

# Severe and regionally-widespread increases in tissue urea in the human brain represent a novel finding of pathogenic potential in Parkinson's disease dementia

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## Research article

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# Abstract

**Background:** Widespread elevations in brain urea have, in recent years, been reported in certain types of age-related dementia, notably Alzheimer's disease (AD) and Huntington's disease (HD). Urea increases in these diseases are substantive, and approximate in magnitude to levels present in uraemic encephalopathy. In AD and HD, elevated urea levels occur across the entire brain, and not only in regions heavily affected by neurodegeneration. However, measurements of brain urea have not hitherto been reported in Parkinson's disease dementia (PDD), a condition defined by changes in thinking and behaviour in someone with a diagnosis of Parkinson's disease, which shares neuropathological and symptomatic overlap with both AD and HD. This study aims to address this gap in the current knowledge of PDD.

**Methods:** Here we report measurements of tissue urea from nine neuropathologically-confirmed regions of the brain in PDD and post-mortem-delay-matched controls, in regions that included the cerebellum, motor cortex, sensory cortex, hippocampus, substantia nigra, middle temporal gyrus, medulla oblongata, cingulate gyrus, and pons, by applying ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). Case-control differences were determined using multiple t-tests followed by correction with 10% false discovery rate.

**Results:** We found urea concentrations to be substantively elevated in all nine regions, the average increase being 3-4-fold. Urea concentrations were remarkably consistent across regions in both cases and controls, with no clear distinction between regions heavily affected by neuronal loss in PDD compared to less severely affected areas. These urea elevations mirror those found in uraemic encephalopathy, where equivalent levels are generally considered to be pathogenic. These urea elevations also reflect those previously reported in AD and HD.

**Conclusions:** Increased urea is a widespread metabolic perturbation in brain metabolism common to PDD, AD, and HD, at levels equal to those seen in uremic encephalopathy. This presents a novel pathogenic mechanism in PDD, which is shared with two other neurodegenerative diseases.

## Background

Parkinson's disease (PD) is the second most common neurodegenerative condition after Alzheimer's disease (AD) (1). PD is characterised mainly by motor dysfunction including bradykinesia, resting tremor, and rigidity. Up to 80% of patients with PD develop cognitive dysfunction during the course of their disease, usually within twenty years from the diagnosis, designated as Parkinson's disease dementia (PDD) (2). Neuropathologically, PD and PDD are characterised by extensive loss of dopaminergic neurons in the substantia nigra, and accumulation and progressive spread of misfolded  $\alpha$ -synuclein with formation of Lewy bodies and Lewy neuropil threads. Deposition of  $\alpha$ -synuclein is thought to begin in the olfactory bulbs and lower brainstem and progress to the midbrain and eventually to the neocortex (3, 4).

The severity of  $\alpha$ -synuclein deposition in the post-mortem brain is assessed using the Braak staging system.

A conclusive clinical diagnosis of PD/PDD can be challenging due to the heterogeneity of the condition, presence of comorbidities and overlap with other forms of movement disorders and dementia (5, 6). The hypothesis that different forms of dementia represent a spectrum with common pathogenetic mechanisms has been suggested (7, 8). Studies that search for perturbations in different areas of the brain across different neurodegenerative conditions represent an approach to unveil potential common pathogenetic mechanisms. Previous metabolomics studies have investigated AD and HD and demonstrated widespread increases in brain-tissue urea in both these conditions despite their different clinical phenotypes and genetic alterations (9–12).

Peripheral blood levels of urea have been reported in PD patients with discordant results including increase in plasma (13), no change in serum (14), decreases in the CSF (15), and decrease in whole blood concentrations (16). Additionally, none of these studies of peripheral urea distinguished between PD with or without dementia. Conclusions on cerebral tissue levels of this metabolite cannot be inferred from these studies but to our knowledge, brain-tissue urea levels have not previously been reported in PDD.

## Methods

### Obtaining Tissue for Urea Quantification

For this study, tissues from nine brain regions from nine cases with definitely diagnosed PDD and nine controls were obtained from the University of Miami Brain Endowment Bank, Miami, FL, USA (part of the National Institute of Health NeuroBioBank network). Tissues were dissected from the following human-brain regions: middle temporal gyrus (MTG); motor cortex (MCX); primary visual cortex (PVC); hippocampus (HP); anterior cingulate gyrus (CG); cerebellum, at the level of the dentate nucleus (CB); substantia nigra (SN); pons; and medulla oblongata (MED). All available patient metadata for cases and controls were obtained and are herein presented in the additional files (see Additional File 1, Tables 1–2).

