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Exploring the putative role of kallikrein-6, calpain-1 and cathepsin-D in the proteolytic degradation of α-synuclein in multiple system atrophy

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Abstract

Aims. There is evidence that accumulation of α-synuclein (α-syn) in Parkinson's disease (PD) and dementia with Lewy bodies (DLB) results from impaired removal of α-syn rather than its overproduction. Kallikrein-6 (KLK6), calpain-1 (CAPN1) and cathepsin-D (CTSD) are among a small number of proteases that cleave α-syn and are dysregulated in PD and DLB. Our aim in this study was to determine whether protease activity is altered in another α-synucleinopathy, multiple system atrophy (MSA), and might thereby modulate the regional distribution of α-syn accumulation.

Methods. mRNA and protein level and/or activity of KLK6, CAPN1 and CTSD were measured and assessed in relation to α-syn load in multiple brain regions (posterior frontal cortex, caudate nucleus, putamen, occipital cortex, pontine base and cerebellar white matter), in MSA (n = 20) and age-matched post-mortem control tissue (n = 20).

Results. CTSD activity was elevated in MSA in the pontine base and cerebellar white matter. KLK6 and CAPN1 levels were elevated in MSA in the putamen and cerebellar white matter. However, the activity or level of these proteolytic enzymes did not correlate with the regional distribution of α-syn.

Conclusions. Accumulation of α-syn in MSA is not due to reduced activity of the proteases we have studied. We suggest that their upregulation is likely to be a compensatory response to increased α-syn in MSA.

Abbreviations

AD	-	Alzheimer's disease
ALP	-	autophagy-lysosomal pathway
α-syn	-	α-synuclein
CAPN1	-	Calpain-1
CSF	-	cerebrospinal fluid
CTSD	-	Cathepsin D
DLB	-	dementia with Lewy bodies
GCIs	-	glial cytoplasmic inclusion
KLK6	-	kallikrein-6
MSA	-	multiple system atrophy
NAC	-	non-amyloid-β component
NCI	-	neuronal cytoplasmic inclusion
NNI	-	neuronal nuclear inclusion
PD	-	Parkinson's disease
SNCA	-	α-synuclein gene
UPS	-	ubiquitin-proteasomal pathway

Introduction

Abnormal aggregates of α-synuclein (α-syn) within neurons, neurites or glial cells are the defining neuropathological hallmark of the α-synucleinopathies. In Parkinson's disease (PD) and dementia with Lewy bodies (DLB), the aggregates form characteristic neuronal and neuritic inclusions (Lewy bodies and neurites), while multiple system atrophy (MSA) is characterised by α-syn-containing glial cytoplasmic inclusions (GCIs) in oligodendrocytes. Smaller numbers of neuronal nuclear or cytoplasmic inclusions (NNI or NCI) are also a feature of the disease. In α-synucleinopathies, α-syn accumulates without increased *SNCA* mRNA expression [1-3], suggesting that impaired protein clearance may play a role in the pathogenesis of these disorders. The ubiquitin-proteasomal pathway (UPS) and the autophagy-lysosomal pathway (ALP) are major routes of α-syn clearance and have been shown to be dysregulated in PD and DLB (reviewed in [4-6]). Cathepsin D (CTSD) is the main lysosomal protease responsible for α-syn cleavage [7-9]. CTSD-mediated α-syn cleavage reduces aggregation and toxicity of α-syn both *in vitro* and *in vivo* [7, 8], and is upregulated, probably as a compensatory response to elevated α-syn, in DLB and PD [10].

