors therefore cannot be excluded. Whereas among DATATOP and PRECEPT participants serum urate was found to be a stronger prognostic predictor in men than in women,[5,6] it is noteworthy that the results of a recent phase 2 randomized trial in patients with early PD suggested that urate elevation may be more effective in women than in men.[32]

We a priori considered *SLC2A9* the primary gene of interest in relation to serum urate levels, so we did not consider potential joint or synergistic effects of a combination of SNPs recently considered by other authors.[19,33] Although a composite genetic score incorporating several loci could be used,[11,19,33] the contribution of the additional genes to serum urate is small relative to *SLC2A9*. The inclusion of numerous genes with modest effects on serum UA could increase the possibility that at least 1 of these genes affects PD progression via mechanisms other than serum urate, thus violating a key assumption of the Mendelian randomization method.[10] In particular, the second strongest genetic predictor of serum urate levels is the ATP-binding cassette, subfamily G, isoform 2 protein (ABCG2), which has been related to the clearance of neurotoxic polypeptides from the brain[34] and neuroregeneration,[35] and whose expression in brain capillaries is altered in an animal model of PD.[36] We cannot therefore exclude the possibility that variations in *ABCG2* could affect PD progression through mechanisms independent from its effects on urate. The validity of the Mendelian randomization approach in our study is supported by the finding that the genotype used as an instrumental variable (*SLC2A9*) is strongly associated with the exposure of interest (serum urate), and is most likely independent of the factors that confound the association between serum urate and PD progression. The finding that other genes involved in urate transport but without sizable effects on serum urate were not related to PD progression indirectly supports this conclusion. Because urate is also inversely associated with PD risk, one might expect that SNPs in *SLC2A9* that predict lower urate levels should have been found to be associated with PD risk in large GWAS. Therefore, its absence[37] may appear to contradict the hypothesis of a genuine protective effect of urate. However, because of the stringent significance criteria imposed by the large number of tests performed, even large GWAS are underpowered to detect the small effects attributable to single *SLC2A9* SNPs.

In summary, we found that patients in the early stages of PD who carry the variant *SLC2A9* alleles

associated with lower urate levels have a faster rate of disease progression than those homozygous for the wild-type alleles. This finding suggests that the previously reported inverse association between higher urate levels and rate of PD progression is not explained by unmeasured confounders and is thus likely to reflect a genuine neuroprotective effect of urate. Genotypic characterization may be useful in identifying those most likely to respond to urate-elevating interventions. These data raise the possibility that modulation of *SLC2A9* might be an equally or even more effective approach to urate elevation, compared to urate precursor administration,[38] as a candidate strategy for slowing PD progression.

Authorship

K.C.S.—research project: execution; statistical analysis: design and review; manuscript: writing. S.E.—statistical analysis: design and execution; manuscript: review and critique. X.G.—research project: execution; manuscript: review and critique. D.O.—research project: conception, organization, and execution; statistical analysis: review and critique; manuscript: review and critique. C.M.T.—research project: conception, organization, and execution; manuscript: review and critique. I.S.—research project: conception, organization, and execution; manuscript: review and critique. S.F.—research project: conception, organization, and execution; manuscript: review and critique. M.A.S.—research project: conception, organization, and execution; manuscript: review and critique. A.A.—research project: conception, organization, and execution; statistical analysis: design and review; manuscript: writing, review, and critique.

Potential Conflicts of Interest

S.E.: grants, NIH, Michael J. Fox Foundation, Biogen Idec, Auspex Pharmaceuticals. C.M.T.: consultancy, IMPAX Pharmaceuticals, Abbvie, Pfizer Pharmaceuticals, ADAMAS Pharmaceuticals. I.S.: US Food and Drug Administration, Parkinson's Disease Foundation, Lundbeck A/S, Michael J. Fox Foundation; consultancy, Auspex Pharmaceuticals, AZTherapies, Biogen, Caravel Group, Chelsea Therapeutics, Edison Pharmaceuticals, Genzyme Corporation, Innovation Consulting, Impax, Ipsen, Isis Pharmaceuticals, Knopp, Lundbeck A/S, Medtronic, Neuroglobe, Omeros Corporation, Orphazyme APS, Pick Research Solutions, Prana Biotechnology (consultant, non-executive director), Salamandra, Seneb Biosciences, Sofinnova Venture Partners, Velocity Pharmaceutical Development, Voyager Therapeutics; periodic honoraria, Banner Alzheimer's Institute, Columbia University, JAMA Neurology, National Institute of Neurological Disorders and Stroke, Partners Health Care, University of California Irvine, University of Rochester. S.F.: safety monitoring board, Merz Pharma; grant, Genervon Biotechnology; consultancy, PixarBio; advisory board, Lundbeck Pharma, AstraZeneca Pharmaceuticals, IMPAX Pharmaceuticals.

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Neuroprotective effects of urate are mediated by augmenting astrocytic glutathione synthesis and release

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ABSTRACT

Urate has emerged as a promising target for neuroprotection based on epidemiological observations, preclinical models, and early clinical trial results in multiple neurologic diseases, including Parkinson's disease (PD). This study investigates the astrocytic mechanism of urate's neuroprotective effect. Targeted biochemical screens of conditioned medium from urate- versus vehicle-treated astrocytes identified markedly elevated glutathione (GSH) concentrations as a candidate mediator of urate's astrocyte-dependent neuroprotective effects. Urate treatment also induced the nuclear translocation of the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) protein and transcriptional activation of its key target genes in primary astrocytic cultures. Urate's neuroprotective effect was attenuated when GSH was depleted in the conditioned media either by targeting its synthesis or release by astrocytes. Overall, these results implicate GSH as the extracellular astrocytic factor mediating the protective effect of urate in a cellular model of PD. These results also show that urate can employ a novel indirect neuroprotective mechanism via induction of the Nrf2 signaling pathway, a master regulator of the response to oxidative stress, in astrocytes.

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1. Introduction

Urate (2,6,8-trioxy-purine; a.k.a. uric acid) has been gaining momentum as a promising candidate therapeutic target for people with Parkinson's disease (PD) based on its antioxidant and neuroprotective properties, and on its identification as a predictor of a reduced PD risk and a favorable rate of disease progression (Cipriani et al., 2010). Several groups including ours have documented protective properties of urate in cellular and rodent models of PD and other neurodegenerative diseases (Bakshi et al., 2015). In dopaminergic cell lines, urate blocked cell death and oxidative stress induced by 6-hydroxydopamine (6-OHDA), dopamine or rotenone (Zhu et al., 2012; Jones et al., 2000; Duan et al., 2002). Urate at physiologically relevant concentrations enhanced function and survival of dopaminergic neurons in primary cultures of rat ventral mesencephalon (Guerreiro et al., 2009). Urate also confers protection in various cellular models of neurotoxicity beyond that of PD. Urate protected cultured spinal cord or hippocampal neurons from excitotoxic (glutamate-induced) (Yu et al., 1998; Du et al., 2007)

or nitrosative (peroxynitrite-induced) cell death (Scott et al., 2005). Similarly, in intact animals urate protects these neurons from ischemic brain or compressive spinal cord injuries (Yu et al., 1998; Scott et al., 2005).

The neuroprotective effects of urate have also been evaluated in vivo in rodent models of PD, and was found to attenuate 6-OHDA toxicity (Gong et al., 2012). Similarly, our group found that mice with a urate oxidase gene (*UOx*) knockout have elevated brain urate levels and are resistant to toxic effects of 6-OHDA on nigral dopaminergic cell counts, striatal dopamine content, and rotational behavior (Chen et al., 2013). Conversely, transgenic over-expression of *UOx* exacerbated these anatomical, neurochemical, and behavioral deficits of the lesioned dopaminergic nigrostriatal pathway.

Although considerable evidence indicates that urate is a powerful direct antioxidant few studies have investigated alternative mechanisms of its protective effect. Previous findings of our group (Cipriani et al., 2012a) and others (Du et al., 2007) have suggested a prominent role of astrocytes in the neuroprotective effects of urate. We demonstrated an essential requirement for astrocytes in order for urate to fully protect dopaminergic cells (Cipriani et al., 2012a) or nigral neurons (Cipriani et al., 2012b). We further determined that in response to urate, astrocytes release a potent neuroprotective factor, which differs from urate because it is insensitive to urate-eliminating incubation with commercial UOx enzyme. In the present study we identify and characterize

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the astrocytic protective factor and signaling pathways mediating urate's neuroprotective effect on dopaminergic cells.

2. Materials and methods

2.1. Cell cultures, drug treatment and conditioned media experiments

Astroglial cultures were prepared from the cerebral cortex of 1- or 2-day-old neonatal mice as described previously (Cipriani et al., 2012a). Our astroglial cultures comprised >95% astrocytes, <2% microglial cells, <1% oligodendrocytes, and no detectable neuronal cells. Astrocyte cultures reached confluence after 7–10 days in vitro. Urate was dissolved in DMEM as 20X concentrated stocks. Enriched astroglial cultures were treated with 100 μM urate or vehicle. Twenty-four hours later conditioned media (CM) were collected and filtered through a 0.2 μm membrane to remove cellular debris and immediately used for experiments. All reagents for cell culture were obtained from Life Technologies.

The rodent MES 23.5 dopaminergic cell line, which was derived from the fusion of a dopaminergic neuroblastoma and embryonic mesencephalon cells (Crawford et al., 1992), was obtained from Dr. Weidong Le at Baylor College of Medicine (Houston, USA). The cells were cultured as described previously (Cipriani et al., 2012a). MES 23.5 cells were incubated with CM from urate- or vehicle-treated astrocytes for 24 h before addition of 200 μM H_2O_2 and incubation for another 24 h.

2.2. RNA isolation and quantitative real-time PCR

RNA was extracted from astrocyte cultures by TRIzol (GIBCO/BRL) extraction. RNA quality was determined by spectrophotometry and by visual inspection of electropherograms using the RNA 6000 NanoChip Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies). cDNA synthesis was performed using Superscript® VILO cDNA synthesis kit (Invitrogen). For quantitative gene expression analysis, SYBR green primers and probes (Applied Biosystems) were used. The specificity of each PCR product was confirmed by melting dissociation curve (T_m) analysis. The comparative threshold cycle method was used for quantitative analysis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and RPL13 ribosomal RNA were used as RNA loading controls. Equal amplification efficiencies were confirmed for target and reference genes. Primer pairs used for quantitative PCR are listed as follows: mouse GCLM (forward 5′-GCA CAG GTA AAA CCC AAT AG-3′, reverse 5′-TTA GCA AAG GCA GTC AAA TC-3′), mouse GCLC (5′-CTA TCT GCC CAA TTG TTA TGG-3′, reverse 5′-ACAGGTAGCTATCTATTGAGTC-3′), mouse NQO1 (forward 5′-CCT TTC CAG AAT AAG AAG ACC-3′, reverse 5′-AAT GCT GTA AAC CAG TTG AG-3′) and mouse HO-1 (forward 5′-CAA CCC CAC CAA GTT CAA ACA-3′, reverse 5′-AGG CGG TCT TAG CCT CTT CTG-3′).

2.3. Western blot analysis

Western blot analysis was performed using lysates from astrocytes. The blots were probed with antibodies against NQO1 (Sigma N5288), GCLC (Abcam ab53179), GCLM (Abcam ab126704) and β-actin (Abcam ab8227) using the dilutions recommended in the product datasheet. Band densities were analyzed with ImageJ software.

2.4. Immunofluorescence microscopy

Primary astrocyte cultures grown on chamber slides were fixed in 4% paraformaldehyde for 20 min at room temperature and then permeabilized for 30 min with 0.1% Tween-20 in phosphate-buffered saline (PBS). After blocking the cells in 3% bovine serum albumin/PBS for 1 h, anti-Nrf2 and anti-glial fibrillary acidic protein (GFAP) antibodies were added at 4 °C overnight, followed by incubation with Cy3-conjugated secondary antibody for GFAP and fluorescein isothiocyanate

(FITC) conjugated secondary antibody for Nrf2 (Molecular Probes) for another 1 h. Slides were treated with Vectashield mounting medium containing DAPI (4′,6-diamidino-2-phenylindole; Vector Laboratories) and mounted. images were collected with a Nikon A1+/A1R+ confocal microscope and were processed with NIS Element confocal imaging software.

Fluorescence intensity of Nrf2 in the nuclear and cytoplasmic regions was quantified using ImageJ (http://rsbweb.nih.gov/ij/) as described (Drake et al., 2010). Background-corrected nuclear:cytoplasmic (N/C) ratios were calculated from mean fluorescence intensities measured within a small circular region of interest placed randomly in a region of uniform staining devoid of any punctate structures within the nucleus and the cytoplasm of each cell.

2.5. Cell viability assays

For evaluation of cell viability, cells were co-stained with propidium iodide (PI)/ annexin V and analyzed using fluorescence activated cell sorting (FACS) as described before (Behbahani et al., 2005). The percentage of apoptotic cells was determined by FACScan Flow Cytometer and CellQuest software (Becton Dickinson).

2.6. Screening for neurotrophic factors

Screening for GDNF, BDNF and IL6 was conducted using commercial ELISA kits as per manufacturer (Abcam) instructions. Total GSH levels (GSH + GSSG) were measured in lysates and CM from urate- and vehicle-treated astrocytes. Levels of GSH were determined by a simple in vitro fluorometric detection assay kit as per manufacturer (Abcam ab65322) instructions. All GSH measurements were normalized to total protein levels.

2.7. GSH depletion assays

MK-571 and BSO were purchased from Sigma and dissolved in DMEM as 100X concentrated stocks. Enriched astroglial cultures were treated with vehicle or urate (100 μM) alone or in combination with BSO (0.25 mM) or MK-571 (50 μM). Twenty-four hours later CM was collected and filtered through a 0.2 μm membrane to remove cellular debris and immediately used for experiments with MES 23.5 cells. To verify the effects of BSO or MK-571, GSH content in the CM was measured. MES 23.5 cells were pretreated with vehicle CM or urate CM, with or without BSO or MK-571, (or were not pretreated) before being exposed to oxidative stressor (H_2O_2) for 24 h. The percentage of dead cells was analyzed using the FACS method described above.

2.8. Statistical analysis

Values were expressed as mean ± standard error of the mean (SEM). Differences between groups were examined for statistical significance using one-way ANOVA or two-tailed Student's t-tests, using GraphPad Prism 5 software. A p value less than 0.05 denoted the presence of a statistically significant difference.

