



Published in final edited form as:

Neurodegener Dis. 2013 ; 12(4): . doi:10.1159/000346370.

Postmortem Brain Levels of Urate and Precursors in Parkinson 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 disease (PD) higher, but still normal, levels of blood and CSF 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 frontal and temporal cortex, striatum, and cerebellum, using HPLC with electrochemical and ultraviolet detection.

Results—Age was well-matched among groups. Mean postmortem interval (PMI) for samples was 16.3 ±9.9 hrs. Urate levels in cortical and striatal tissue trended lower in PD and AD compared to control in males only. These findings correlated with increased urate in male vs. 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.

Keywords

Uric acid; purines; neurodegeneration; parkinsonism; Lewy body disease; Alzheimer's disease

Introduction

Increasing clinical, epidemiological, and laboratory evidence suggests that urate (or uric acid) may play a role in neurodegenerative disease, and Parkinson 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 (Figure 1) and circulates at high plasma concentrations. Urate's antioxidant capacity 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 provided initial, post-mortem evidence that nigrostriatal levels of urate (but not ascorbate) as well as dopamine are reduced in PD.[7] 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 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 MassGeneral Alzheimer Disease Reserch 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 frontal and temporal cortex, striatum, and cerebellum using 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 MassGeneral 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 tissue (~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 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 <24hrs) and matched across the disease and control groups (Table 1). 5–10 samples for each disease state, gender, and specified brain region were examined.

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 μ M methyl dopa and 1 μ M 3,4-dihydroxybenzylamine as internal standards, and then centrifuged at 16K \times g for 15 min. The supernatant was filtered through a 0.22 μ m 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 mV 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, MA) with autorange gain enabled. Measurements for each sample were normalized to the methyl dopa 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, $\alpha = 0.05$) with post-hoc Dunn's multiple 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 GLM (MANCOVA) with disease, brain region, and gender as independent variables and with age and PMI as covariates. Posthoc pairwise comparisons were performed with Bonferonni correction. Data in graphs are expressed as mean \pm 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 age for all groups being 79.2 years (SD \pm 9.0). Overall mean PMI was 16.1 hours (SD \pm 10.4) and differed among groups ($H = 8.6$, $df = 3$, $p = 0.035$) with PMI for AD (12 hours \pm 7.5) being significantly lower than for controls (22.7 hours \pm 12.4). 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 (Figure 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 (Figure 2B).[25, 26] Among precursors only xanthine showed a significant correlation and decline in levels with age.

Urate and Precursor Levels in Parkinson Disease 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), consistent with that reported in serum.[27, 28] Urate levels in PD trended lower than control ($p=0.096$) among males, whereas there was no difference among females (Figure 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) than in males (32.9 ± 2.3), whereas in control and DLB levels were higher in females (42.0 ± 4.1 , $p=0.002$ and 43.1 ± 2.9 , $p<0.000$; respectively). Additionally, among females xanthine was reduced in PD compared to DLB ($p<0.0005$) and to control ($p=0.004$). 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 , respectively). Among males, inosine levels in AD were also increased (137.8 ± 10.8 , $p=0.025$) 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 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 (Figure 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 ($F[3,162] = 8$ in striatum.83, $p < 0.0005$; Figure 4A, B) on postmortem brain levels. Simple effects analysis indicated that cerebellar urate levels were higher than that in striatum ($p=0.041$). Among disease groups, urate was elevated in DLB brains vs. control, AD, and PD ($p = 0.029$, <0.0005 , and <0.0005 , respectively). Posthoc analyses similarly showed significant increase in urate in cerebellum for DLB compared to AD ($p=0.004$). Though urate in PD and AD appeared lower than control, 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 striatum compared to frontal and temporal cortices ($p<0.0005$), as well as cerebellum compared to 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 vs. control ($p = 0.20$), 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 ($p=0.006$).

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 vs. females, mirroring similar gender differences reported in serum.[29] Our findings are generally in agreement with Church and Ward (1994), who also found lower levels of urate in PD substantia nigra and striatum compared to control.[7] 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 cells 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 derivitization 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 reduced risk of dementia 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 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 epidemiology 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 [125 I]-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 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 their 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 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 MGH-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.