A small number of non-lysosomal proteases including kallikrein-6 (KLK-6) (neurosin, zyme, protease M) [11-13] and calpain-1 (CAPN1) [14] have been shown to cleave and regulate α-syn level, both *in vitro* and *in vivo*. KLK6 and CAPN1 proteases cleave monomeric α-syn within the non-amyloid-β component (NAC) region, are required for α-syn aggregation [15, 16], and generate cleavage fragments that are less neurotoxic and inhibit α-syn aggregation *in vitro* [8, 11, 12]. KLK6 is expressed in neurons and oligodendrocytes [17-19]; it is found within Lewy bodies and GCIs [18, 20] and degrades extracellular α-syn [13]. Brain-targeted, viral vector-mediated upregulation of KLK6 protects against α-syn pathology in

animal models of DLB [21] and MSA [22]. CAPN1 is expressed neuronally and co-localises with α-syn in the cytosol [23]. CAPN1 is capable of degrading both monomeric and fibrillar α-syn [14]. Degradation of fibrillar α-syn by CAPN is, however, associated with accelerated α-syn aggregation, suggesting a complex relationship between CAPN1 activity and α-syn pathogenicity. We, and others, demonstrated that the expression and activity of KLK6 and CAPN1 are reduced in DLB and PD in human tissue and CSF [21, 23, 24] and in animal models [21], and that the extent of reduction correlates closely with α-syn accumulation [23].

In this study we have explored the hypothesis that a reduction in proteolytic cleavage of α-syn is responsible for the elevated α-syn level in MSA. We have measured KLK6, CAPN1 and CTSD in MSA and assessed their association with α-syn load in brain regions with varied predilection for MSA pathology. Unlike in DLB, our findings do not support the hypothesis that the accumulation of α-syn in MSA is associated with reduced proteolytic clearance. The upregulation of the three proteases in regions with high α-syn load is probably a physiological (if ineffective) response to the increase in α-syn.

Materials and Methods

Case selection

20 MSA and 20 control brains were used for biochemical assessment of enzyme protein/activity and measurement of α-syn load (See Table 1). Subsets of 6 control and 6 mixed MSA cases were used for NanoString nCounter mRNA expression studies and 7 control and 7 mixed MSA cases for western immunoblotting. Mixed MSA cases were

selected, based on Ozawa criteria [25]. MSA can be subdivided according to the distribution of pathology into three subcategories: striatonigral degeneration (SND), olivopontocerebellar atrophy (OPCA), and a combination of the two known as mixed MSA. For the current study, we chose to use mixed MSA cases (hereafter referred to simply as MSA cases) to allow us to compare the findings in multiple brain regions within a single dataset.

Human brain tissue

All brains had been donated to the Queen Square Brain Bank for Neurological Disorders, UCL Institute of Neurology. The brain donation programme and protocols have ethical approval from the NRES Committee London–Central, and tissue is stored for research under a licence issued by the Human Tissue Authority (No. 12198).

The right hemisphere was sliced, and the slices flash frozen and stored at -80°C. Tissue was dissected from the frontal lobe, caudate nucleus, putamen, pontine base, occipital lobe and cerebellar white matter and homogenised in lysis buffer: 50mM Tris, 175 mM NaCl, protease and phosphatase inhibitor tablet and 1% Triton-X (all reagents from Roche/Merck, UK), in a Precellys 24 homogeniser with ceramic beads (Bertin technologies, France). The left half-brain was fixed in 10% buffered formalin, sliced, tissue blocks selected for paraffin wax embedding, and 8 µm sections prepared for histology.

NanoString nCounter analysis

We used NanoString nCounter Human Inflammation panel v2 mRNA Expression Assay (NanoString Technologies, Seattle, WA) to quantify transcripts of the three α-syn-degrading enzymes and α-syn. Total mRNA was isolated from frontal lobe and cerebellar white matter