### Diagnosis & Severity of PDD Cases

Cases and controls were diagnosed by the referring neuropathologists of the Miami Brain Endowment Bank. All cases were diagnosed to be of the alpha-synucleinopathy neocortical type, consistent with the clinical phenotype of PDD. Controls did not show any features of neurodegeneration or vascular pathology. The brains were assessed using either Braak staging (3) and/or McKeith's staging criteria for Lewy body dementias (17) (see Additional File 1).

### Tissue Dissection

Brain samples were cut into sections of 50 mg ( $\pm$  5 mg) for urea quantification using a metal-free ceramic scalpel. Samples were stored in 'Safe-Lok' microfuge Eppendorf tubes (Eppendorf AG; Hamburg, Germany) and stored at  $-80^{\circ}\text{C}$  prior to extraction.

# Urea Quantification

Urea was quantified in brain sample by UHPLC-MS/MS. Brain samples were extracted in 50:50 (v/v) methanol:chloroform containing labelled urea internal standard (Urea-<sup>15</sup>N<sub>2</sub> 98 atom % <sup>15</sup>N, 99% (CP), 316830 Sigma-Aldrich, MO, USA). Extraction blanks containing only the methanol:chloroform:internal standard solvent were prepared. Samples were lysed in a TissueLyser batch bead homogeniser (Qiagen, Manchester, UK). LC-MS grade water was then added to samples before separation of polar and non-polar phases by centrifugation at 2400 x g for 15 minutes. The polar methanol phase was transferred to a fresh tube before being dried overnight in a Speedvac centrifugal concentrator (Savant Speedvac, Thermo Scientific, UK).

Once dried, 0.1% formic acid was added to samples. The resulting solution was transferred to 300- $\mu$ l autosampler vials, with two blanks containing only 0.1% (v/v) formic acid also prepared. Standard solutions containing a labelled urea internal standard and corresponding unlabelled urea external standards (urea analytical standard, 56180 Supelco, PA, USA) were prepared, containing concentrations of 0–5000  $\mu$ M unlabelled urea in 0.1% v/v formic acid. Three QC samples were also prepared containing 10  $\mu$ M labelled urea and 20, 200, and 2000  $\mu$ M unlabelled urea in 0.1% v/v formic acid.

Urea quantification was performed on a TSQ Vantage triple quadrupole mass spectrometer coupled with an Accela UHPLC system (Thermo Fisher Scientific, MA, USA). Separation was carried out on a Hypersil Gold AQ column with a diameter of 2.1 mm, length of 100 mm, and particle size of 1.9  $\mu$ m (Thermo Fisher Scientific) maintained at 25°C with a 0.5  $\mu$ m pre-column filter (Thermo Fisher Scientific). Gradient elution was performed using 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) at 300  $\mu$ l/min. Urea and labelled urea internal standard were detected using electrospray ionisation in positive ionisation mode.

## UHPLC-MS/MS Data Analysis

UHPLC-MS/MS data were analysed using LCQuan software (Thermo Fisher Scientific, MA, USA). Chromatographic peaks were identified based on expected retention time (RT), and compared against labelled urea internal standard peak retention times for each individual QC/standard/sample. Each peak was manually checked for correct identification.

Quantification of urea in samples was performed using the ratio of urea peak area to internal standard peak area and comparison to the standard curve. These concentrations were corrected for sample wet-weight and analysed in GraphPad Prism v8.1.2. (Prism; La Jolla, CA). A non-parametric Mann-Whitney-U test was used to determine the significance of case-control differences due to the relatively small sample sizes.

The minimum sample size required to confidently determine case-control differences at a significance level of  $p < 0.05$  and  $p < 0.01$  was calculated using the sample size calculator from SPH Analytics, Alpharetta, USA (<https://www.sphanalytics.com/sample-size-calculator-using-average-values/>).