3. Results

3.1. Urate induces GSH release from cultured primary astrocytes

First we confirmed the neuroprotective effect of conditioned medium (CM) from urate-treated astrocytes by assessing dopaminergic cell death with a method complementary to those of our previous report (Cipriani et al., 2012a). Using a PI/annexin dual staining method to evaluate cell viability we observed complete protection of dopaminergic MES 23.5 cells from oxidative stressor (H_2O_2)-induced cell death after their incubation with CM from astrocytes treated with 100 μM urate (Fig. 1A).
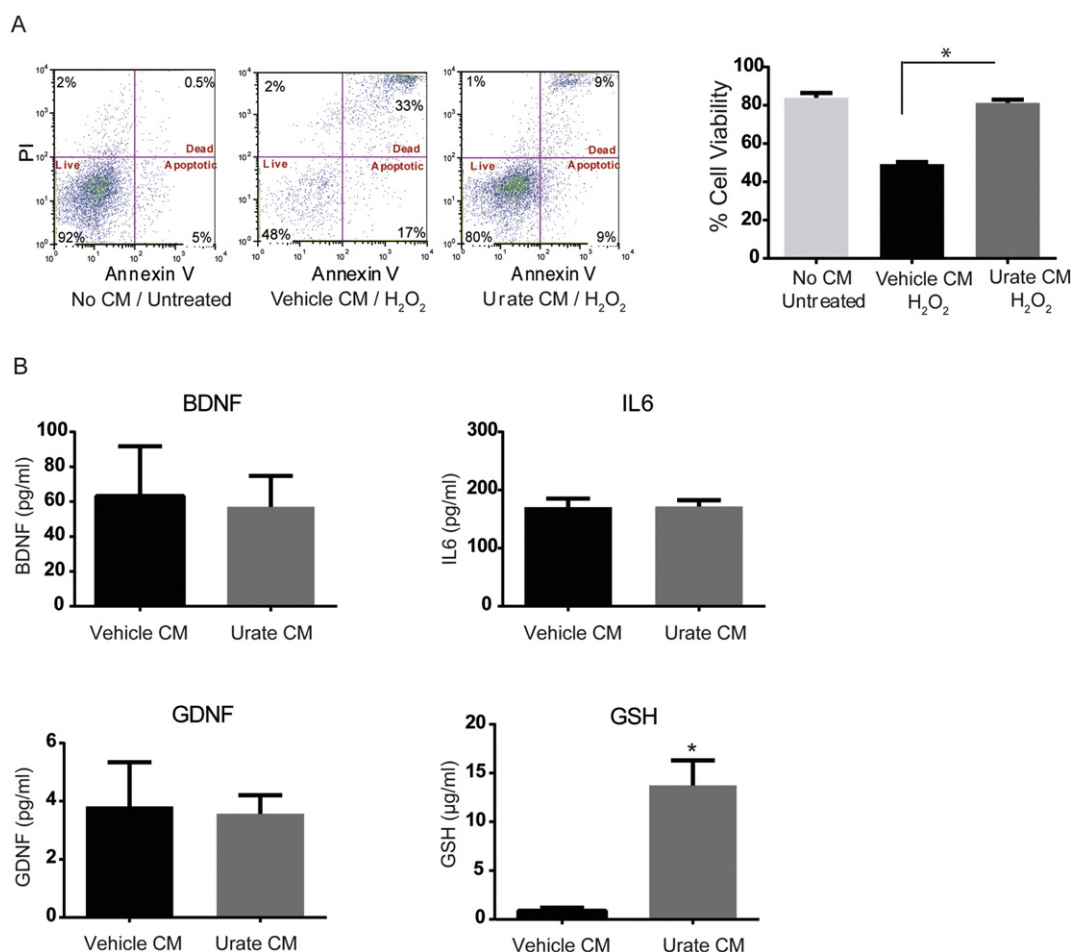
Fig. 1. Marked elevation in GSH release from astrocytes treated with urate. (A) Conditioned medium (CM) from urate-treated astrocytes protects dopaminergic cells from H_2O_2-induced cell death. Representative graphs of FACS analysis show cell viability using propidium iodide (PI)/AnnexinV staining. Percentages of PI+/AnnexinV+ (dead), PI−/AnnexinV+ (apoptotic) and PI−/AnnexinV− (vital) staining are shown for untreated MES 23.5 cells, or those treated with CM from vehicle- or urate (100 μM)-treated astrocytes a day before and during 200 μM H_2O_2 treatment for 24 h. (B) Targeted screening of several neurotrophic factors in the CM. There was no change detected in levels of BDNF, GDNF and IL6 factors in the urate versus vehicle CM. GSH content was significantly increased in CM from urate- (versus vehicle-) treated astrocyte. * denotes p value < 0.001; (n = 4 independent experiments).

To identify putative inducible factors secreted by urate-treated astrocytes we conducted a targeted screen of their CM for prominent neurotrophic factors known to be released by astrocytes. Using commercial assays we measured levels of glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), interleukin-6 (IL6) and GSH in urate- and vehicle-treated glial CM. No significant difference was found in the levels of BDNF, IL6 and GDNF in urate- versus vehicle-treated CM (Fig. 1B). In contrast, GSH levels were significantly higher in CM from urate- compared to vehicle-treated astrocytes.

3.2. Urate induces GSH levels and Nrf2-targeted gene in astrocytes

To determine the mechanism of increased extracellular GSH levels after urate treatment, we examined changes in GSH levels as well as GSH synthesis within primary astrocytes. Urate treatment for 24 h significantly increased GSH levels in the astrocytes compared to those in control astrocytes (Fig. 2A). We also examined the protein and transcript levels of two subunits of the γ-glutamyl cysteine ligase (GCL), the rate-limiting enzyme of GSH synthesis. Both the protein and mRNA expression levels of the modifier subunit (GCLM) were significantly induced by urate treatment (Fig. 2B and C). Because GCL is transcriptionally regulated by nuclear factor (erythroid-derived 2)-like 2 (Nrf2), we also measured the mRNA and protein expression of other key Nrf2-regulated genes including NAD(P)H:quinone oxidoreductase 1 (NQO1) and heme oxygenase-1 (HO-1). Urate treatment for 24 h led to a significant increase in mRNA levels of NQO1 in astrocyte

cultures (Fig. 2C) and a trend towards an increase in its protein levels (Fig. 2B). Transcript levels of HO-1 also appeared increased, though not significantly, with urate treatment (Fig. 2C). The protein levels of HO-1 were not detected by the western blot analysis.

3.3. Urate induces nuclear localization of Nrf2 in astrocytes

Activation of the Nrf2 pathway involves translocation of the Nrf2 protein from the cytoplasm into the nucleus where it can transactivate its targets. Nrf2 is known to bind to the antioxidant-response element (ARE) in promoter regions of Nrf2-responsive genes leading to their transcriptional activation in response to oxidative stress or related external stimuli. Here, the subcellular distribution of Nrf2 was studied by immunofluorescence confocal microscopy. We observed significant redistribution of Nrf2 immunoreactivity from its relatively balanced cytoplasmic and nuclear localization in vehicle-treated astrocytes to a predominantly nuclear localization after treatment with urate (Fig. 3). Cultures were also stained with DAPI to visualize nuclei and immunostained with astrocyte-specific anti-GFAP antibody.

3.4. GSH depletion attenuates neuroprotection by conditioned medium from urate-treated astrocytes

To determine whether GSH mediates urate's neuroprotective effect we reduced the GSH concentration of CM from urate-treated astrocytes. This was accomplished by two strategies. First, GSH synthesis in urate-
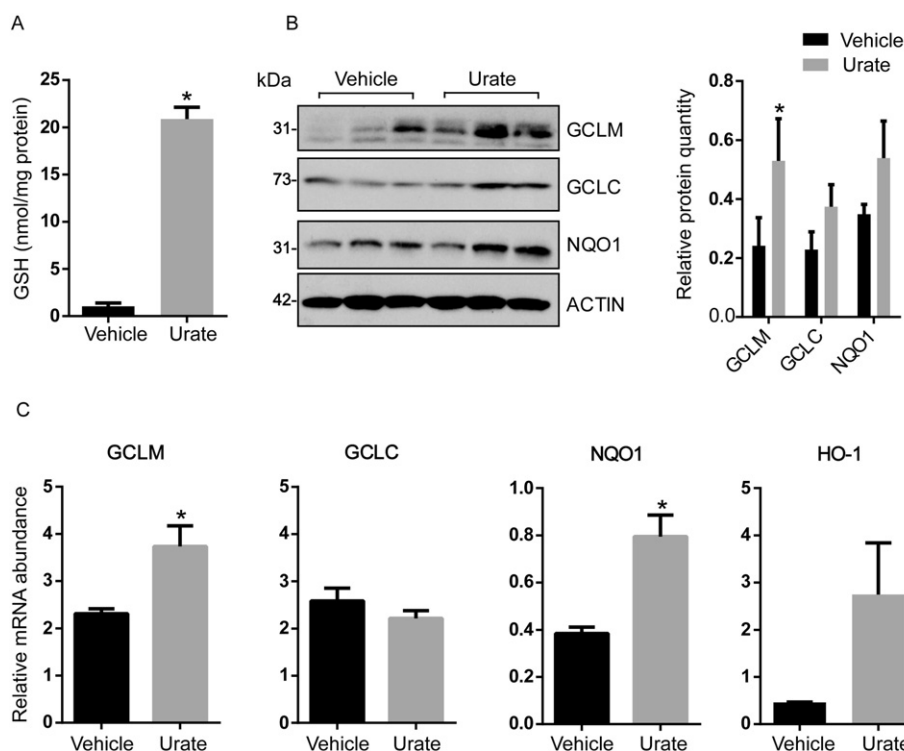
Fig. 2. Urate induces the Nrf2 pathway in astrocytes.(A) GSH levels are elevated in lysates from 100 μM urate- (versus vehicle-) treated astrocytes. (B) Urate induces protein products of Nrf2-targeted genes as shown by western blots of GCLM, GCLC and NQO-1 proteins in urate-treated astrocytes compared to controls. The approximate molecular weight of the indicated protein in kDa is indicated at Left. The graph represents densitometric analysis of the western blots to semi quantify the protein levels. (C) Quantitative PCR analysis of Nrf2 target genes in urate- (versus vehicle-) treated cells. Mean ± SEM are shown (n = 3). * denotes p value < 0.05.

treated astrocytes was inhibited by buthionine sulfoximine (BSO), which inhibits GCL, the rate-limiting enzyme of GSH synthesis (Drew and Miners, 1984). Second, we blocked GSH release from the astrocytes by inhibiting multidrug resistance protein 1 (MRP1) transporter with its competitive inhibitor MK-571 (Hirrlinger et al., 2002). We performed cell viability experiments in MES 23.5 dopaminergic cells treated with CM from astrocytes treated with vehicle or urate in combination with BSO (0.25 mM) or MK-571 (50 μM). These drugs were added to the astrocytes concurrently with urate or vehicle 24 h prior to the collection of the CM. GSH levels were undetectable in urate or vehicle CM from astrocytes treated with either BSO or MK-571 (Fig. 4A). There was no significant effect of BSO or MK-571 treatment alone on MES 23.5 cell viability

in the absence of H_2O_2 (Fig. 4B and C). Neuroprotection by CM from urate-treated astrocytes was significantly reduced by BSO or MK-571 treatment as depicted by increased cell death compared to urate CM alone (Fig. 4B and C), indicating that the presence of GSH in the CM is critical for urate's protective effect.

4. Discussion

Its well-documented direct antioxidant properties notwithstanding, urate can produce much of its neuroprotective effect indirectly via astrocytes (Cipriani et al., 2012a; Du et al., 2007). They in turn release a potent neuroprotective factor, which differs from urate because incubation of medium conditioned by urate-treated astrocytes with commercially obtained UOx eliminates urate but not the protective influence (Cipriani et al., 2012a). Here we identified GSH as a primary candidate for the putative neuroprotective factor that is released from urate-treated astrocytes based on its markedly higher concentration in CM and lysates from urate-treated compared to control astrocytes. The glutathione system is very important for cellular defense and protects against a variety of different reactive oxygen species (ROS). The total GSH content of astroglial cultures measured in the astrocytes and the extracellular media was in the same physiological range as reported previously (Raps et al., 1989; Dringen et al., 1999). GSH is a tripeptide that is synthesized by two successive enzymatic reactions. The first, rate-limiting step in GSH biosynthesis is catalyzed by GCL (Lu, 2013). In its catalytically most active form, GCL comprises a catalytic subunit (GCLC) and a modifier subunit (GCLM). Urate significantly increased the transcript and protein levels of the modifier (but not the catalytic) subunit of the enzyme in astrocytes, likely contributing to their increased GSH synthesis given that GCLM increases V_{max} of GCLC and its affinity for its substrates (Franklin et al., 2009). Although the significance of differential induction of GCLM versus GCLC genes is unclear, it is consistent with similarly discordant GCL subunit regulation by other extracellular stimuli (Cai et al., 1995, 1997; Franklin et al., 2009; Moellering et al., 1998).
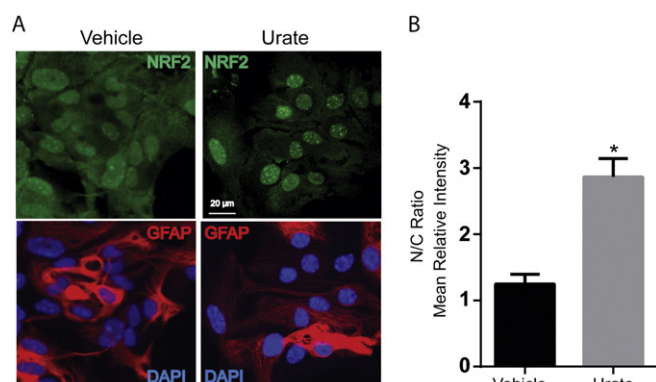

Fig. 3. Urate induces nuclear translocation of the Nrf2 protein. (A) Astrocytes were incubated with urate (100 μM) or vehicle control for 8 h. Cells were immunostained for astrocyte-specific marker GFAP (red) and nuclei were stained with DAPI (blue). Nrf2 was detected using FITC (green) staining. These representative images show a predominant nuclear distribution of the Nrf2 protein after urate treatment, in contrast to the greater proportion of cytoplasmic expression of Nrf2 in vehicle-treated astrocytes. (B) The graph represents the quantification of the nuclear:cytoplasmic (N/C) ratios of Nrf2 staining intensity using ImageJ software. 20–30 cells from 4 independent experiments were counted. * denotes p value < 0.05.
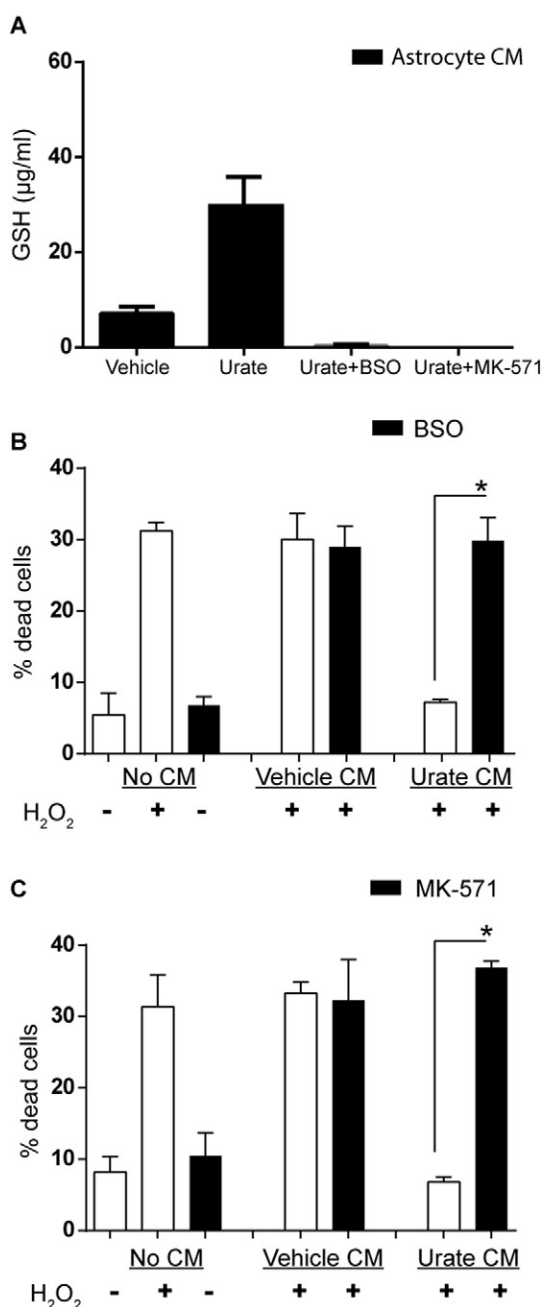
Fig. 4. GSH depletion attenuated the protective effects of urate. (A) GSH content was undetectable in CM from astrocytes that were treated with urate as well as either BSO (0.25 mM) or MK-571 (50 µM). (B,C) MES 23.5 cells were exposed to an oxidative stressor (200 µM H_2O_2) after pretreatment with CM. The CM was from vechicle- or urate-treated astrocytes, with the latter also treated with or without BSO (0.25 mM) or MK-571 (50 µM) as indicated. The protective effect of CM from urate-treated astrocytes against H_2O_2 toxicity (% dead cells) was significantly reduced by astrocyte incubation with BSO (B) or MK-571 (C) (n = 3). * denotes p value < 0.05.