of 6 control and 6 MSA cases (Table 1). 150 ng of total RNA from each sample was analysed. Probes were designed according to the manufacturer's design principles [26], including screening for inter- and intra-reporter and capture probe interactions, and selection for probes with optimal melting temperature [26]. The laboratory running the assay was blinded to the diagnosis. To avoid run-order bias, samples of cases or controls were randomly assigned to plates. Raw counts were subjected to a technical normalisation and normalized to the geometric mean using nSolver Analysis Software v2.0 (NanoString). For biological normalization, we used the geometric mean for all house-keeping genes (*CLTC, GAPDH, GUSB, HPRT1, PGK1, TUBB*) included in the CodeSet. The NanoString data were analysed using NanoString nSolver software. Data from NanoString nCounter expression counts were inspected and any samples determined to be outliers relative to negative controls were excluded from further analysis. The count data were normalised to negative controls and to positive controls according to the manufacturer's instructions, correcting for differences in hybridization efficiency and processing variables including purification and RNA/reporter complex immobilisation. Two-tailed Student's *t* tests were used to test for significant difference in gene expression between MSA and control frontal lobe and cerebellar white matter. The Benjamini-Yekutieli method was used to exclude false positive results.

Quantification of α-syn load

Immunohistochemistry was performed with anti-α-syn (Catalogue number ab15530 at 1:1300) (Abcam, UK) for 1 h and used a modified method for automated Intellipath FLX staining system (Menarini Diagnostics, Wokingham, UK) as per the manufacturer's instructions. Sections were pre-treated in the Menarini unit with an excess of super buffer

and mounted in DPX with glass coverslips. Immunolabelled sections were processed by digital slide scanner and images acquired using a Leica Aperio Imagescope. The percentage section area immunopositive for α-syn was determined by applying an Image J colour-thresholding macro.

Western immunoblotting

Protein determination was performed by BCA assay (Pierce) as per the manufacturer's instructions. For SDS-PAGE and western immunoblotting we used the XCell surelock blot system (Thermo Fisher Scientific). Samples diluted 20 µg/20 µl in MES buffer (Thermo Fisher Scientific) were run at 120 V. Proteins were transferred from the gel to nitrocellulose membrane (GE healthcare) in 1X transfer buffer (Thermo Fisher Scientific) at 37 V for 80 min on ice. The blots were analysed using the Odyssey system. The integrated area-density of immunoreactive bands at the predicted molecular weight for each protein of interest was quantified using Image J. Statistical analysis was performed using a two-tailed student *t* test in which significance was set at $p < 0.05$.

Kallikrein-6 (KLK6) sandwich ELISA

KLK6 level was determined in brain tissue homogenates by sandwich ELISA that was developed in-house, as previously described [23]. Mouse monoclonal anti-KLK6 antibody (Sigma Aldrich, Dorset, UK) diluted in PBS (1:200) was used to coat wells in clear Costar™ high-binding 96-well microplates (R&D systems, Abingdown, UK) and was left overnight at room temperature (RT). The plates were washed in PBS 0.01% tween-20 five times and blocked for 3 h in 1%BSA/PBS at 26°C. Following a further wash step, tissue homogenates (6.5 µg total protein) and human recombinant standards (R&D systems, UK) diluted in PBS

containing 1% BSA were added overnight at 4°C. After another wash, biotinylated goat polyclonal anti-KLK6 antibody (1 µg/ml) (R&D systems, Cambridge, UK) diluted in 1 % BSA/PBS, was added for 2 h at 26°C. After further washes, streptavidin:HRP (1:200) (R&D systems, UK) diluted in PBS/0.01% tween-20 was added for 1 h at 26^{o}C, the plate was washed, and chromogenic substrate (TMBS, R&D systems, UK) added for 20 min in the dark. The reaction was stopped with 2 N sulphuric acid, and absorbance read at 450 nM in a FLUOstar Optima plate reader. KLK6 concentration was determined by interpolation against measurements on serial dilutions of recombinant human KLK6 (720-11.25 ng/ml) included on each plate. All sample measurements were made in duplicate.