## Results & Discussion

### Cohort Characterisation

Metadata were obtained for all cases and controls, including sex, age, race, ethnicity, post-mortem delay (PMD), brain weight, comorbidities, and cause of death (see Additional File 1 for individual data). There were no significant case-control differences in sex, age at death, PMD or brain weight (Table 1). All samples had a PMD of 26 hours or less, with an average of 19.8 hours in controls and 14.6 hours in cases.

Due to a lack of available SN tissue for two of the cohort samples, two alternate SN samples were obtained from different donors. These samples were age- and sex-matched, but the cases had a lower PMD than controls (~ 6 hours,  $p = 0.03$ ; Table 2). The impact of this is discussed in the section on brain tissue urea findings below.

Table 1  
Cohort Characteristics

	Gender (% male)	Age at death (years)	PMD (hours)	Brain Weight (g)
<b>Controls (n = 9)</b>	44	70 (61–79)	19.8 (12.5–26.0)	1285 (1135–1371)
<b>Cases (n = 9)</b>	66	73 (61–81)	14.6 (4.3–21.9)	1291 (1187–1520)
Mean (range) age, PMD, and brain weight. No case-control differences were significant.				

Table 2  
SN Cohort Characteristics

	Gender (% male)	Age at death (years)	PMD (hours)	Brain Weight (g)
<b>Controls (n = 9)</b>	44	70 (62–79)	20.6 (10.8–26.0)	1335 (1135–1550)†
<b>Cases (n = 9)</b>	66	73 (61–81)	14.6 (4.3–21.9)*	1291 (1187–1520)
Mean (range) age, PMD, and brain weight. * $p < 0.05$ between cases and controls as determined by Mann-Whitney-U Test; all other differences were not significant. †Brain weight not available for one control.				

### Brain-tissue Urea Findings in PDD

Urea levels were increased in PDD cases compared to controls in every region analysed (Table 3 and Fig. 1). There was an average 5.5-fold increase in tissue-urea concentrations in the CG of cases compared with controls, and an average ~ 3.5 to 4.5-fold increase in cases in every other region.

Inter-regional concentrations of urea were highly consistent in both controls and cases, with no significant differences between any two regions. Control tissue-urea concentrations averaged ~ 9.4 mmol/kg and case concentrations 40.0 mmol/kg, showing a substantial, ~ 4-fold increase in case-control urea levels across the brain overall.

Neither of the substituted SN samples showed significant differences in urea concentrations compared with other cases in the cohort (see Additional File 2 for individual values). Case-control differences in SN-urea levels remained significant with exclusion of substituted SN controls C10 and C11.

Table 3  
Tissue Urea Concentrations in PDD Cases and Controls

Region	Controls (n = 9) (mmol/kg)	PDD Cases (n = 9) (mmol/kg)	Fold-Change	P-Value
<b>CB</b>	9.6 (2.7–16.4)	35.2 (15.4–55.0)	<b>3.7</b>	<b>0.003</b>
<b>MCX</b>	9.2 (2.5–16.0)	37.7 (15.7–59.7)	<b>4.1</b>	<b>0.002</b>
<b>PVC</b>	8.1 (2.3–13.9)	34.9 (14.6–55.2)	<b>4.3</b>	<b>0.001</b>
<b>HP</b>	9.0 (2.6–15.4)	37.6 (10.7–64.4)	<b>4.2</b>	<b>0.004</b>
<b>SN</b>	11.6 (3.4–19.9)	45.8 (17.3–74.2)	<b>3.9</b>	<b>0.006</b>
<b>MTG</b>	11.0 (2.3–19.6)	46.9 (17.9–76.0)	<b>4.3</b>	<b>0.002</b>
<b>MED</b>	8.5 (2.3–14.7)	38.9 (14.9–62.9)	<b>4.6</b>	<b>0.002</b>
<b>CG</b>	8.8 (3.0–14.6)	48.4 (17.6–79.1)	<b>5.5</b>	<b>0.0008</b>
<b>PONS</b>	8.8 (2.2–15.5)	34.2 (15.4–53.0)	<b>3.9</b>	<b>0.003</b>

Mean tissue-urea concentrations  $\pm$  95% confidence intervals expressed in mmol/kg. Significance of case-control differences was determined using the Mann-Whitney-U test wherein  $P < 0.05$  has been considered significant. Fold-changes are cases compared to controls.