Acknowledgments

Special thanks to Karlotta Fitch and Miriam Greenstein for help with brain tissue samples. This study was supported by the Harvard NeuroDiscovery Center, NIH K08NS067024 (N.R.M.), NIH K24NS060991, DoD W81XWH-11-1-0150 and the RJG Foundation

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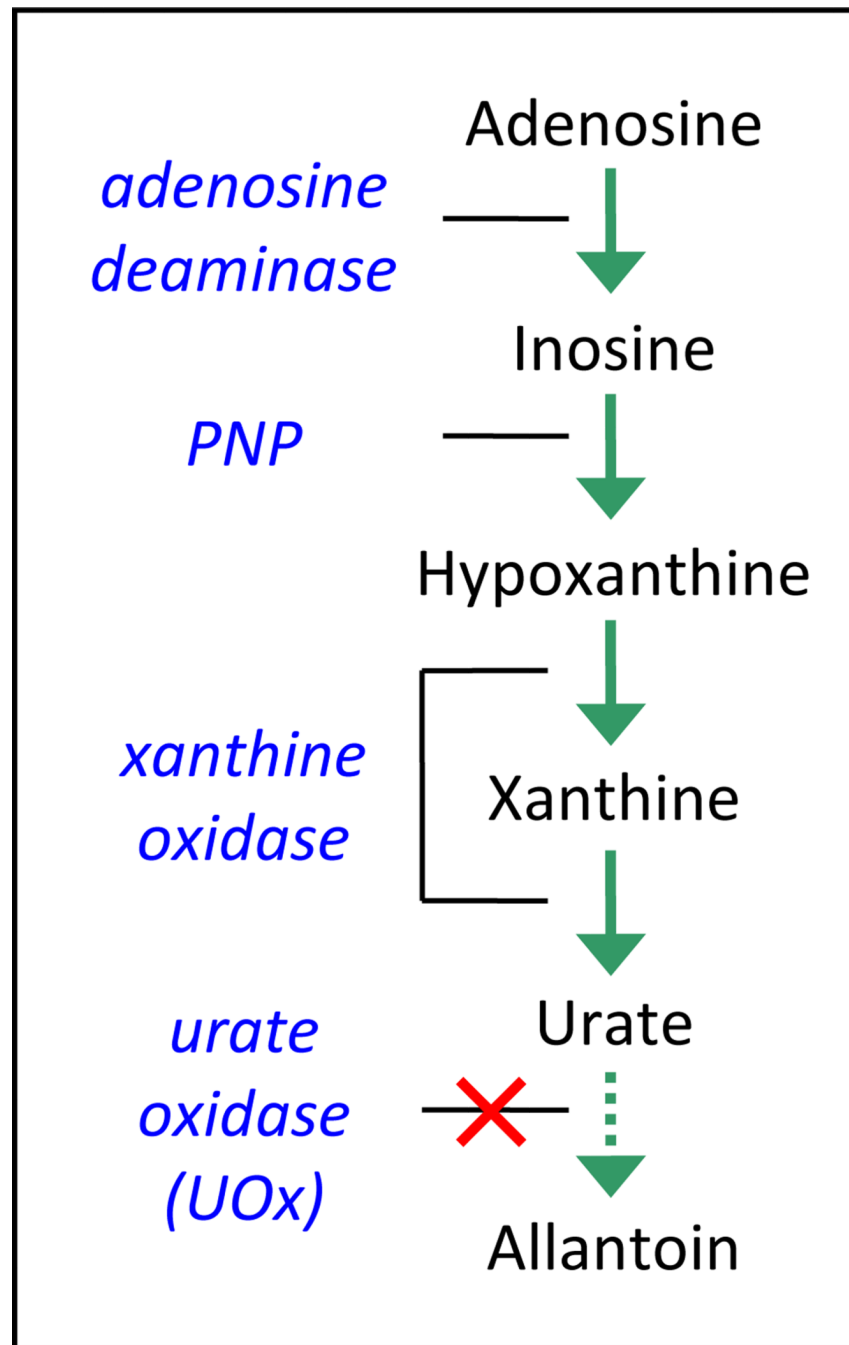


Figure 1. Metabolism of purines in humans

Loss of urate oxidase (UOx) function results in elevated urate. (PNP, purine nucleoside phosphorylase)