The GCL genes are part of a broader cellular antioxidant pathway that controls a set of effector genes through a unique cis-acting transcriptional regulatory sequence, termed the antioxidant response element (ARE). Several lines of evidence suggest that Nrf2 is a transcription factor responsible for upregulating ARE-mediated gene expression (Kensler et al., 2007). The Nrf2 pathway has been known to be activated by both oxidative stress as well as antioxidants (Ma, 2013). From our findings, urate appears to be a key activator of Nrf2 signaling and its downstream targets that guard against oxidative stress. Astrocytes are known to interact with surrounding neurons and their neuroprotective

properties are well documented (Maragakis and Rothstein, 2006; Belanger and Magistretti, 2009; Sidoryk-Wegrzynowicz et al., 2011). Here we demonstrate an unanticipated requirement for a neuroprotective factor released from astrocytes in order for urate to fully protect dopaminergic cells in a cellular model of PD. Interestingly, others have recently reported Nrf2 involvement in urate's neuroprotective effects on dopaminergic cells (Zhang et al., 2014). They reported that urate protected dopaminergic cell lines in the absence of glial cells but, as noted by the authors, at much (~100-fold) higher concentrations of urate than are needed in the presence of astrocytes (Cipriani et al., 2012a; Zhang et al., 2014). They found urate to be protective at concentrations of 200 µM and above, whereas CNS concentrations of endogenous urate are typically 10- to 100-fold lower (Ascherio et al., 2009; Chen et al., 2013). Thus activation of astrocytic Nrf2 signaling by urate and its indirect neuroprotective effects may be more pathophysiologically relevant than are the direct effects of urate on neuronal cells. The importance of astrocytic Nrf2 is in agreement with earlier demonstrations that Nrf2 induction in astrocytes boosts their production of GSH, which in turn can protect glial cells and neighboring neurons (Kraft et al., 2004; Shih et al., 2003). The protective effects of astrocytic Nrf2 against neurodegeneration have been suggested by neuroprotective effects of Nrf2 overexpression in astroglial cells in mouse models of amyotrophic lateral sclerosis (ALS) and PD (Chen et al., 2009; Vargas et al., 2008). Moreover, astrocytes have greater antioxidant potential than neurons (Makar et al., 1994; Raps et al., 1989) and many studies also provide evidence for efflux of GSH from astrocytes as a key factor in neuroprotection (Iwata-Ichikawa et al., 1999; Dringen et al., 2000; Dringen and Hirrlinger, 2003). Nrf2/ARE activation in astrocytes leading to increased levels of GSH seems to be a major component of the protection conferred by urate.

In addition to the extensive preclinical data supporting a key role of Nrf2 disruption in PD neurodegeneration, recent epidemiological studies have suggested that Nrf2 genetic variants modify PD susceptibility and onset (Todorovic et al., 2015; von Otter et al., 2014). From a therapeutic standpoint this astrocytic Nrf2-orchestrated defense system may offer an attractive drug target in several neurodegenerative diseases and other neurological disorders. For example, another small molecule dimethyl fumarate (DMF), which has been found effective and approved for use as a disease-modifying treatment of multiple sclerosis, may confer its cytoprotective effects via activation of the Nrf2 pathway (Scannevin et al., 2012). Urate has been gaining momentum as a promising target or agent for neuroprotection based on accumulating epidemiological observations, laboratory data, and encouraging early clinical (including phase II) trial results for several neurological conditions, most actively for PD (PSG et al., 2014) and stroke (Chamorro et al., 2014). The present findings implicating a discrete astrocytic antioxidant response signaling cascade in the protective actions of urate, in addition to its established but nonspecific direct antioxidant properties, strengthen the biological plausibility of its protective potential and support its further clinical development.

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Appendix P

REVIEW

Purines: forgotten mediators in traumatic brain injury

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Abstract

Recently, the topic of traumatic brain injury has gained attention in both the scientific community and lay press. Similarly, there have been exciting developments on multiple fronts in the area of neurochemistry specifically related to purine biology that are relevant to both neuroprotection and neurodegeneration. At the 2105 meeting of the National Neurotrauma Society, a session sponsored by the International Society for Neurochemistry featured three experts in the field of purine biology who discussed new developments that are germane to both the pathomechanisms of secondary injury and development of therapies for traumatic brain injury. This included presentations by Drs. Edwin Jackson on the novel 2′,3′-cAMP pathway in neuroprotection, Detlev Boison on adenosine in post-traumatic seizures and epilepsy, and Michael Schwarzschild on the potential of urate to treat central nervous system injury. This mini review summarizes the important findings in these three areas and outlines future directions for the development of new purine-related therapies for traumatic brain injury and other forms of central nervous system injury.

Keywords: adenosine, cyclic-AMP, neuroprotection, seizure, urate, uric acid.

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In the last 5–10 years, the topic of traumatic brain injury (TBI) has garnered incredible attention in both the scientific community and lay press. This has resulted from the emerging recognition of the importance and consequences of both repetitive mild traumatic brain injury in the civilian population (repeated sports concussion) and blast-induced TBI in combat casualty care and terrorist attacks. The potential linkage of TBI to a variety of neurodegenerative diseases such as chronic traumatic encephalopathy, among others has further fueled this interest. There have been exciting developments in the field of purine biology that may have relevance to TBI. At the 2015 meeting of the National Neurotrauma Society, a session sponsored by the International Society for Neurochemistry featured three experts in the field of purine biology who discussed new developments

germane to the pathomechanisms and development of therapies in TBI. This included presentations by Drs. Edwin Jackson on the novel 2′,3′-cAMP pathway in neuroprotection, Detlev Boison on adenosine in post-traumatic seizures and epilepsy, and Michael Schwarzschild on the potential of urate to treat CNS injury.

The 2′,3′–cAMP pathway in neuroprotection

### Discovery of nucleoside 2′,3′-cyclic monophosphates

Liquid chromatography-tandem mass spectrometry is a powerful tool that couples the resolving capability of ultra-performance liquid chromatography with the sensitivity and specificity of tandem mass spectrometry. Jackson and coworkers measured, using liquid chromatography-tandem mass spectrometry, purines in the renal venous outflow from isolated, perfused rat kidneys (Ren *et al.* 2009). They observed a large chromatographic peak that was due to a 330 m/z precursor ion and 136 m/z product ion consistent with what would be expected for 3′,5′-cAMP. Surprisingly, however, the retention time of the compound was shorter than that for authentic 3′,5′-cAMP, thus eliminating the possibility that the signal was because of 3′5′-cAMP. They identified the unknown compound as adenosine 2′,3′-cAMP, and these studies were arguably the first unequivocal identification of 2′,3′-cAMP in any biological system. The discovery of 2′,3′-cAMP in the rat kidney was rapidly followed by other publications providing evidence for the existence in biological systems of not only 2′,3′-cAMP, but other nucleoside 2′,3′-cyclic monophosphates (2′,3′-cNMPs) (Pabst *et al.* 2010; Van Damme *et al.* 2012, 2014; Burhenne *et al.* 2013; Bähre and Kaever 2014; Bordeleau *et al.* 2014; Jia *et al.* 2014). It is now clear that there exist a family of

non-canonical cNMPs with 2′,3′-cyclic, rather than 3′,5′-cyclic, phosphodiester bonds (Fig. 1).

The 3′,5′-cAMP-adenosine pathway

Extracellular adenosine biosynthesis occurs via several pathways activated by diverse stimuli to produce adenosine for different purposes. The classical pathway is catalyzed by the ecto-enzyme CD39 working in tandem with the ecto-enzyme CD73; a pathway that produces adenosine in the interstitium as follows: ATP → ADP → 5′-AMP → adenosine. Hypoxia and inflammation activate the 'CD39/CD73 pathway' which produces large amounts of extracellular adenosine that restore tissue perfusion and down-regulate inflammation (Eltzschig 2009, 2013; Eltzschig and Carmeliet 2011; Eltzschig *et al.* 2012). The 'extracellular 3′,5′-cAMP-adenosine pathway' is another mechanism for producing adenosine in the interstitium. It involves: (i) the intracellular conversion of ATP to 3′,5′-cAMP by adenylyl cyclases; (ii) rapid export of 3′,5′-cAMP from cells by cyclic nucleotide transporters such as MRP4 (Cheng *et al.* 2010); (iii) conversion of extracellular 3′,5′-cAMP to 5′-AMP by ecto-3′,5′-cyclic nucleotide 3′-phosphodiesterases; and (iv) metabolism of extracellular 5′-AMP to adenosine by CD73 and tissue non-specific alkaline phosphatase (TNAP; an ecto-enzyme structurally similar to CD73). The extracellular adenosine produced by the extracellular 3′,5′-cAMP-adenosine pathway engages adenosine receptors to expand/modulate the initial effects of adenylyl cyclase activation. The first explicit formulation of the extracellular 3′,5′-cAMP-adenosine pathway was postulated in 1991 (Jackson 1991); evidence for this mechanism and the role of 3′,5′-cAMP as a '3rd messenger' is extensive (Mi *et al.* 1994; Mi and Jackson 1995, 1998; Dubey *et al.* 1996, 1998, 2000a,b, 2001, 2010;

Fig. 1 Chemical structures of nucleoside 2′, 3′-cyclic monophosphates (2′,3′-cNMPs). 2′, 3′-cAMP (center) was discovered to exist in biological systems in 2009. Subsequently, a number of other 2′, 3′-cNMPs were discovered including 2′, 3′-cUPM, 2′, 3′-cCMP, 2′, 3′-cGMP, 2′, 3′-cIMP. The absence of 2′, 3′-cTMP is consistent with the concept that all 2′, 3′-cNMPs derive from RNA, rather than DNA, degradation. That 2′, 3′-cNMPs exist in bacteria, plants, animals, and humans indicate that these molecules are ancient and conserved.
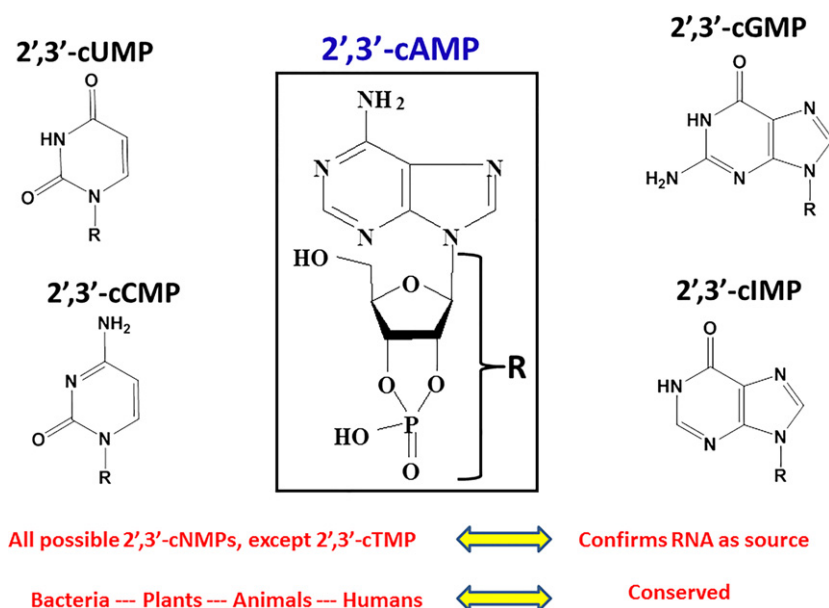
Jackson *et al.* 1997, 2003, 2006, 2007; Hong *et al.* 1999; Jackson and Mi 2000, 2008; Do *et al.* 2007; Chiavegatti *et al.* 2008; Giron *et al.* 2008; Müller *et al.* 2008; Kuzhikandathil *et al.* 2011; Duarte *et al.* 2012; Sciaraffia *et al.* 2014).

The 2′,3′-cAMP-adenosine pathway

Since biological systems can express an extracellular 3′,5′-cAMP-adenosine pathway, Jackson and coworkers (Jackson *et al.* 2009) considered whether an analogous 'extracellular 2′,3′-cAMP-adenosine pathway' may also exist: intracellular synthesis of 2′,3′-cAMP→ egress of 2′,3′-cAMP→ extracellular conversion of 2′,3′-cAMP to 2′-AMP plus 3′-AMP→ extracellular catabolism of 2′-AMP and 3′-AMP to adenosine. The rationale for this hypothesis was based on four observations. First, intracellular ribonucleases (RNases) degrade RNA by facilitating the hydrolysis of the P-O$^{5'}$ bond of RNA via transphosphorylation of RNA to yield 2′,3′-cNMPs (Wilusz *et al.* 2001). NMR spectroscopy (Thompson *et al.* 1994) demonstrated that 2′,3′-cNMPs formed by transphosphorylation of RNA are released form RNases as intact 2′,3′-cNMPs. Consequently, most 2′,3′-cNMPs, such as 2′,3′-cAMP, likely are formed from nucleotide bases in RNA via RNase catalyzed transphosphorylation (Thompson *et al.* 1994). Recent studies (Gu *et al.* 2013; Sokurenko *et al.* 2015) using a variety of approaches reveal a detailed molecular mechanism for 2′,3′-cNMP biosynthesis from RNA. Inasmuch as mRNA has a large number of adenosine monophosphates in the poly-A tail (Alberts *et al.* 1989), mRNA degradation can generate large amounts of 2′,3′-cAMP.

Second, nucleotide transporters, for example, MRP4 and MRP5, both rapidly and actively export a diverse number of nucleotides (linear and cyclic), into the extracellular space (Kruh *et al.* 2001; van Aubel *et al.* 2002; Deeley *et al.* 2006; Borst *et al.* 2007). Likely then nucleotide transporters would also export 2′,3′-cAMP; release of endogenous 2′,3′-cAMP into the renal circulation (Jackson *et al.* 2009, 2011a; Ren *et al.* 2009) unquestionably indicates that 2′,3′-cAMP reaches the extracellular compartment.

Third, there exist enzymes that could catalyze the pathway. For example, 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) can metabolize 2′,3′-cAMP to 2′-AMP *in vitro* (Vogel and Thompson 1988; Sprinkle 1989; Thompson 1992). Some secreted RNases can hydrolyze 2′,3′-cAMP to 3′-AMP (Sorrentino and Libonati 1997; Sorrentino 1998). Rao *et al.* (2010) report that six different phosphodiesterases containing three different families of hydrolytic domains can generate 3′-AMP from 2′,3′-cAMP. Consistent with the existence of ecto-2′,3′-cyclic nucleotide 2′-phosphodiesterases and ecto-2′,3′-cyclic nucleotide 3′-phosphodiesterases are the findings that: 1) 3′-AMP is present in rat spleen, kidney, liver, heart, and brain (Bushfield *et al.* 1990; Fujimori and Pan-Hou 1998; Fujimori *et al.* 1998; Miyamoto *et al.* 2008); and 2) 2′-AMP and 3′-AMP are present in human cerebrospinal fluid (CSF)

and their concentrations correlate with the concentrations of 2′,3′-cAMP (Verrier *et al.* 2012).