## Comparisons with Brain-urea Concentrations in AD and in HD

Our group has previously reported severe and regionally-widespread increases in brain-tissue urea concentrations in cases with AD dementia (9), and with HD dementia (10, 11). In these studies, AD and HD brain-tissue urea values were reported from multiple regions compared between cases and matched controls (Table 4, Fig. 2; refs (8–10)).



Although there are some differences in the methodologies used in the two former studies compared with the current one (such as previous use of gas chromatography-mass spectrometry, and some differences in the regions studied; Fig. 2), case-control tissue-urea fold-changes can be compared between PDD, HD, and AD (Table 4) across several brain regions.

Cases and controls in the HD study were slightly younger than those in the current PDD cohort, at an average of around three years for controls and eight years for cases (10, 11). Moreover, PMD was significantly lower than in the current PDD cohort, at an average of ~ 11 hours in both cases and controls. Cause of death in the HD cohort was most commonly bronchopneumonia in cases and heart failure in controls, but data on comorbidities and other vascular pathology were unavailable. Brain tissue-urea concentrations showed a similar average fold-increase to that measured in PDD of 3.5 in HD cases compared to controls (Table 4, Fig. 2). As in PDD, the increase was observed in the SN, CB, HP, MTG, CG, SCX, and MCX, as well as in other regions not included in the current PDD study, such as the putamen, globus pallidus, middle frontal gyrus, and entorhinal cortex.

The AD cases whose brain-tissue urea levels we measured were similar in age to the current PDD cohort, with an average of ~ 70 years in both cases and controls (9). PMD was also significantly shorter in this cohort, at an average of 9 hours in controls and 7 hours in cases. Causes of death were varied in both cases and controls, most commonly being due to heart or lung complications. Comorbidity and vascular pathology metadata were not available. Although absolute tissue-urea concentrations cannot be directly compared to the previous studies in AD brains, there was a higher average case-control fold-change in AD than that observed here in PDD, the average being ~ 5.3-fold, but with increases as high as 6.5-fold in the HP (9) (Table 4). Tissue-urea elevations in this AD cohort were observed in the HP, MTG, SCX, MCX, CG, and CB, as well as in the entorhinal cortex (which was not included in the current study). The AD cohort was similar in age to the current PDD cohort.

Table 4  
Urea fold-changes in PDD, AD, and HD

Region	Fold-Change in PDD (this study)	Fold-Changes in AD (Xu et al, 2016)	Fold-Changes in HD (Patassini et al, 2015)
CB	3.7	4.9	3.6
MCX	4.1	5.0	3.4
PVC	4.3	4.9	3.4
HP	4.2	6.5	3.6
SN	3.9	-	3.5
MTG	4.3	4.7	3.4
MED	4.6	-	-
CG	5.5	5.3	3.5
PONS	3.9	-	-
PUT	-	-	3.7
GP	-	-	3.6
MFG	-	-	3.0
ENT	-	5.6	2.8
<b>Overall</b>	4.3 (3.9–4.6)	5.3 (4.8–5.8)	3.5 (3.2–3.6)

Comparisons of case-control fold-changes in human brain urea levels between PDD, AD and HD. Case-control differences were significant for every region in every disease. Overall shows mean overall fold-change with  $\pm 95\%$  confidence intervals.

Additional studies have also confirmed urea increases in AD (18) and HD (12) brains, although one study reported decreases in the HD striatum and no change in the HD frontal lobe (19). Such discrepancies may be contributed to by differences in methods used or between cohorts, such as differences in PMD. However, we have previously observed that PMD does not affect urea levels for up to 72 hours in rat brains (20), although this may not necessarily also be the case in human brains.

Together, the observations in PDD, AD, and HD suggest a shared pathogenic mechanism in these three diseases, despite apparent differences in causative processes and symptomology. There appear to be regional differences between diseases with respect to tissue-urea elevations. For example, AD showed the highest increases in the severely-affected HP (6.5-fold), whereas in HD the putamen showed slightly higher-than-average elevations (3.7-fold). These regional differences may contribute to differences in severity and pathogenesis in the different conditions. Interestingly, the CG showed the highest urea

increase in PDD (5.5-fold); the anterior CG is involved in emotive states and emotionally-coded memories. All other studied regions of the PDD brain showed urea increments of around 3.5- to 4.5-fold. However, there were no statistically significant differences between different PDD-control regions or between different PDD-case regions. Greater power (by increasing the sample size), would probably be required to determine whether the fold-elevation observed in the CG is significantly greater than that of other regions of the PDD brain. If so, it is possible that the prominence of both motor and cognitive dysfunction in PDD correlates with similar tissue urea elevations in areas involved in both cognition and motor control processes – a future investigation into regional tissue urea dysregulation in PD brains without dementia could help elucidate this and would probably be a logical next step.