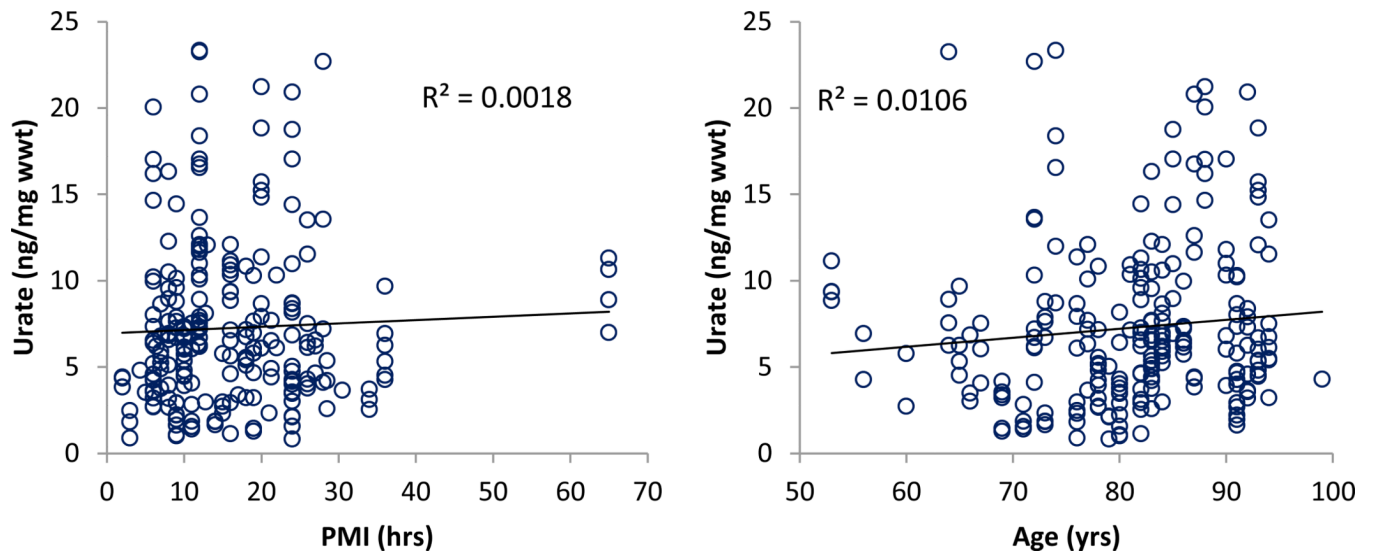


Figure 2. Regression analysis of A) PMI vs. urate levels ($\beta = -0.042$, $p = .576$), and B) age vs. urate ($\beta = .114$, $p = .128$). No significant relationships were found.