Calpain-1 (CAPN1) fluorogenic activity assay

CAPN1 activity was measured in brain tissue homogenates by use of an internally quenched fluorogenic substrate for CAPN1, as previously described [23]. The substrate included the CAPN1-specific cleavage site in α-spectrin (1 µM, H-K(FAM)-EYY ~ GMMK(DABCYL)-OH (Millipore, Hertfordshire, UK)) and the assays were performed with or without the addition of a calpain-1-specific inhibitor (10 µM Tocris, Bristol, UK). Brain tissue homogenates and purified human CAPN1 (Sigma Aldrich, Dorset, UK) were diluted in assay buffer (10 µl of homogenate in 40 ul assay buffer, containing 50 mM Tris-HCL, 50 mN NaCL, 5 mM β-mercaptoethanol, 5 mM CaCl2, 1 mM EGTA, 1 mM EDTA) pre-warmed to 37°C as described by Mittoo et al [27]. Each sample and standard was added in duplicate to wells, with or without inhibitor, and left for 10 min at 37°C, prior to addition of the fluorogenic substrate which was also diluted in assay buffer. The reaction was left to proceed at 37°C for 3 h in the dark after which fluorescence was read in a FLUOstar Optima plate reader with excitation at 490 nm and emission at 518 nm. For each sample the fluorescent signal in the inhibited

wells was subtracted from that in the uninhibited wells. Measurements were repeated on two separate occasions. The calpain-1 activity of each sample was interpolated from a standard curve derived from measurements on serial dilutions of calpain-1 (20-1.25 µg/ml); these were included in each plate to control for variation across plates.

Cathepsin D (CTSD) fluorogenic activity assay

CTSD enzyme activity was measured in brain tissue homogenates by adding a fluorogenic peptide substrate, Mca-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH2 [Mca= (7-methoxycoumarin-4-yl)acetyl; Dnp=dinitrophenyl] (BML-P-145-001) at 20 µM (Enzo Life Sciences, Exeter, UK) in the presence or absence of a specific cathepsin-D inhibitor (Pepstatin-A at 4µM). Brain tissue homogenates (1.25 µl in 1 ml assay buffer) or purified cathepsin-D from liver (BML-SE199, Enzo Life Sciences, Exeter, UK) was diluted in assay buffer made up of 0.1 M sodium acetate and 0.1 M sodium chloride at pH 3.5. Each sample or standard was added in duplicate and incubated with or without pepstatin-A for 10 min at 37°C prior to addition of the fluorogenic substrate. All components were diluted in assay buffer. The reaction was left to proceed at 37°C for 3 h in the dark after which fluorescence was read using a FLUOstar Optima plate reader with excitation at 320-340 nm and emission at 393-420 nm. For each sample the fluorescent signal in the inhibited wells was subtracted from that in the uninhibited wells. Each sample was measured in duplicate and measurements were repeated on two separate occasions. CTSD activity was interpolated from measurements on serial dilutions of serially recombinant CTSD that were included on each plate.

Statistical analysis

Parametric statistical tests were used for comparisons between groups, and ANOVA with Bonferroni post-hoc test was used for multiple-group comparisons. In some cases, the data required logarithmic transformation to obtain a normal distribution. For variables that were not normally distributed even after transformation, Kruskall-Wallis test was used, followed by Dunn's test for pairwise intergroup comparisons. Pearson analysis was used to assess the correlation between pairs of variables. Statistical tests were performed using SPSS version 16. P-values < 0.05 were considered statistically significant.

Results

KLK6, CTSD and CAPN1 mRNA

We examined KLK6, CAPN1 and CTSD mRNA level in frontal lobe and cerebellar white matter from MSA and control brains. KLK6 mRNA level was significantly elevated in frontal lobe in MSA (4.53-fold increase) ($P < 0.01$). KLK6 mRNA level in the cerebellar white matter did not differ between MSA and control brains. CTSD and CAPN1 mRNA were unchanged in MSA compared to controls in any of the regions studied (Fig. 1). As in previous studies ([2, 3], α-syn (*SNCA*) mRNA expression was not altered in MSA.

KLK6, CTSD and CAPN1 protein

We assessed KLK6, CAPN1 and CTSD protein level in a subset of brains, by western blot, in MSA and controls. KLK6 protein level was significantly elevated in the putamen ($P < 0.05$) but was unchanged in all other regions examined (Fig. 2). CAPN1 level, measured by western blot, was significantly increased in MSA in the putamen ($P < 0.05$) (Fig. 3) and cerebellar white matter ($P < 0.01$) (Fig. 3). CTSD level was unchanged in MSA in all regions (Fig. 4).