Urea's main metabolic function is to provide a route for the excretion of nitrogen-containing moieties derived from toxic nitrogen-containing compounds. In the urea cycle, ammonia is converted into urea, which is then excreted from the body via the urine. Urea is mainly produced in the liver and is carried to other parts of the body via the blood stream. However, it is slow to cross the blood-brain barrier (BBB) (21), leading to the question as to whether cerebral urea does in fact enter the brain through the BBB, or whether it is also produced in the brain. There is little evidence for the presence of intracerebral urea cycle activity, although there have been limited observations of partial activity in some studies. For example, ornithine transcarbamylase, an enzyme essential to the urea cycle was initially reported to be expressed only in AD brains (22), but has also been observed in healthy control brains in limited amounts (23, 24). However, studies attempting to investigate the levels of other urea cycle enzymes in HD sheep model brains for example were unable to identify either ornithine transcarbamylase or carbamoyl phosphate synthetase I in the striatum (12). Additionally, a more recent large-scale proteomic study of six regions of human AD and control brains was unable to identify the presence of either of these urea cycle enzymes at the protein level (25). Some other urea cycle components such as adenosine (9, 26, 27), citrulline (28), and ornithine (18, 28) have been reported in the AD brain, as well as the HD brain (11, 19) – however, as these metabolites are also involved in other metabolic pathways, this does not necessarily indicate urea cycle activity. For example, adenosine is a core component of several widespread co-enzymes such as adenosine triphosphate (ATP), diphosphate (ADP), and monophosphate (AMP) which are crucial to a wide variety of metabolic pathways including the ETC (29), TCA cycle (30), and purine metabolism (31). As such, it seems likely that cerebral urea may be formed by an alternative process and not the urea cycle itself.

Several urea cycle intermediates have also been reported to be dysregulated in PD serum, plasma, and CSF. Arginine has been reported by some investigations to be decreased in PD serum (32) and CSF (33), although other reports have observed no changes in either the serum (14), CSF (32, 34), or plasma (33). There is one report of increased citrulline in PD serum (35), although another investigation reported no change (14), and further reports indicated no change in the CSF (33, 34) or plasma (33, 36). Increases in PD serum ornithine have been reported (14), with varying reports in the CSF of decrease (33), increase (37), or no change (34). Moreover, no changes have been reported by several groups in studies of plasma ornithine in PD (33, 36, 37). None of these urea cycle components have been reported on in the PD/PDD brain itself, and the investigations of peripheral levels in the plasma, CSF, and serum have reported

inconsistent results. As such, this entire pathway, and the possibility of partial or whole urea cycle activity in the brain, presents a novel area for future investigation of PDD.

Protein dysregulation and neuronal death may lead to greater protein breakdown, and so to increased urea production. Disruptions to the BBB, as observed in PDD (38), may result in defective urea clearance from the brain or increased entry of urea from the bloodstream via urea transporters. Urea transporters are responsible for regulating movement of urea by facilitating urea diffusion, and are expressed in astrocytes and the BBB as well as outside the brain. Urea transporter B has been shown to be upregulated in the HD CB, which may reflect attempts to clear elevated urea levels in the HD brain (12). Urea transporters have not yet been investigated in PD/PDD, but may show similar perturbations to those reported in HD.

Urea accumulation caused by kidney failure is known to be toxic to the brain, leading to a condition called uraemic encephalopathy (39). High urea levels lead to synaptic loss and inhibition of long-term potentiation via carbamylation of mTOR in a chronic kidney disease mouse model (40). Carbamylation is a post-translational modification involving the addition of isocyanate, usually derived from urea, to protein-bound amino-acid residues, causing alterations in the structure and function of the affected proteins. Increased carbamylation has been observed in aging humans (41), and as a result of chronic kidney disease (42) as well as in AD with cerebrovascular disease (43). It has been shown that tau, which is aggregated in AD and also to a lesser degree in PDD, can be carbamylated, resulting in increased amyloid formation and tau accumulation (44). Whether  $\alpha$ -synuclein may also be carbamylated is unknown, but it does contain several potentially-susceptible amino-acid residues, which might serve as target sites for carbamylation.