Fourth, there are ecto-nucleotidases that process extracellular 2′-AMP and 3′-AMP to adenosine. Ohkubo *et al.* (2000)showed that in NG108-15 cells TNAP can release orthophosphate from 2′-AMP or 3′-AMP. Moreover, in kidneys TNAP metabolizes 2′-AMP and 3′-AMP to adenosine (Jackson, E.K.; unpublished observations). Also, adenosine levels in human CSF correlate with those of 2′,3′-cAMP, 2′-AMP, and 3′-AMP (Verrier *et al.* 2012) – consistent with the metabolism of these compounds to adenosine in the extracellular compartment.

Jackson and coworkers confirmed the existence of the extracellular 2′,3′-cAMP-adenosine pathway. In kidneys, arterial infusions of 2′,3′-cAMP dramatically increased renal venous 3′-AMP, 2′-AMP, and adenosine (Jackson *et al.* 2009, 2011a); and infusions of 2′-AMP and 3′-AMP augmented secretion of adenosine similar to that achieved by 5′-AMP (prototypical adenosine precursor). Energy depletion can activate the degradation of RNA (Akahane *et al.* 2001a,b; Almeida *et al.* 2004) and should engage the extracellular 2′,3′-cAMP-adenosine pathways. As predicted, treatment of kidneys with metabolic inhibitors increased renal venous 2′,3′-cAMP, 2′-AMP, 3′-AMP, and adenosine (Jackson *et al.* 2009, 2011a). The extracellular 2′,3′-cAMP-adenosine pathway may exist in many cells, tissues, and organs. For example, pre-glomerular vascular smooth muscle cells (Jackson *et al.* 2010), pre-glomeular vascular endothelial cells (Jackson and Gillespie 2012), glomerular mesangial cells (Jackson *et al.* 2010), renal epithelial cells (Jackson and Gillespie 2012, 2013), aortic vascular smooth muscle cells (Jackson *et al.* 2011b), coronary artery vascular smooth muscle cells (Jackson *et al.* 2011b), microglia (Verrier *et al.* 2011), astrocytes (Verrier *et al.* 2011), oligodendrocytes (Verrier *et al.* 2013), neurons (Verrier *et al.* 2013), Schwann cells (Verrier *et al.* 2015), and intact brain *in vivo* (Verrier *et al.* 2012) metabolize exogenous 2′,3′-cAMP, 2′-AMP, and 3′-AMP to downstream purines.

CNPase and the role of the 2′,3′-cAMP-adenosine pathway in neurotrauma

CNPase is the most abundant protein in non-compact CNS myelin, and is the 3rd most abundant protein overall in CNS myelin (Raasakka and Kursula 2014). Yet, the role for CNPase remained an enigma from the time of its discovery (Whitfeld *et al.* 1955) until the discovery of 2′,3′-cAMP in rat kidneys in 2009 (Ren *et al.* 2009). Before 2009, 2′,3′-cAMP was not known to exist in biological systems; therefore the ability of CNPase to convert 2′,3′-cAMP to 2′-AMP *in vitro* was viewed as an epiphenomenon (Vogel and Thompson 1988; Thompson 1992; Schmidt 1999). With the discovery of 2′,3′-cAMP pathway, the role of the enzymatic activity of CNPase is being reconsidered (Raasakka and Kursula 2014).

Jackson and coworkers (Verrier *et al.* 2012) were the first to reveal an important role for the enzymatic activity of CNPase. Employing microdialysis to infuse 2′,3′-cAMP and 2′-AMP into the extracellular compartment of the mouse brain they demonstrated that the brain converts exogenous 2′,3′-cAMP to 2′-AMP and adenosine and metabolizes exogenous 2′-AMP to adenosine. Notably the increase in interstitial levels of 2′-AMP and adenosine following delivery of 2′,3′-cAMP to the brain interstitial compartment caused significant increases in 2′-AMP and adenosine within 30 min. Similarly the increase in interstitial levels of adenosine following delivery of 2′-AMP also occurred within 30 min. However, the conversion of exogenous 2′,3′-cAMP to 2′-AMP was attenuated in brains from CNPase knockout (KO) mice. In wild-type mice, TBI increased brain interstitial levels of 2′,3′-cAMP, 2′-AMP, 3′-AMP, adenosine, and inosine (adenosine metabolite) within a time frame of 30 min. In CNPase KO mice, TBI induced higher levels of interstitial 2′,3′-cAMP, yet lower levels of 2′-AMP, adenosine, and inosine. Thus, deficiency of CNPase impairs the 2′,3′-cAMP-adenosine pathway (Verrier *et al.* 2012) and activity of CNPase importantly participates in this system. Furthermore, histology suggested greater hippocampal neuronal injury in CNPase KO versus wild-type and functional outcomes were worse in CNPase KO. This is consistent with observations by others that CNPase KO mice have enhanced astrogliosis, microgliosis, axon degeneration, and defects in working memory following brain injury (Wieser *et al.* 2013); and an aging associated psychiatric disease (catatonia-depression syndrome) (Hagemeyer *et al.* 2012).

Neuroprotective mechanism of CNPase and the 2′,3′-cAMP-adenosine pathway
Adenosine is neuroprotective (Kochanek *et al.* 2013) and 2′,3′-cAMP rapidly (within min) opens mitochondrial permeability transition pores (mPTPs) (Azarashvili *et al.* 2009, 2010) possibly triggering apoptosis, necrosis, and autophagy (mitophagy). Therefore, metabolism of endogenous 2′,3′-cAMP to 2′-AMP by CNPase would rid the brain cells of an intracellular neurotoxin (2′,3′-cAMP), whereas metabolism of 2′-AMP and 3′-AMP to adenosine would provide a neuroprotectant/anti-inflammatory agent (adenosine). With regard to inflammation, 2′,3′-cAMP, 3′-AMP, and 2′-AMP inhibit the release of proinflammatory TNF-α and CXCL10 in murine microglia via production of adenosine leading to activation of A_{2A} receptors (Newell *et al.* 2015). It is important to note that the effects of brain injury on components of the 2′,3′-cAMP-adenosine pathway occurs within 30 min. Thus, the pathway is activated in a time frame rapid enough to affect early events following brain injury. Indeed, in TBI patients, CSF levels of 2′,3′-cAMP are increased for 12 h after injury and correlate with CSF 2′-AMP, 3′-AMP, adenosine, and inosine (Verrier *et al.* 2012).

These clinical findings suggest that the 2′,3′-cAMP-adenosine pathway is engaged post-TBI in humans in a time frame consistent with affecting early events after injury. Although activation of the 2′,3′-cAMP-adenosine pathway requires RNA metabolism, this does not mean that the pathway is activated only in dying cells. In addition, it would be expected that even cells destined to die could produce extracellular adenosine via the 2′3′-cAMP-adenosine pathway, and this adenosine could increase the survival rate of surrounding cells via paracrine effects.

Studies by Lappe-Siefke *et al.* (2003) suggested that myelin and axonal morphology are normal in CNPase$^{-/-}$ mice up to about 3.5 months of age. Then axonal pathology begins to emerge and gradually increases with age. In aged CNPase$^{-/-}$ mice, the main axonal pathology is axonal swellings, whereas the myelin sheath remains relatively normal (only minor changes at the paranodal regions). However, subsequent studies by Edgar *et al.* (2009) indicated early changes (i.e., swelling) in the inner tongue of the myelin sheath in paranodal regions of small (but not large) axons associated with some degeneration of small axons. However, these changes were not associated with a significant reduction in axon number until about 6 months of age. In preliminary studies, we examined with transmission electron microscopy white matter tracts of CNPase KO mice and CNPase wild-type mice that were about 3 months old. We did not detect any changes in axonal morphology, autophagosome number and area or mitochondrial number and area. Because our TBI experiments were performed in CNPase KO mice that were about 3 months of age, it is unlikely that background white matter pathology accounted for the differential response to TBI in CNPase KO versus CNPase wild-type mice. Although, we cannot completely rule out this possibility, it is important to consider that subtle changes in background axonal health may indeed be mediated by chronic deficiency of the 2′,3′-cAMP-adenosine pathway. That is to say, not only may acute changes in the pathway determine the response to an acute injury, it is conceivable that chronic deficiency causes underlying pathology that determines the response to acute TBI as well as the risk of neurodegenerative processes such as chronic traumatic encephalopathy.

Brain cells that mediate the 2′,3′-cAMP-adenosine pathway
The aforementioned findings suggest that: (i) the 2′,3′-cAMP-adenosine pathway exists *in vivo* in the CNS of mice and humans; (ii) brain CNPase converts endogenously generated 2′,3′-cAMP to 2′-AMP; and (iii) the 2′,3′-cAMP-adenosine pathway and CNPase are neuroprotective. What CNS cell types mediate the 2′,3′-cAMP-adenosine pathway? Although astrocytes, microglia, oligodendrocytes, and neurons all can metabolize 2′,3′-cAMP to 2′-AMP, oligodendrocytes are pre-eminent in this regard (Verrier *et al.* 2013); likely because oligodendrocytes are enriched in CNPase. In oligodendrocytes from CNPase KO mice, the metabolism of 2′,3′-cAMP to 2′-AMP is impaired (Verrier *et al.* 2013). In

contrast, microglia are the most efficient at converting 2′-AMP to adenosine (Verrier *et al.* 2011). Although brain injury increases extracellular 2′,3′-cAMP levels, the major sources of 2′,3′-cAMP have yet to be identified. Likely a collaboration among CNS cell types is required to constitute a complete brain 2′,3′-cAMP-adenosine pathway.

Summary

Evidence is mounting that 2′,3′-cAMP is an important molecule that is metabolized to adenosine. TBI activates the 2′,3′-cAMP-adenosine pathway, and this mechanism is a determinant of outcome. The challenge going forward is to discover ways to manipulate this pathway to benefit patients after TBI. In this regard, there are a number of feasible strategies for using the knowledge generated by studying the 2′,3′-cAMP-adenosine pathway in the brain to treat TBI. For example, using the structure of 2′,3′-cAMP as a starting point, it is feasible to develop antagonists that block the effects of 2′,3′-cAMP on mPTPs thus preventing 2′,3′-cAMP-induced apoptosis, necrosis, and autophagy (mitophagy). Also, inhibitors of RNases that manufacture 2′,3′-cAMP could be developed to temporarily reduced 2′,3′-cAMP production. Other approaches would be to induce the expression (with pharmacological agents) of transporters that mediate cellular egress of 2′,3′-cAMP, CNPase, or TNAP so as to increase the rate at which 2′,3′-cAMP is exported and converted to adenosine. In addition to treating TBI, polymorphisms in CNPase, the relevant transport proteins, and TNAP may serve to identify individuals susceptible to TBI so that they can be advised not to participate in contact sports or other activities that increase the risk of TBI or chronic traumatic encephalopathy.

Role of adenosine in post–traumatic seizures and epilepsy

Post-traumatic epilepsy accounts for ~ 10–20% of all symptomatic epilepsies in the general population (Englander *et al.* 2003). Predicting persons who might develop epilepsy and preventing its development are consequently of utmost importance. Adenosine is a well-known endogenous anticonvulsant and seizure terminator. Since adenosine deficiency, caused by enhanced metabolic clearance through reactive astrocytes and over-expression of the adenosine removing enzyme adenosine kinase (ADK), is a hallmark of epilepsy, therapeutic adenosine augmentation is a rational approach to suppress seizures in the epileptic brain (Boison and Aronica 2015; Boison *et al.* 2002). Seizure suppression by adenosine is mediated by increased activation of adenosine A_1 receptors, whereas a lack of A_1 receptors is associated with lethal seizures after exposure of the brain to trauma or an excitotoxin (Fedele *et al.* 2006; Kochanek *et al.* 2006). Although the receptor-dependent effects of adenosine are well characterized and have been the subject of drug development efforts (Chen *et al.* 2013a,b) new findings demonstrate that adenosine has additional, adenosine receptor independent, unprecedented properties to prevent the development of epilepsy through an epigenetic mechanism.

Epileptogenic brain areas in chronic epilepsy, in the clinic and in rodent models, are characterized by over-expression of ADK in reactive astrocytes (Aronica *et al.* 2011; Boison 2012) and a hypermethylated state of DNA (Kobow *et al.* 2013; Williams-Karnesky *et al.* 2013; Miller-Delaney *et al.* 2015). As stated in the 'methylation hypothesis of epileptogenesis' originally proposed by Kobow and Blumcke (2011) seizures by themselves may induce epigenetic chromatin modifications, thereby aggravating the epileptogenic condition. Consequently, hypermethylation of DNA was considered a driving force for the progression of epilepsy (Kobow and Blumcke 2012). DNA methylation is an epigenetic modification whereby *S*-adenosylmethionine (SAM) contributes a methyl group to the formation of 5-methylcytosine bases in the DNA. This leads to the formation of *S*-adenosylhomocysteine (SAH), which is cleaved by SAH hydrolase into adenosine and homocysteine. Since the thermodynamic equilibrium of the SAH hydrolase reaction is on the side of SAH formation and since SAH is a product inhibitor of DNA methyltransferases (DNMTs), DNA methylation can only occur if adenosine is effectively removed by ADK. Consequently, increased expression of ADK, as occurs in epilepsy, drives increased DNA methylation, whereas therapeutic adenosine augmentation blocks DNA methylation and induces a hypomethylated status of DNA (Williams-Karnesky *et al.* 2013) (Fig. 2).