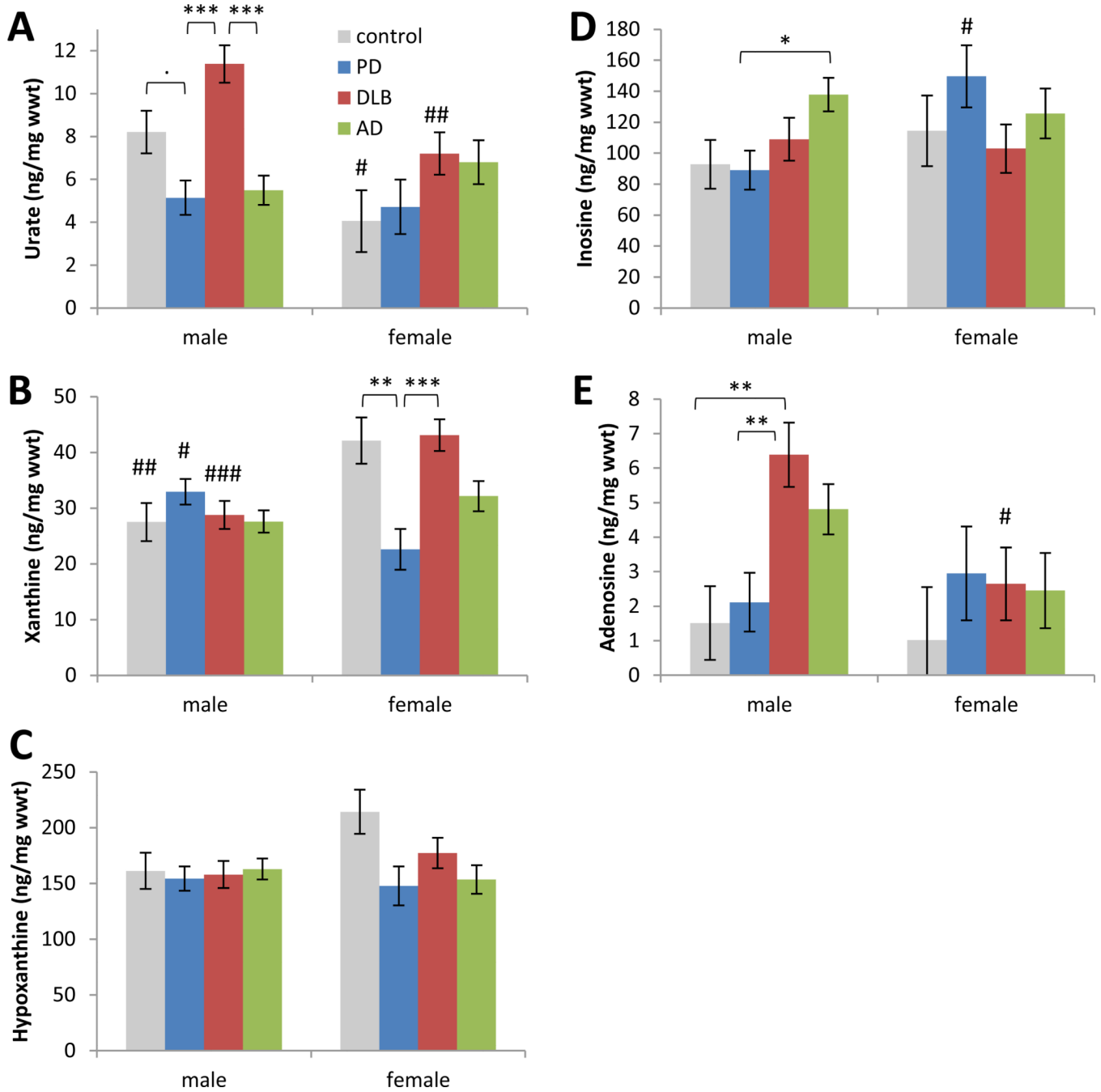


Figure 3. Disease versus gender differences in brain purine levels for non-neurodegenerative disease control, PD, DLB, and AD tissue

Mean levels are expressed as ng per wet weight tissue (mg). Urate levels (A) in controls are significantly lower in female vs male. 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 ($p < 0.0005$). Among precursors, xanthine (B) in PD is decreased in females vs males, whereas increased in DLB females. Xanthine in DLB females is also significantly elevated compared to PD. Inosine (D) levels are increased in AD compared to PD. In PD tissues, inosine is also significantly elevated in female vs male samples. Adenosine is increased in DLB relative to control and

PD among males. (\cdot $p < 0.1$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$) (gender comparison: $\#p < 0.05$, $\#\#p < 0.01$)