High urea levels in chronic kidney disease and renal failure have also been linked to increased oxidative stress (45). Oxidative stress is a well-recognised feature of PD, AD, and HD (19, 46) and is linked to other pathogenic mechanisms including mitochondrial dysfunction and proteinopathy (47), dysregulated glucose metabolism (48), insulin resistance and inflammation (49), and  $\alpha$ -synuclein accumulation, oligomerisation, and phosphorylation (50, 51). As such, it is possible that elevated urea levels in PDD could contribute to one or more of these known pathogenic mechanisms.

## Conclusions

In conclusion, this investigation shows widespread urea accumulation throughout the PDD brain, similar to that previously observed in AD and HD, with concentrations comparable to those seen in uremic encephalopathy. This suggests a novel shared pathogenic insult across multiple neurodegenerative conditions and may indicate a common mechanism in the development of cognitive impairment.

## Abbreviations

AD Alzheimer's disease

ADP Adenosine diphosphate

AMP Adenosine monophosphate

ATP Adenosine triphosphate

BBB Blood-brain barrier

CB Cerebellum

CG Cingulate gyrus

CSF Cerebrospinal fluid

ENT Entorhinal cortex

ETC Electron transport chain

GP Globus pallidus

HD Huntington's disease

HP Hippocampus

MED Medulla oblongata

MCX Motor cortex

MFG Middle frontal gyrus

MTG Middle temporal gyrus

mTOR Rapamycin

PD Parkinson's disease

PDD Parkinson's disease dementia

PMD Post-mortem delay

PUT Putamen

PVC Primary visual cortex

SCX Sensory cortex

SN Substantia nigra

TCA Tricarboxylic acid cycle (AKA Kreb's cycle or citric acid cycle)

UHPLC-MS/MS Ultra-high performance liquid chromatography-tandem mass spectrometry

## **Declarations**

### **Ethics Approval and Consent to Participate**

The studies involving human participants were reviewed and approved by Manchester REC (09/H0906/52+5). The patients/participants or their families provided their written informed consent to participate in this study.

### **Consent for Publication**

Not applicable.

### **Availability of Data and Materials**

The datasets supporting the conclusion of this article are included within the article and Additional File 2. Additional patient metadata is available upon request.

### **Competing Interests**

The authors declare that they have no competing interests.

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### **Authors' Contributions**

MS participated in the conception and design of this study, and was responsible for acquisition, analysis, and interpretation of the data, as well as writing and editing of the manuscript. SJC participated in design

of the methodologies, acquisition of data, and editing of the manuscript. JX was responsible for acquisition and analysis of AD Auckland cohort data. SP was responsible for acquisition and analysis of HD Auckland cohort data. FR, NMH, and RDU participated in conception of this study and editing of the manuscript. GJSC participated in conception and design of this study, as well as editing of the manuscript. All authors read and approved the final manuscript.