If increased DNA methylation is functionally implicated in epilepsy progression, then therapeutic adenosine augmentation, by reducing the methylation status of DNA should block epileptogenesis. To test this hypothesis we used a rat model of status epilepticus-induced progressive temporal lobe epilepsy and silk-based brain implants engineered to release a defined dose of adenosine (250 ng adenosine/day/per ventricle) only transiently for 10 days (Williams-Karnesky *et al.* 2013). Transient drug use followed by a drug-free 'washout' period is a standard strategy to distinguish between acute antiictogenic effects of a drug and longer lasting antiepileptogenic effects (Silver *et al.* 1991). Adenosine-releasing polymers, or corresponding silk-only control rods, were implanted into the lateral brain ventricles of rats after the onset of epilepsy. Compared to naïve controls, hippocampal DNMT activity was elevated in the epileptic controls prior to the adenosine delivery, whereas local silk-based adenosine delivery almost completely abrogated any DNMT activity. Consistent with those findings, DNA in the epileptic controls was hypermethylated versus healthy controls, however, the transient delivery of adenosine for only 10 days reverted the DNA methylation status in the epileptic animals back to normal; Importantly, normal methylation was maintained even weeks after cessation of adenosine release from the polymer. To assess epilepsy progression following silk-polymer implantation, the animals
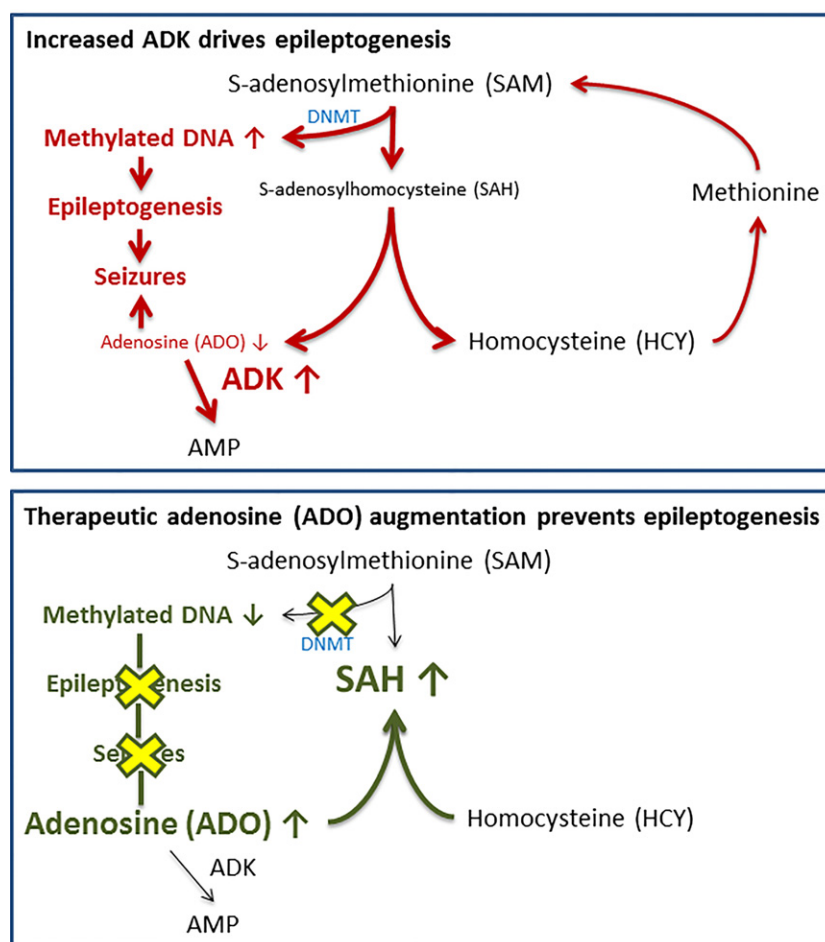
Fig. 2 The epigenetics of epileptogenesis. Increased adenosine kinase (ADK) expression (top) drives increased DNA methylation as a prerequisite for progressive epileptogenesis and seizure generation. Conversely, adenosine therapy (bottom) restores normal DNA methylation preventing epileptogenesis. ADO, adenosine; ADK, adenosine kinase; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; DNMT, DNA-methyltransferase.

were monitored for an additional 3 months after expiration of active adenosine delivery. Sham treated controls and those that received control silk implants progressed in frequency and severity of seizures. Conversely, animals receiving a transient dose of adenosine for 10 days did not progress further in epilepsy development. Three month after treatment the seizure rate stabilized at ~ 2 per week, whereas controls progressed to at least eight seizures per week and some controls died from excessive seizure activity. Consistent with those findings a transient dose of adenosine halted mossy fiber sprouting, a characteristic marker for epileptogenesis. Methylated DNA immunoprecipitation arrays and bisulfite sequencing revealed distinct sets of genes whose methylation status increased during epileptogenesis and was corrected by adenosine therapy. Among the targets with reduced DNA methylation during adenosine therapy several interact with DNA, or play a role in gene transcription or translation (*PolD1, Polr1e, Rps6kl1, Snrpn, Znf524, Znf541, Znf710*), making them candidates for mediating adenosine-dependent changes in major homeostatic functions (Williams-Karnesky *et al.* 2013). Further research into epigenetically regulated antiepileptogenic mechanisms may reveal transcriptional activators as epigenetic meta-regulators such as those linked to the mTOR pathway (Cho 2011). In further support of an antiepileptogenic role of hypermethylated DNA, we kindled rats in the presence of the DNMT inhibitor 5-Aza2dC (5AZA). Under those conditions, kindling epileptogenesis was suppressed, and when re-stimulated after an 11 day drug-free washout period, rats kindled in the presence of 5AZA showed a robust reduction in the seizure phenotype (stage 3 instead of stage 5 seizures) compared to control animals kindled in the absence of 5AZA. Thus, changes in DNA methylation patterns are a key determinant of epilepsy progression and adenosine augmentation may reverse DNA hypermethylation and break the cycle of increasing seizure severity (Williams-Karnesky *et al.* 2013).

On the basis of our new findings, we propose an amended version of our original 'ADK hypothesis of epileptogenesis' (Boison 2008) by including a biphasic response of the DNA methylome in response to an epileptogenesis triggering insult: Acute DNA hypomethylation, also seen within 24 h after TBI and associated with microglial activation (Zhang *et al.* 2007), may contribute to the initiation of epileptogenesis, whereas chronic DNA hypermethylation associated with astroglial activation and adenosine deficiency (Williams-Karnesky *et al.* 2013) might be required to

maintain the epileptic state and to promote disease progression; this biphasic response may be directly related to biphasic expression changes of ADK during the course of epileptogenesis (Boison 2008; Li *et al.* 2008; Williams-Karnesky *et al.* 2013). We acknowledge that at this time our hypothesis is largely based on correlative evidence. Although key data, such as long-term epigenetic and antiepileptogenic effects of a short-term adenosine dose and antiepileptogenic activity of a conventional DNMT inhibitor, strongly support a causal relationship between increased DNA methylation and increased epileptogenesis, more research is needed to identify relevant epigenetic targets and mechanisms.

Intriguingly, genetic variants of ADK were associated with the development of post-traumatic epilepsy in humans (Diamond *et al.* 2015). Therefore, changes in adenosine metabolism, such as those triggered by pathological over-expression of ADK or by genetic mutations, emerge as an attractive biomarker for the prediction of epileptogenesis and a therapeutic target to prevent post-traumatic epilepsy.

Rehabilitating urate – the maligned and forgotten purine

Urate's generally bad reputation

Urate (a.k.a. uric acid) is often referred to as a waste product of purine metabolism (Johnson *et al.* 2009; Rock *et al.* 2013). It circulates at high levels in humans and other hominoids because of mutations in the gene encoding the urate-catabolizing enzyme urate oxidase during primate evolution (Wu *et al.* 1992; Oda *et al.* 2002). In our species its circulating concentrations are so high they approach the limits of solubility. Urate is best known clinically for the pain and damage that results when these limits are exceeded and urate crystallizes. When this occurs in joints it results in gout, a form of inflammatory arthritis triggered by urate crystals. Similarly, when urate (or more typically its acid form, uric acid) crystallizes in the urine then it can cause kidney stones.

In addition to its direct, causal contributions to these crystallopathic disorders, higher blood levels of urate have been found to carry an increased risk of heart disease, hypertension, kidney disease and diabetes (Feig *et al.* 2008; Edwards 2009; Johnson *et al.* 2013). Although these adverse associations are partially explained by other co-morbidities of elevated urate such as obesity (Palmer *et al.* 2013), they have fostered concerns that higher urate may mediate as well as mark major classes of human disease. The advent of multiple new urate-lowering therapies may be adding to the unfavorable image of urate even in the absence of gout or stones (Gaffo and Saag 2012; Bach and Simkin 2014; Borghi *et al.* 2014).

Urate's protective potential for Parkinson's and other neurodegenerative diseases

Despite these known and theoretical adverse effects of higher urate levels, the evolutionary biology and biochemistry of urate have suggested that its salubrious actions may offset and possibly outweigh is detrimental effects (Álvarez-Lario and Macarrón-Vicente 2010). Because the urate-elevating inactivation of the urate oxidase enzyme in chimpanzees, gorillas and humans can be attributed to multiple independent mutations in urate oxidase during the speciation of primates (Wu *et al.* 1992; Oda *et al.* 2002), it is reasonably presumed that urate elevation conveyed a critical survival advantage to our ancestors. The discovery that urate possesses strong antioxidant properties, with a comparable or greater activity than ascorbate at their physiological concentrations in humans (Ames *et al.* 1981), suggested potential benefits of protection against oxidative stress.

The findings for urate's antioxidant actions converged with evidence that neurodegenerative diseases like Parkinson's disease (PD) result from excessive oxidative damage to neurons (Jenner 2003). They hypothesized that higher levels of urate may help protect the brain from PD and prompted epidemiologists to investigate the relationship between blood urate levels and the risk of PD. Studies of prospectively followed healthy cohorts have repeatedly demonstrated that higher but 'normal' blood urate among healthy individuals conveys a reduced risk for developing PD later in life in men (Davis *et al.* 1996; de Lau *et al.* 2005; Weisskopf *et al.* 2007; Chen *et al.* 2009), with findings less consistent in women (O'Reilly *et al.* 2010; Jain *et al.* 2011) who have lower serum urate levels on average. For example, men in the top quartile of plasma urate levels had a significantly (55%) lower risk of later developing PD than men in the bottom quartile in a rigorously followed cohort of health professionals (Weisskopf *et al.* 2007). The decrease in risk was even greater (with an 80% PD risk reduction in highest compared to the lowest quartile; $p < 0.01$ for trend) in those with blood collected at least 4 years before diagnosis, suggesting that the lower urate in those who develop PD precedes symptom onset and is thus unlikely to be a consequence of changes in diet, activity or medical treatment early in the clinical course of PD. Complementary epidemiological findings that a urate-elevating diet (Gao *et al.* 2008) and gout (Alonso *et al.* 2007; De Vera *et al.* 2008) are also associated with a lower risk of PD strengthen the link. Similarly, many cross-sectional studies have reported lower urate levels (Bakshi *et al.* 2015b) or urate-lowering genotypes (Gonzalez-Aramburu *et al.* 2013) are more likely in PD than controls. The link appears robust as it has been demonstrated across nationalities and races (Jesus *et al.* 2012; Sun *et al.* 2012), and in community (Winquist *et al.* 2010) as well as academic center-based (Cipriani *et al.* 2010) cohorts.

This epidemiological association between urate and PD *risk* in healthy populations prompted investigation of whether urate might also be linked to PD *progression* among those already diagnosed with PD. In multiple prospectively followed PD cohorts, higher blood (or CSF)

urate was strongly associated with a slower clinical progression (Ascherio *et al.* 2008; Schwarzschild *et al.* 2009; Moccia *et al.* 2015). A similarly significant, inverse association between serum urate and subsequent rates of radiographic progression was measured by serial measurement of dopamine transporter binding sites from the striatum using dopamine transporter brain scan imaging (Schwarzschild *et al.* 2009). Similarly, lower urate levels have been linked to the development or more rapid progression of other neurodegenerative diseases including amyotrophic lateral sclerosis and Huntington's disease (Bakshi *et al.* 2015b).

Pre-clinical studies of pharmacological (Gong *et al.* 2012) or genetic (Chen *et al.* 2013a,b) strategies to elevated brain urate levels in animal models of PD provided biological evidence of protection by urate against the dopaminergic neuron degeneration characteristic of PD. Interestingly, an indirect antioxidant effect of urate, via its activation of the Nrf2 antioxidant response pathway in astrocytes (Zhang *et al.* 2014; Bakshi *et al.* 2015a), may account for much of urate's protection potential in PD.

Together these epidemiological, clinical and neurobiological data identified urate as a promising molecular biomarker and possible mediator of favorable clinical progression of PD. They prompted an initial clinical trial of the urate precursor inosine as a potential urate-elevating strategy for disease modification in PD. The Safety of Urate Elevation in PD (SURE-PD) study (Parkinson Study Group SURE-PD Investigators *et al.* 2014) assessed three primary outcomes for safety, tolerability and urate-elevating ability of oral inosine in early PD. Despite known risks of gout and uric acid kidney stones, inosine demonstrated overall good safety and tolerability and significant elevation of both CSF and serum urate, supporting further clinical development of inosine for PD.

Prospect for protection against acute neuronal injury: from stroke to TBI

Urate elevation has emerged as a neuroprotective strategy in acute neuronal injury, as well as in chronic neuronal degeneration. As with targeting urate in PD, that in stroke has undergone rapid translation to phase 2/3 clinical trials based on a combination of laboratory and clinical data. Building on its well-established antioxidant properties (Ames *et al.* 1981), stroke biologists administered urate just before or during transient unilateral cerebral ischemia and found reduced striatal or cortical damage as well as preserved neurological function (Yu *et al.* 1998; Romanos *et al.* 2007). Clinical epidemiology studies found that people with higher serum urate levels when presenting with an ischemic stroke had better clinical outcomes upon hospital discharge ~2 weeks later (Chamorro *et al.* 2002).

Based on these human and animal data Chamorro *et al.* (2014) conducted a phase 2b/3 trial of intravenous urate in acute ischemic stroke. Although it did not demonstrate a statistically significant overall benefit of urate, it was sufficiently suggestive to warrant for fuller phase 3 clinical testing. Interestingly, post hoc analysis stratifying by gender indicated significantly better anatomical and clinical outcomes after urate treatment in women, who at baseline have substantially lower serum urate levels than do men (Llull *et al.*, 2015). Interestingly, this sex difference appeared to be mirrored for PD in the SURE-PD study (Parkinson Study Group SURE-PD Investigators *et al.* 2014; Schwarzschild *et al.* 2014) and warrants further attention in future studies.

TBI like stroke entails a sudden profound metabolic (as well as mechanical) injury of neurons, and thus may trigger a common cascades of excitotoxic, inflammatory and oxidative factors that contribute to functional disability. Thus the rapid clinical translation and potential of urate as a therapeutic target in stroke as well as neurodegenerative disease warrants consideration of its 'lateral translation' to TBI. Although urate itself has not been systematically investigated in TBI models, its precursor inosine (which rapidly metabolized to urate, and is currently in clinical development for PD) has been found to improve outcomes in rodent models of TBI (Dachir *et al.* 2014) and spinal cord injury (SCI) (Kim *et al.* 2013). Evidence that astrocytic Nrf2 pathway activation confers protection against neuronal cell death in TBI/SCI (Mao *et al.* 2012; Miller *et al.* 2014) and neurodegeneration, and that urate confers protection in PD models via this pathway (Zhang *et al.* 2014; Bakshi *et al.* 2015a), lends support to the rationale for investigating urate in TBI and SCI.

Alternatively, inosine may have direct beneficial effects independent of its metabolism to urate (Cipriani *et al.* 2014) as it can also protect via extracellular engagement of adenosine receptors (Gomez and Sitkovsky 2003; Shen *et al.* 2005) and intracellular activation of the Mst3b signaling cascade (Kim *et al.* 2013). However, the metabolism of inosine to urate by way of peripheral xanthine oxidase could in theory generate deleterious oxidative stress via its hydrogen peroxide byproduct (Kelley *et al.* 2010), potentially offsetting some of its putative benefits. Thus, in traumatic CNS injury, as in acute stroke, the intravenous administration of urate itself may be the most effective as well as expedient strategy to take advantage of its benefits. Urate elevation represents a readily testable candidate neuroprotective strategy across disorders of neurodegeneration and acute neuronal injury.