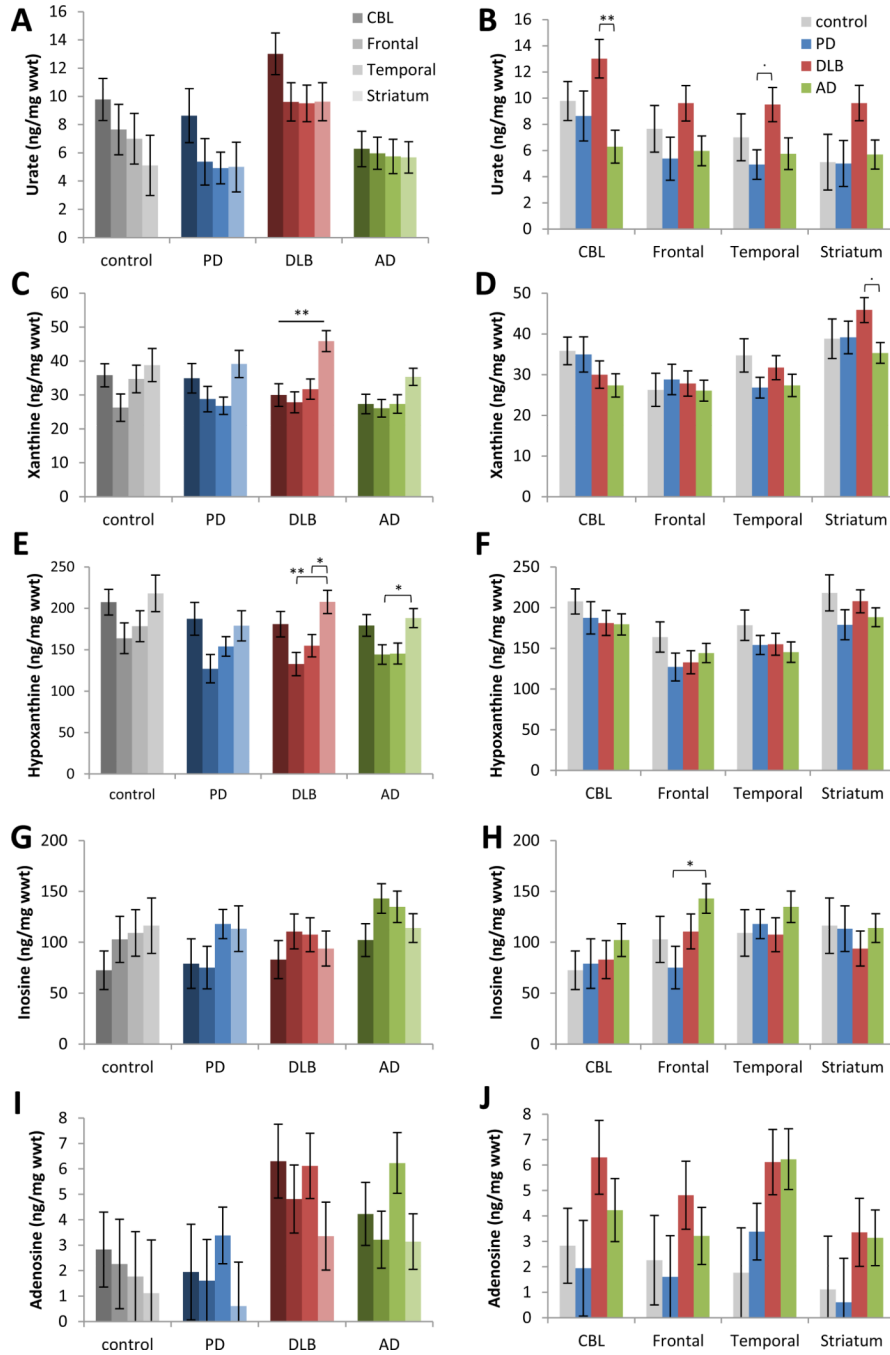


Figure 4. Region versus disease group differences in brain purine levels

Graphs of both disease vs. region (left) and region vs. 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 striatum. Similarly, hypoxanthine (E,F) levels are greater in striatum and cerebellum (CBL) compared to frontal 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 that 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 ($\cdot p < 0.1$, $*p < 0.05$, $**p < 0.01$).

Table 1**Group Statistics**

Values in same row for age and PMI not sharing the same subscript are significantly different ($p < .05$) for two-sided test of column means (Kruskal-Wallis test of disease versus age or PMI). PMI for control and AD are significantly different ($p = 0.023$).

	Control	PD	DLB	AD	P
Total (n)	13	17	13	19	-
M/F	8/5	8/9	7/6	12/7	-
Mean Age in yrs (SD)	78.3 (11.3) _b	79.2 (7.5) _a	75.6 (9.8) _a	82.2 (7.6) _b	0.342
Mean PMI in hrs (SD)	22.7 (14.7) _a	16.0 (9.1) _{a,b}	15.5 (7.9) _{a,b}	12.0 (7.5) _b	0.035*

Table 2

Regression data for Age and PMI vs. urate and precursors

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 limit of detection and violate homoscedasticity assumptions).

Regression data	Age		PMI		
	t	P	t	P	
Urate	0.114	1.528	0.042	0.560	0.576
Xanthine	-0.172	-2.323	0.243	3.349	0.001*
Hypoxanthine	-0.053	-0.713	0.137	1.845	0.067
Inosine	-0.026	-0.344	-0.135	-1.816	0.071
Adenosine	0.039	0.519	-0.235	-3.227	0.001*