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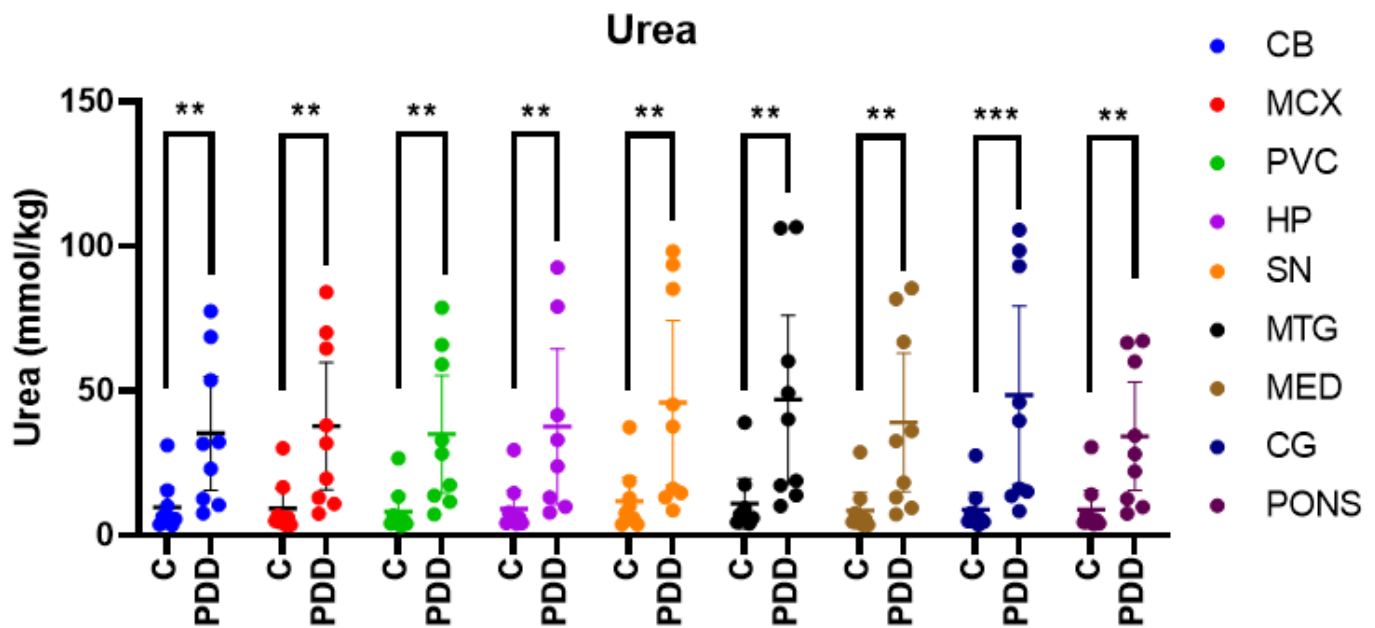
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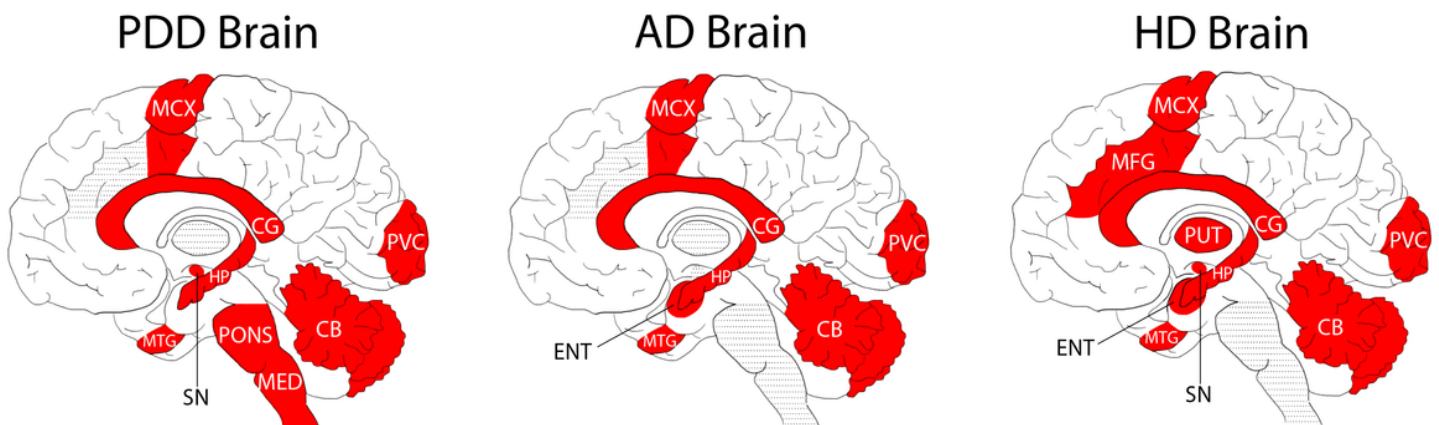
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## Figures



**Figure 1**

Urea Concentrations in PDD Cases and Matched Controls. Mean tissue-urea concentrations  $\pm$  95% confidence intervals expressed in mmol/kg. Case-control differences were determined by applying the Mann-Whitney-U test. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . C = control; PDD = Parkinson's disease dementia case



**Figure 2**

Regional Distribution of Measured Tissue Urea Increases in the PDD, AD, and HD Brain. Areas shaded in red denote significant increases in urea compared to intra-cohort controls. Areas shaded in grey were not investigated in the illustrated studies.

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