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Nevertheless, these results are important for research that recruits PD patients with borderline or MCI. They suggest the need to attend to deficits in memory and executive function, given that subtle disruption in these areas increases the likelihood that a patient is not capable of giving consent. Indeed, people with PD MCI may also have reduced awareness of executive function impairment, potentially compromising safety and judgment in naturalistic settings.[15] In such cases, study precautions should be considered, including a structured assessment of capacity and asking the patient to designate a study partner. We remind readers and investigators that a brief measure of executive function, such as the DRS-2 *Initiation-Perseverance* subscale, *Visuospatial/Executive* subscale of the MoCA, or even a brief screening instrument such as the MoCA, is not a sufficient measure of capacity. Low performance on these scales, however, may serve as a prompt to consider additional protections to guard against the possibility of mistakenly judging a patient who is not capable as capable. ●

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Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Oral Inosine Persistently Elevates Plasma Antioxidant Capacity in Parkinson's Disease

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ABSTRACT

Introduction: Higher serum urate predicts slower progression in PD. The aim of this work was to assess whether oral inosine alters antioxidant capacity of plasma or CSF or urinary markers of oxidative injury in early PD.
Methods: We assayed plasma and CSF antioxidant capacity by ferric-reducing antioxidant power and measured DNA oxidation adduct 8-hydroxydeoxyguanosine from urine in Safety of URate Elevation in PD, a randomized, placebo-controlled trial of oral inosine assessing safety of elevating serum urate from <6 mg/dL to 6.1–7.0 or 7.1–8.0 mg/dL in patients with early PD.
Results: At 6 months, antioxidant capacity was 29% higher among mild and 43% higher among moderate group participants compared to placebo and correlated with change in serum urate ($r = 0.86$) and inversely with rate of clinical decline ($r = -0.26$). CSF antioxidant capacity and urine 8-hydroxydeoxyguanosine did not differ.

Conclusions: The findings demonstrate a dose-dependent, persistent elevation of plasma antioxidant capacity from oral inosine of potential therapeutic relevance. © 2016 International Parkinson and Movement Disorder Society

Key Words: Parkinson's disease; antioxidant; uric acid/urate; inosine

Higher levels of serum urate are a predictor of decreased incidence of Parkinson's disease (PD) and slower progression in early PD.[1-6] Whether these associations reflect a causally protective role of urate is unclear. In rodent models of PD, raising or lowering urate levels protects or exacerbates PD phenotypes, respectively.[7,8] In human beings, many characteristic biochemical features of PD, such as mitochondrial dysfunction, decreased nigral glutathione levels, and increased nigral iron load, are associated with increased oxidative stress.[9-11] Urate, which has both direct and indirect antioxidant effects, is hypothesized to be neuroprotective in PD by alleviating oxidative nigral injury.[12,13]

The Safety of URate Elevation in PD (SURE-PD) study was a randomized, double-blind, placebo-controlled phase II trial testing the safety, tolerability, and feasibility of raising serum and cerebrospinal fluid (CSF) urate with inosine (an orally bioavailable metabolic precursor of urate) in patients with early PD not requiring symptomatic therapy.[14] The results showed that inosine was well tolerated and safely raised serum and CSF urate levels.[14] In this exploratory biomarker study, we report on ferric-reducing antioxidant power (FRAP; a measure of antioxidant capacity) in plasma and CSF and on 8-hydroxydeoxyguanosine (8-OHdG; a measure of nucleic acid oxidative injury) in urine.[15,16]

Patients and Methods
Biospecimens

As detailed previously,[14] the SURE-PD trial enrolled 75 patients with early PD not yet requiring symptomatic antiparkinsonian treatment (except for a stable

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dose of monoamine oxidase type B inhibitor) with serum urate <6 mg/dL. They were randomized at 16 sites in 1:1:1 distribution to three treatment groups: (1) placebo, or oral inosine titrated to (2) mildly elevated serum urate (6.1–7.0 mg/dL), or (3) moderately elevated serum urate (7.1–8.0 mg/dL). Inosine dosing was adjusted based on serum urate at scheduled study visits. Participants remained on study drug for up to 24 months (average, 18; range, 9–24). Plasma was collected in heparinized tubes at baseline, 6-month-visit, and final visit on drug. Urine was collected at baseline and 6-month-visit. CSF was collected once at 12-week visit. Samples were obtained with institutional review board–approved consent procedures and frozen at –80°C. Investigators were blinded to treatment assignments.

FRAP Assay

Antioxidant capacity was measured by FRAP, as previously described.[17] In this colorimetric assay, when ferric tripyridyltriazine (Fe(III)-TPTZ) complex is reduced to the ferrous form by the added sample, a blue color develops—absorption at 560 nm is proportional to the degree of antioxidant power of the sample. Antioxidant capacity is expressed as equivalent concentrations of the standard ferrous (II) chloride (ranging from 0.03 to 1.0 mM). Plasma and CSF (sixth 3-mL lumbar puncture collection tube) were assayed in triplicate in three 96-well assay plates with interassay coefficient of variation (CV) <5%.

Urine 8-OHdG

The oxidative DNA adduct, 8-OHdG, was measured in urine by competitive enzyme-linked immunosorbent assay (ELISA; Japan Institute for the Control of Aging [JaICA], Fukuroi, Japan) and normalized to urinary creatinine, which was assayed using a colorimetric kit (R&D Systems, Minneapolis, MN). Each urine sample was measured in triplicate in three 96-well assay plates with CV <10%. 8-OHdG is expressed as ratio of concentrations of 8-OHdG and creatinine.

Statistical Analysis

Treatment- and visit-specific mean biomarker levels were estimated from shared-baseline linear mixed models with fixed effects for visit and treatment × postbaseline visit interaction and random participant-specific intercepts and slopes with unstructured covariance. Linear contrasts were used to test for treatment-dependent differences in mean change from baseline. Participant-specific rates of change of UPDRS III motor scores were estimated from a similar shared-baseline, random-slopes, mixed-effects model with time treated as continuous and censoring observations after initiation of dopaminergic therapy. Associations

TABLE 1. Baseline characteristics of participants with plasma FRAP measurements

	Overall (n = 68)	Placebo (n = 23)	Mild (n = 21)	Moderate (n = 24)
Age in years (SD)	61.5 (10.7)	60.8 (11.4)	62.1 (10.1)	61.7 (10.8)
Male (n)	45.1% (32)	50.0% (12)	36.4% (8)	48.0% (12)
UPDRS Part III motor score (SD)	15.8 (7.6)	17.1 (8.3)	14.7 (7.2)	15.6 (7.4)
UPDRS Parts I–III total score (SD)	21.8 (9.7)	23.5 (10.4)	20.4 (9.1)	21.4 (9.7)
Years of symptoms (SD)	2.4 (1.8)	2.3 (1.4)	2.7 (2.0)	2.2 (2.0)
Diabetes mellitus (n)	4.2% (3)	4.2% (1)	0.0% (0)	8.0% (2)
Smoker, ever (n)	32.4% (23)	33.3% (8)	36.4% (8)	28.0% (7)
Mean systolic BP in mm Hg (SD)	130 (14.2)	127 (15.0)	131 (13.5)	132 (14.0)
BMI in kg/m^2 (SD)	27.5 (5.2)	28.1 (5.3)	27.5 (6.5)	27.0 (3.9)
Baseline serum creatinine in mg/dL (SD)	0.82 (0.14)	0.84 (0.15)	0.82 (0.13)	0.81 (0.15)
Baseline FRAP in mmol Fe(II)/L (SD)	0.84 (0.13)	0.82 (0.12)	0.84 (0.17)	0.86 (0.11)
Baseline urine 8-OHdG in ng/mg creatinine (SD)	11.0 (4.2)	10.7 (3.6)	10.9 (2.8)	11.3 (5.6)
Baseline serum urate in mg/dL (SD)	4.47 (0.76)	4.63 (0.57)	4.31 (0.92)	4.47 (0.74)
Baseline CSF urate in mg/dL (SD)	0.54 (0.18)	0.43 (0.19)	0.55 (0.15)	0.60 (0.18)

None of the characteristics differed for the mild or moderate group compared to placebo.
BMI, body mass index; BP, blood pressure; SD, standard deviation.

between baseline biomarker levels, changes in biomarkers levels from baseline, and biomarker levels and rates of change of UPDRS III motor scores were estimated as simple, unadjusted Pearson correlations. With the achieved sample size, the study had 80% power to detect correlations as small as $\rho = 0.33$ or 0.41 for blood or CSF biomarkers, respectively.

Results
Plasma FRAP

Baseline plasma FRAP values were available from 23 of 25 subjects in placebo, 21 of 24 in mild, and 24 of 26 in moderate groups. Baseline characteristics were balanced among the arms (Table 1). Paired baseline and follow-up plasma FRAP values were unavailable for 1 subject each in placebo, mild, and moderate groups. Inadequate sample collection or plasma separation accounted for missing values. At six months, plasma FRAP was 29% higher among mild (1.17 mM) and 43% higher among moderate (1.30 mM) group participants compared to placebo (0.90 mM) members ($P < 0.001$ for each; Fig. 1A). At the final visit on study drug (∼18 months), FRAP values among mild and moderate group participants remained elevated ($P < 0.001$ for each vs. placebo). The slightly higher FRAP values at 6 months compared to final visit matches a similar spike in serum urate values at 6-month visit (caused by one-time trough measurement of serum urate at preceding visit and compensatory increase in inosine dosing). The moderate group had higher FRAP levels compared to the mild group at 6-month visit and beyond ($P = 0.025$). Change in plasma FRAP correlated strongly with change in serum urate ($r = 0.86$; $P < 0.001$; Fig. 1B). The data also indicate an inverse correlation between the extent of plasma FRAP increase and rate of clinical decline

(as assessed by UPDRS Part III motor score slope estimate; $r = -0.26$; $p = 0.034$; Fig. 1C).

CSF FRAP

CSF FRAP values were available from 11 of 25 in placebo, 15 of 24 in mild, and 18 of 26 in moderate groups (lumbar puncture being optional or technically inadequate for some participants). CSF FRAP did not differ significantly among groups and did not correlate with serum urate concentration ($r = -0.04$; 95% confidence interval [CI]: –0.34 to 0.26; $P = 0.78$) or plasma FRAP ($r = 0.04$; 95% CI: –0.27 to 0.34; $P = 0.81$).

Urine 8-OHdG

Baseline and 6-month urine 8-OHdG values were available from 21 of 25 patients in placebo, 22 of 24 in mild, and 25 of 26 in moderate groups. Baseline values were similar across the groups. At 6 months, urine 8-OHdG values did not differ significantly among placebo, mild, and moderate groups (Table 2). Change in urine 8-OHdG did not correlate significantly with change in serum urate ($r = -0.11$; 95% CI: –0.34 to 0.13; $P = 0.37$).

Discussion

Long-term oral administration of inosine resulted in sustained, dose-dependent increases in plasma antioxidant capacity. This increase correlated tightly with increase in serum urate consistent with the major contribution (∼60%) urate makes to plasma antioxidant capacity measured by FRAP.[16,17] The findings suggest that homeostatic mechanisms do not attenuate the increase in plasma antioxidant capacity attributed to urate elevation. Although such homeostatic control phenomena may limit the long-term effects of
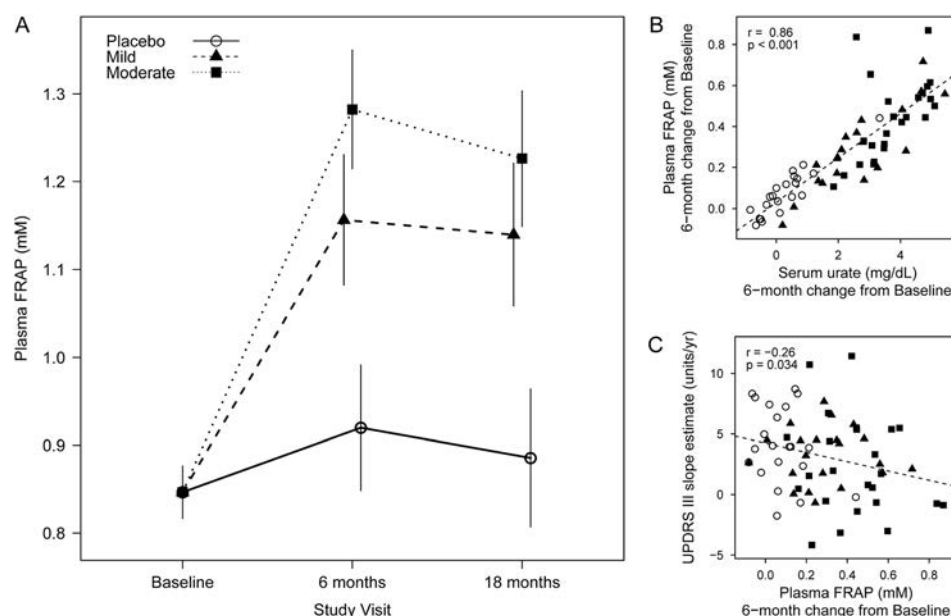
FIG. 1. (A) Plasma FRAP increased in mild (triangle) and moderate (square) groups compared to placebo (circle). (B) Plasma FRAP and serum urate changes are correlated. (C) Increase in plasma FRAP correlated with slower progression of motor symptoms of PD.

treatment with other specific antioxidants like ascorbate,[18,19] oral inosine titrated to a urate level can produce long-term elevations in peripheral antioxidant capacity.

Whereas many assays measure total antioxidant capacity, FRAP has been assessed in multiple relevant clinical studies. Decreased FRAP levels have been found in diseases with increased oxidative stress, such as cardiovascular disease,[20] chronic kidney disease,[21] and Alzheimer's disease.[22] In the PREDIMED diet trial, Mediterranean diet supplemented with olive oil or nuts resulted in early increased plasma FRAP level at 1 year and improved cardiovascular outcome in longer follow-up.[23,24] Taken together, plasma antioxidant levels measured by FRAP may be of potential therapeutic significance.

The lack of apparent effect of inosine on CSF FRAP may suggest that the antioxidant actions of inosine dosing do not extend to the CNS, or at least to its CSF compartment. Alternatively, an inosine-induced increase in CSF FRAP may have been more difficult to detect. Urate concentration in the CSF is 8- to 10-fold lower than in serum and comprises a smaller fraction of CSF antioxidant power.[14,25,26] Thus, a proportional increase in urate in both compartments would have a smaller impact on CSF FRAP. Our study was also markedly limited by lack of baseline CSF samples and a small number of subjects consenting for lumbar puncture.

We attempted to assess systemic antioxidant effect by measuring urine 8-OHdG, which is produced by hydroxyl radical attack on nucleosides in DNA. Urine 8-OHdG in a cross-sectional study increased with progression of PD, suggesting that progressive nucleic acid oxidative injury accompanies disease progression.[27] At 6 months, we did not detect differences in urine 8-OHdG among the three groups. This may indicate that increased antioxidant *capacity* does not result in greater antioxidant *effects*, and urate may not confer neuroprotection in PD or may do so by another mechanism. Alternatively, 8-OHdG in urine may inadequately reflect antioxidant effects in remote organs such as the brain. Finally, different methods of measuring 8-OHdG (such as high-performance liquid chromatography vs. ELISA) can yield values orders of magnitude apart, limiting comparison of studies.

The finding that rates of clinical decline (in the motor UPDRS Part III) were slower among participants with larger increases in plasma FRAP suggests a role for plasma FRAP as biomarker of target engagement.[28] There are several limitations of this interpretation. First, this is an exploratory analysis and requires replication in a study powered for treatment-dependent differences in rates of disease progression. Second, mechanistic significance is tempered by the absence of demonstrable CSF FRAP elevation. Last, serum urate itself is simpler and less expensive to measure than FRAP and is more directly relevant to safety than FRAP for titrating inosine dose to avoid hyperuricemic adverse events.[11]

In conclusion, chronic oral inosine administration in people with early PD patients produced a substantial, dose-dependent, persistent elevation of plasma antioxidant capacity in parallel with its urate elevating effects. The findings provide additional evidence for a

possible antioxidant mechanism of inosine and suggest a potential therapeutic benefit from urate's antioxidant properties. ∎

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NEUROPROTECTION BY CAFFEINE IN THE MPTP MODEL OF PARKINSON'S DISEASE AND ITS DEPENDENCE ON ADENOSINE A2A RECEPTORS

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Abstract—Considerable epidemiological and laboratory data have suggested that caffeine, a nonselective adenosine receptor antagonist, may protect against the underlying neurodegeneration of parkinson's disease (PD). Although both caffeine and more specific antagonists of the A2A subtype of adenosine receptor (A2AR) have been found to confer protection in animal models of PD, the dependence of caffeine's neuroprotective effects on the A2AR is not known. To definitively determine its A2AR dependence, the effect of caffeine on 1-methyl-4-phenyl-1,2,3,6 tetra-hydropyridine (MPTP) neurotoxicity was compared in wild-type (WT) and A2AR gene global knockout (A2A KO) mice, as well as in central nervous system (CNS) cell type-specific (conditional) A2AR knockout (cKO) mice that lack the receptor either in postnatal forebrain neurons or in astrocytes. In WT and in heterozygous A2AR KO mice caffeine pretreatment (25 mg/kg ip) significantly attenuated MPTP-induced depletion of striatal dopamine. By contrast in homozygous A2AR global KO mice caffeine had no effect on MPTP toxicity. In forebrain neuron A2AR cKO mice, caffeine lost its locomotor stimulant effect, whereas its neuroprotective effect was mostly preserved. In astrocytic A2AR cKO mice, both caffeine's locomotor stimulant and protective properties were undiminished. Taken together, these results indicate that neuroprotection by caffeine in the MPTP model of PD relies on the A2AR, although the specific cellular localization of these receptors remains to be determined. © 2016 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: adenosine A2A receptors, MPTP, caffeine, neuroprotection, parkinson's disease.

INTRODUCTION

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

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

Laboratory studies have supported a true neuroprotective effect of caffeine in PD by demonstrating its biological plausibility in multiple animal models of the disease. When caffeine is co-administered with MPTP to mice at doses comparable with those of typical human exposure, it dose-dependently attenuates the loss of

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Abbreviations: 6-OHDA, 6-hydroxydopamine; MPTP, 1-methyl-4-phenyl-1,2,3,6 tetra-hydropyridine; PD, parkinson's disease; SNpc, substantia nigra pars compacta; WT, wild-type.

striatal dopamine triggered by MPTP using different exposure paradigms and in several mouse strains (Chen et al., 2001b; Xu et al., 2002). It is shown that caffeine can be given up to two hours before or after MPTP and still confer protection against MPTP-induced dopamine loss. Moreover, caffeine's metabolites, paraxanthine and theophylline, also provide protection against MPTP neurotoxicity (Xu et al., 2010). Other preclinical studies have shown that caffeine protects against dopaminergic neuron degeneration, dopamine loss and/or associated behavioral changes induced by 6-hydroxydopamine (6-OHDA) in rats (Joghataie et al., 2004; Aguiar et al., 2006; Kelsey et al., 2009) and by the pesticide combination of paraquat and maneb in mice (Kachroo et al., 2007). Interestingly, the neuroprotective effect of caffeine could be dissociated from its psychomotor stimulant properties (Xu et al., 2002; Yu et al., 2008); whereas the latter showed tolerance to repeated administration, under the same conditions caffeine's neuroprotective action persisted unabated (Xu et al., 2002).

Although the convergence of caffeine's clinical correlations and protective effects in animal models supports a true beneficial effect in reducing PD risk, the mechanisms underlying neuroprotection by caffeine remain a matter of debate. Pharmacological studies indicate that its central nervous system (CNS) effects are mediated primarily by its antagonistic actions at the A_1 and A_{2A} subtypes of adenosine receptors (Fredholm and Persson, 1982; Nehlig et al., 1992; Fredholm and Lindström, 1999; Fisone et al., 2004; Ferré, 2008). Interestingly, $A_{2A}R$ blockade, but not A_1R blockade mimics caffeine's protective effects in several experimental models of PD (Schwarzschild et al., 2006). In rodents, selective $A_{2A}R$ antagonists attenuate the loss of dopaminergic neurons in the SNpc or dopamine depletion in the striatum induced by either systemic administration of MPTP or acute infusion of 6-OHDA in the medial forebrain bundle (Chen et al., 2001b; Ikeda et al., 2002; Pierri et al., 2005). A critical role of the $A_{2A}R$ in the MPTP model of PD was confirmed by the phenotype of global $A_{2A}R$ KO mice, which show preserved striatal dopamine content after acute MPTP multiple dose administration (Chen et al., 2001b).

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

EXPERIMENTAL PROCEDURES

Transgenic animals

Breeding and characterization of global $A_{2A}R$ knockout mice (A_{2A} KO), CaMKIIα gene promoter-driven forebrain neuron $A_{2A}R$ cKO mice (CaMKIIα-$A_{2A}R$ KO) as well as the GFAP gene promoter-driven astrocyte $A_{2A}R$ cKO (gfap-$A_{2A}R$ KO) mice have been described previously (Chen et al., 1999, 2001a,b, 2002; Bastia et al., 2005; Yu et al., 2008; Matos et al., 2015). Briefly, chimeric $A_{2A}R$ KO mice (F0) that were derived from 129-Steel embryonic stem cells were bred to C57BL/6 mice, resulting in mice of mixed C57BL/6 × 129-Steel background. The mixed line was then repeatedly backcrossed to pure C57BL/6 mice over 10 generations, yielding an $A_{2A}R$ KO line congenic for the C57BL/6 background. $A_{2A}R$ KO, heterozygous and wild-type (WT) littermates (male, 4–14 months old) were used in the global KO study. Both fb-$A_{2A}R$ KO and astro-$A_{2A}R$ KO mice were generated using a Cre/loxP strategy. Cre recombinase gene expression was controlled by either the forebrain (cortical and striatal) neuron-specific CaMKII-α promoter (Bastia et al., 2005) or astrocyte-specific GFAP gene promoter, as previously described elsewhere (Bajenaru et al., 2002). Both transgenic cre mice and "floxed" $A_{2A}R$ gene ($A_{2A}^{flox/flox}$) mice were backcrossed for 10–12 generations to C57Bl/6 mice (Charles River; Wilmington, MA). Homozygous floxed ($A_{2A}^{flox/flox}$) mice were crossed with cre(+) mice, and female cre(+) ($A_{2A}^{flox/+}$) offspring were then crossed with $A_{2A}^{flox/+}$ males. Their cre(+) $A_{2A}^{flox/flox}$ and cre(−) $A_{2A}^{flox/flox}$ offspring (male and females, 2–9 months old) were used in this study. Mice were housed five in each cage in temperature- and humidity-controlled rooms with a 12-h dark:light cycle and had free access to food and water. All experiments were conducted in accordance with Massachusetts General Hospital and NIH Guidelines on the ethical use of animals.

Drug treatment

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

Locomotor activity

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

photobeam breaks (ambulation), was determined as described before (Xu et al., 2002).

Measurement of dopamine

One week after MPTP treatment, mice were killed by rapid cervical dislocation. The striatum was dissected out from the right cerebral hemisphere, frozen on dry ice and stored at −80 °C until use. Each striatum was weighed, homogenized with 150 mM phosphoric acid and 0.2 mM EDTA and centrifuged at 12,000g for 15 min at 4 °C. Supernatants were analyzed for dopamine content using standard reverse-phase HPLC with electrochemical detection, according to our previously published protocol (Chen et al., 2001b; Xu et al., 2006). The dopamine content was calculated as picomoles per milligram of tissue, and these values are presented within the figures as percentage of change from respective saline–saline-treated controls.

Statistics

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

RESULTS

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

We first evaluated the effect of global genetic deletion of A$_{2A}$R on caffeine's neuroprotection against MPTP-induced dopamine depletion. MPTP (35 mg/kg ip single injection) significantly depleted striatal dopamine content measured one week later in WT, heterozygous and global A$_{2A}$R KO mice. As reported previously (Xu et al., 2006), caffeine (25 mg/kg single ip injection 10 min before MPTP) significantly attenuated MPTP-induced dopamine depletion in WT mice. Similarly, caffeine also significantly attenuated MPTP-induced dopamine loss in heterozygous mice. However, caffeine pretreatment did not attenuate MPTP-induced dopamine loss in global A$_{2A}$R KO mice (Fig. 1). Although the global A$_{2A}$R KO can have protective phenotype in multi-dose MPTP toxicity paradigms (Chen et al., 2001b; Yu et al., 2008) no appreciable effect of the KO itself was observed here using a single MPTP injection paradigm (69% vs 75% loss of striatal dopamine induced by MPTP in A$_{2A}$R KO vs WT mice, respectively; $p > 0.05$).

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

We next examined the contribution of the A$_{2A}$R in forebrain neurons to the neuroprotection by caffeine in
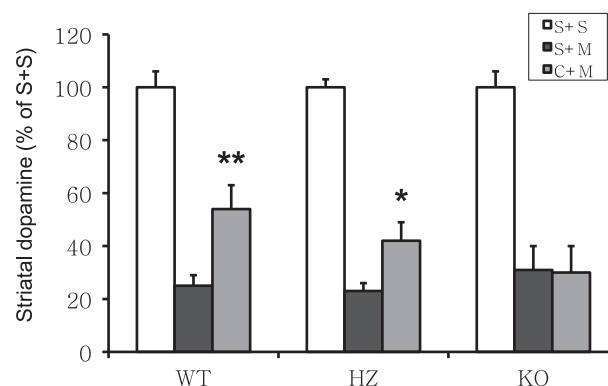

Fig. 1. Caffeine attenuated MPTP-induced striatal dopamine loss in WT and HZ, but not global A$_{2A}$R KO male mice. Saline or Caffeine (25 mg/kg ip) were administered 10 min before saline or MPTP (35 mg/kg ip single injection). Dopamine content was determined one week after drug treatments. S, saline;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

MPTP-induced neurotoxicity. MPTP induced significant and similar dopamine loss in fb-A$_{2A}$R KO mice and their non-transgenic littermate controls. In contrast to our findings with the global A$_{2A}$R KO, caffeine pretreatment significantly attenuated MPTP-induced dopamine depletion in both fb-A$_{2A}$R KO and control mice (Fig. 2. The amount of residual dopamine with caffeine pretreatment was ∼180% greater than without caffeine in control mice, whereas it was only ∼64% greater in
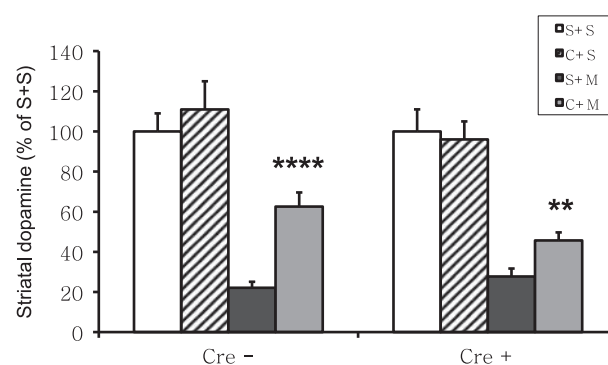

Fig. 2. Caffeine's attenuation of MPTP-induced striatal dopamine loss is at least partially independent of forebrain neuronal A$_{2A}$Rs. Saline or Caffeine (25 mg/kg ip) was administered 10 min before saline or MPTP (35 mg/kg ip single injection). Striatal dopamine was determined one week after drug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

fb-A_{2A}R KO mice ($p = 0.052$, *t*-test; comparing the 'Caffeine + MPTP' mice without and with the *cre* transgene as shown in Fig. 2). These data would suggest that caffeine's attenuation of MPTP-induced striatal dopamine loss is at least partially independent of forebrain neuronal A_{2A}R, though it may contribute partially to caffeine's protective effect. In contrast to what was found for caffeine's neuroprotective effect, caffeine stimulated locomotor activities in fb-A_{2A}R WT but not fb-A_{2A}R KO mice (Fig. 3).

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

Finally, we investigated the roles of A_{2A}R in astrocytes in caffeine's neuroprotection and motor stimulant effect.

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

DISCUSSION

The main finding of our study is the demonstration that caffeine's neuroprotection depends on adenosine A_{2A}R. Although the use of a global A_{2A}R KO helps establish an essential role of this receptor in caffeine's
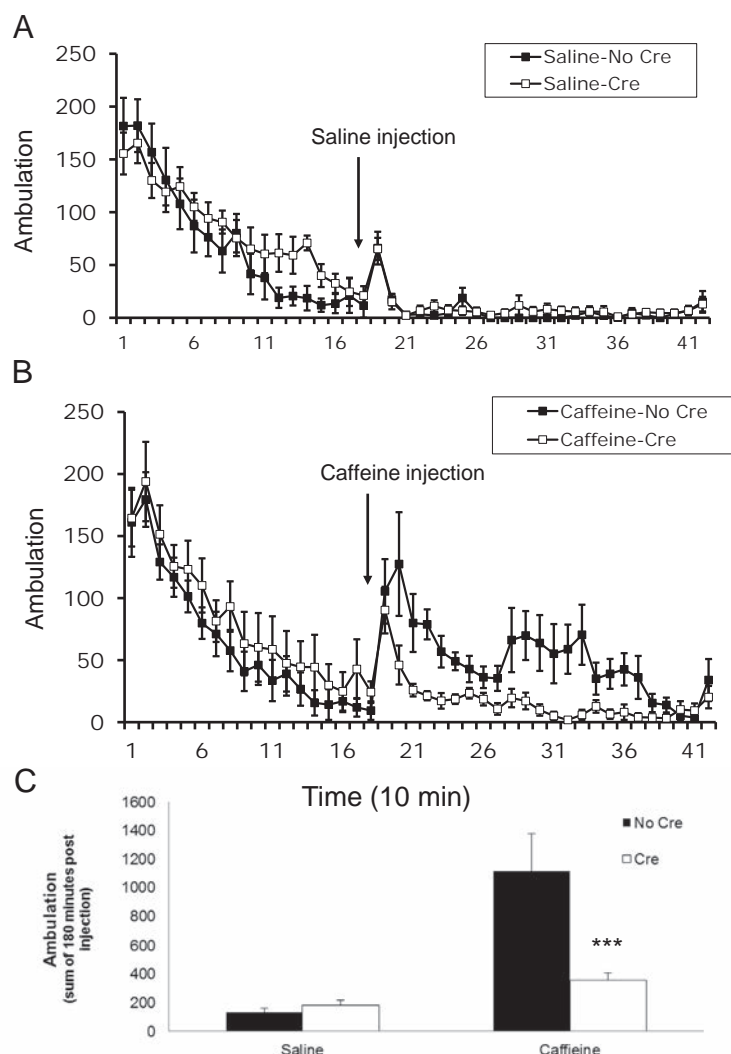

Fig. 3. Dependence of caffeine-induced locomotion on neuronal A_{2A}Rs. Ambulation was scored as the number of adjacent photobeam breaks (mean ± SEM, $N = 8$ for each group). Mice were habituated overnight and basal spontaneous locomotion was recorded for at least 180 min. Ambulation was recorded for another 240 min after caffeine (25 mg/kg ip) or saline injection. (A) Locomotion after saline injection is similar in WT and forebrain neuron A_{2A}R cKO (*CaMKIIα-cre*, $A_{2A}^{flox/flox}$) mice. (B) Caffeine-stimulated locomotion is significantly reduced in forebrain neuron A_{2A}R cKO mice compared to that of WT mice. (C) Bars represent peak locomotion (mean ± SEM) 180 min after caffeine or saline injection. Statistically significant differences among the peak locomotions were determined by a two-way analysis of variance followed by Fisher's Least Significant Difference test. There is a significant difference in Cre versus No Cre mice ($F[1,28] = 6.77$, $p < 0.05$). There is also a significant difference in caffeine versus saline-treated groups ($F[1,28] = 18.46$, $p < 0.001$). ***$p < 0.001$ compared with caffeine-treated group No Cre mice.
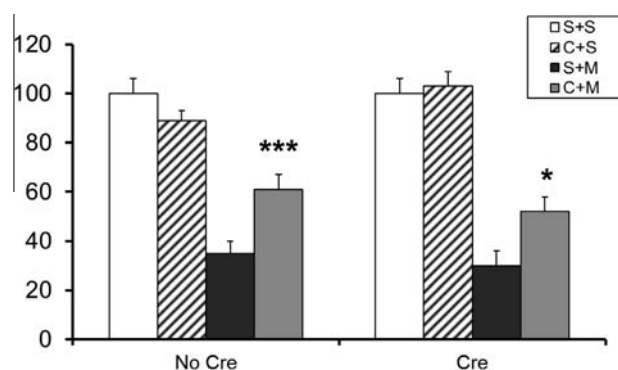
Fig. 4. Caffeine attenuated MPTP-induced striatal dopamine loss in both WT and astrocyte-directed $A_{2A}R$ cKO mice. Saline or Caffeine (25 mg/kg ip) was administered 10 min before saline or MPTP (35 mg/kg ip single injection). Striatal dopamine was determined one week after drug treatments. $N = 3$–6 for saline treatments and $N = 9$–20 for MPTP treatments. Bars represent striatal dopamine levels (mean ± SEM) calculated as percentage of their respective control (i.e., *saline + saline treatment* group). Statistically significant differences among the means of dopamine content in MPTP-treated mice were determined by a two-way analysis of variance followed by Fisher's Least Significant Difference test. There is a significant difference in caffeine- versus saline-treated groups ($F[1,51] = 17.69$, $p = 0.0001$). $^*p < 0.05$, $^{***}p < 0.001$ compared with respective S + M group.

neuroprotective effect, constitutive KO methodology has limitations such as its inability to distinguish between a role for the receptor during development or adulthood (Bockamp et al., 2002). Our results also suggest that caffeine's protective effect is mostly dependent on $A_{2A}R$ other than those located on forebrain (striatal and cortical) neurons and on astrocytes.

Adenosine receptor antagonism has been shown to be the main mechanism of action responsible for the CNS effects of caffeine. Several studies have indicated that caffeine exerts its psychostimulant effects acting as a nonselective adenosine A_1R and $A_{2A}R$ receptor antagonist (Fredholm and Persson, 1982; Nehlig et al., 1992; Fredholm and Lindström, 1999; Fisone et al., 2004; Ferré, 2008). Interestingly, the expression of these two adenosine receptors in the brain displays very different patterns: whereas A_1R are present throughout the brain, the expression of the $A_{2A}R$ is largely restricted to the striatum. Although brain $A_{2A}R$ were initially thought to be exclusively located in this region, several studies provided evidence also for the presence of $A_{2A}R$ in the hippocampus and neo-cortex (Rebola et al., 2005; reviewed in Cunha, 2001) at a lower density (Lopes et al., 2004).

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

Previous reports have suggested that adenosine may contribute to the pathological changes of PD by triggering the activation of surrounding glial cells (Hirsch et al., 1999). $A_{2A}R$ activation is in fact partially responsible for cerebral inflammation and excitotoxicity (Popoli et al., 1995; Sullivan and Linden, 1998; Okusa et al., 1999) and $A_{2A}Rs$ located on glial cells might play a role in neuroprotection mediated by $A_{2A}R$ antagonists against acute (Yu et al., 2008) and chronic MPTP-induced striatal dopamine depletion (Sonsalla et al., 2012) MPTP-induced striatal dopamine depletion.

Whereas several studies have reported a clear role of A_1R in the behavioral effects of acutely administered caffeine with involvement of $A_{2A}R$ preferentially under conditions of chronic caffeine treatment (Karcz-Kubicha et al., 2003; Antoniou et al., 2005; Orrú et al., 2013), we have confirmed (Fig. 3) that caffeine's acute locomotor stimulant effect also requires the $A_{2A}R$, specifically those expressed in forebrain (e.g., striatal) neurons (Yu et al., 2008).

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

While our main results are generally in agreement with the findings of Chen et al. (2001b) and Yu et al., 2008, the depletion of $A_{2A}R$ in the global $A_{2A}R$ KO mice in the current study was not sufficient to attenuate MPTP neurotoxicity in contrast to the earlier studies. This apparent discrepancy could be related to the different MPTP·HCl doses and dosing paradigms used across these experiments (a single injection of 35 mg/kg ip here compared to multiple injections of 20 mg/kg ip hours apart in the earlier studies). Differences in MPTP dose amounts and timing are known to induce different patterns of neuronal death. (e.g., apoptotic vs necrotic; Meredith and Rademacher, 2011), and thus potentially different dependencies on the $A_{2A}R$. Interestingly, recent evidence that caffeine may produce some CNS effects through inverse agonism (rather than competitive antagonism of endogenous adenosine) at the $A_{2A}R$ (Fernández-Dueñas et al., 2014) suggests a possible explanation for how caffeine's protective effect could be $A_{2A}R$-dependent, without $A_{2A}R$ depletion having an effect of its own, in the MPTP paradigm employed in our study.

Selective deletion of $A_{2A}R$ in forebrain neurons showed that caffeine's attenuation of MPTP-induced striatal dopamine loss is at least partially independent of forebrain neuronal $A_{2A}Rs$, although locomotion appeared
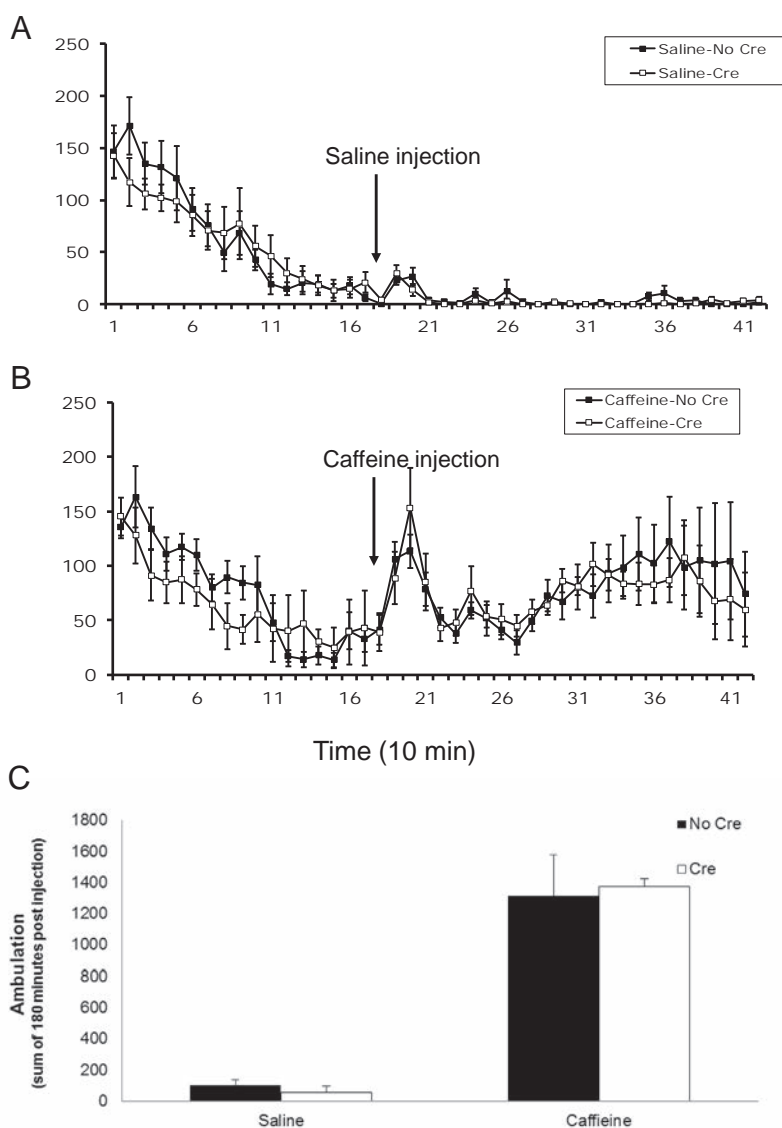
Fig. 5. Lack of dependence of caffeine-induced locomotion on astrocytic A_{2A}Rs. Ambulation was scored as the number of adjacent photobeam breaks (mean ± SEM, $N = 8$ for each group). Mice were habituated overnight and basal spontaneous locomotion was recorded for at least 180 min. Ambulation was recorded for another 240 min after caffeine (25 mg/kg ip) or saline injection. (A) Locomotion is similar after saline injection in WT and astrocyte-directed A_{2A}R cKO (*gfap-cre*, $A_{2A}^{flox/flox}$) mice. (B) Caffeine also stimulated similar locomotion in WT and astrocyte-directed A_{2A}R cKO mice. Bars represent peak locomotion (mean ± SEM) 180 min after caffeine or saline injection. There is no statistical difference in locomotion after either saline or caffeine injection between Cre and No Cre mice.

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

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

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

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

Other possible cellular sources of A_{2A}Rs should be considered in addition to striatal and cortical neurons and astrocytes. These include A_{2A}R-expressing microglial cells (Fiebich et al., 1996; Hasko et al., 2005) and oligodendrocytes (Stevens et al., 2002). Caffeine or selective A_{2A}R antagonists were shown to attenuate microglia recruitment to sites of injury and to reduce the production of pro-inflammatory cytokines (Brambilla et al., 2003; Brothers et al., 2010; Rebola et al., 2011). Microglial activation during inflammatory processes observed in the brains of older rats is partially reversed by chronic caffeine (Brothers et al., 2010). Furthermore, activation of A_{2A}Rs has been associated with release of brain-derived neurotrophic factor (BDNF) and proliferation of microglial cells, which are intrinsically related events of neuroinflammation. There is also evidence that non-CNS cells expressing the A_{2A}R, such as bone marrow cells, may contribute to ischemic brain cell injury, as suggested by Yu et al. (2004). In addition, it seems that these receptors in peripheral cells may be modulators of inflammatory cytokine production (Ran et al., 2015).

CONCLUSION

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

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

None reported.

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Appendix S

NEUROSCIENCE
2011

Presentation Abstract

	Add to Itinerary
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Program#/Poster#: 244.13/K3

Presentation Title: Does chronic 2,4-dichlorophenoxyacetic acid (2,4-D) exposure in mice produce a model of Parkinson's disease?

Location: Hall A-C

Presentation time: Sunday, Nov 13, 2011, 1:00 PM - 2:00 PM

Authors: *L. G. MCCLURG, A. KACHROO, M. A. SCHWARZSCHILD;
Neurol., Massachusetts Gen. Hosp., Charlestown, MA

Abstract: Growing epidemiological evidence suggests a link between occupational exposure to the herbicide 2,4-D and subsequent development of Parkinson's disease (PD). However, few studies have addressed the biological plausibility of a causal toxicological basis. To assess the effect of prolonged, systemic exposure to 2,4-D in male C57BL/6 mice, animals were intraperitoneally administered 100 or 200 mg/kg of 2,4-D (n=21 per dosage group), or saline (n=14) twice a week for 7 weeks. Locomotion was measured in an automated open field apparatus at baseline (no prior injections) and at least 3-4 days after the 4th, 8th and 12th injections. Locomotion was measured for a 16 hour period that included the 12 hr dark (overnight) phase of their daily light:dark cycle. Mice were sacrificed one week after the 14th injection. Brain and peripheral tissues were collected for analysis. A significant behavioral effect was only observed in the 200 mg/kg treatment group compared to vehicle group, with these toxin-treated mice moving significantly less. A significant loss of body weight (not more than 2.5g) was observed in both the 200 mg/kg and 100 mg/kg toxin-treatments compared to the vehicle after the 6th and 10th injection, respectively. There were no significant differences in kidney weights between any of the groups, suggesting atrophic kidney toxicity did not likely contribute to a behavioral effect. HPLC analysis of striatal dopamine and serotonin content and their metabolites DOPAC and 5HIAA, respectively, in 2,4-D treated mice at either dose, showed no differences between 2,4-D and control groups. The absence of neurochemical deficits after two months of biweekly 2,4-D exposure may reflect a lack of neurotoxicity, or the presence of compensatory mechanisms in striatal nerve terminals. Pending stereological cell counts of nigral neurons will directly address the possibility that 2,4-D produces dopaminergic neuron degeneration.

Disclosures: L.G. McClurg: None. A. Kachroo: None. M.A. Schwarzschild: None.

Keyword(s): PARKINSON'S DISEASE

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Association of α-synuclein gene expression with Parkinson's disease is attenuated with higher serum urate in the PPMI cohort

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*presenting author

Objective: To explore how urate may modulate Parkinson's disease (PD)-specific pathogenic mechanisms using clinical biomarker data. Urate is the end product of purine metabolism in humans, but also possesses potent antioxidant and neuroprotective properties. Lower serum urate is a reproducible risk factor both for developing PD and for a more rapid rate of its clinical progression.

Methods: Data were analyzed from the Michael J. Fox Foundation's Parkinson's Progression Markers Initiative (PPMI), which enrolled 218 people with early, untreated PD and 153 healthy control (HC) subjects for whom baseline blood levels of urate and α-synuclein gene (SNCA) transcript were available.

Results: SNCA transcript counts are substantially reduced (p=0.0001) in PD compared to HC among those with lower urate (below the median HC value of 5.4 mg/dL), but not appreciably different among those with higher urate (p=0.5). In further analysis fully adjusting for relevant covariates, the odds of having PD were markedly lower among individuals with more SNCA transcripts only if they also had lower urate (OR = 0.61 / 10^3 mRNA, p=0.002), not higher urate (OR = 0.91 / 10^3 mRNA, p=0.6), with a significant interaction between urate and SNCA transcripts (p=0.02).

Table: Levels of SNCA transcript (UTR-1) in blood are reduced in PD among those with lower but not higher serum urate in PPMI

		Lower Urate (≤ 5.3 mg/dL)***		Higher Urate (> 5.3 mg/dL)****		p for interaction
SNCA mRNA counts	HC	2188		1941		
	(SD)	(1182)		(1227)		
	PD	1598		1810		
	(SD)	(874)		(1086)		
	PD/HC	0.73	p = 0.0001	0.93	p = 0.5	
Odds Ratio per 10³ mRNA	PD vs HC*	0.62		0.96		0.03
	(95% CI)	(0.46, 0.83)	p = 0.002	(0.72, 1.39)	p = 0.8	
	PD vs HC**	0.61		0.91		0.02
	(95% CI)	(0.44, 0.83)	p = 0.002	(0.66, 1.27)	p = 0.6	

*adjusted for age and gender

**adjusted for age, gender, BMI and transcript-related labs (WBC, RBC, Plt)

***n=187 of PPMI cohort with urate and SNCA mRNA at or below the serum urate median; n =182 for OR analysis.

****n=184 of PPMI cohort with urate and SNCA mRNA above the serum urate median; n=153 fro OR analysis.

Conclusions: These preliminary data suggest that the impact of α-synuclein on PD is attenuated in the presence of higher concentrations of urate.