Regional distribution of α-syn load in MSA

We determined the α-syn load (percentage section area immunopositive for α-syn) as an indicator of regional variation in severity of GCI pathology in MSA. The level of α-syn was highest in the pontine base and putamen, intermediate in the cerebellar white matter, frontal cortex, and caudate nucleus, and lowest in the occipital cortex (Fig. 5).

Brain region variation in KLK6, CAPN1 and CTSD level or activity in MSA

We measured KLK6 protein level by ELISA, and CAPN1 and CTSD enzyme activities, in all regions, in the complete MSA and control cohorts. KLK6 level was significantly elevated in MSA in the putamen ($P < 0.05$) and cerebellar white matter ($P < 0.0001$) (Fig. 6A). CAPN1 enzyme activity was significantly elevated in the putamen ($P < 0.0001$) and cerebellar white matter ($P < 0.001$) (Fig. 6B). CTSD activity was significantly elevated in the pontine base and cerebellar white matter in MSA (Fig. 6C).

Brain region variation in KLK6, CAPN1 and CTSD in relation to α-syn load in MSA

Lastly, we assessed whether regional differences in α-syn load were related to KLK6, CAPN1 or CTSD level or activity. Across the six regions examined, α-syn load did not correlate significantly with KLK6 level, or with CAPN1 or CTSD level or activity. KLK6 was generally lower in regions with higher α-syn load (Fig. 7A), and CAPN1 and CTSD activity tended to be elevated (Fig. 7B-C), but none of these trends reached statistical significance.

Upregulation of KLK6, CTSD and CAPN1 in MSA

In cerebellar white matter from the combined cohort of controls and MSA cases, KLK6 level correlated positively with CTSD activity ($r = 0.64$, $p < 0.0001$) (Fig. 8A) and CAPN1 activity ($r = 0.44$, $p < 0.01$) (Fig. 8B), and CAPN1 activity correlated positively with CTSD activity ($r = 0.37$, $p < 0.05$) (Fig. 8C). No significant correlation between KLK6 level, CTSD activity and CAPN1 activity was observed in any of the other regions that we examined.

Discussion

We have examined the expression of three proteases, previously shown to regulate α-syn homeostasis, in MSA, in relation to the α-syn load in different regions of brain. KLK6, CAPN1 and CTSD were increased in MSA within the putamen, pontine base and cerebellar white matter – regions of high GCI burden and α-syn load in MSA. However, differences in α-syn load between regions were not correlated with the level or activity of KLK6, CAPN1 or CTSD. The findings contrast with our previous observations in PD and DLB [23], and indicate that accumulation of α-syn in MSA is unlikely to result from reduced activity of the proteases we have tested. The upregulation of these α-syn degrading enzymes in regions of high GCI burden and α-syn load is likely to be a compensatory response to elevated α-syn. It should be noted, of course, that we have only tested a small number of potential α-syn proteases, and it remains possible that others, such as matrix metalloproteinase-3 (MMP-3) [28] and endothelin-converting enzyme (ECE) [29], may be downregulated in MSA.

Abnormal accumulation of α-syn in neurons and glial cells is a characteristic hallmark of PD, DLB and MSA. There is no evidence that α-syn mRNA level is increased in α-synucleinopathies [1-3] suggesting that failure of proteolysis or removal by other means

may contribute to the pathogenesis of MSA. The endosomal-lysosomal and autophagic pathways are major routes of α-syn clearance and have been shown to be dysregulated in PD and DLB [4-6, 30] and MSA [31-34]. A small number of proteases have been identified that cleave and degrade intracellular and extracellular pools of α-syn *in vitro* and are down-regulated in PD and DLB [23]. KLK6, CAPN1 and CTSD are expressed neuronally, and within oligodendrocytes and are likely to impact on α-syn load in PD, DLB and MSA. KLK6, CAPN1 and CTSD all have major cleavage sites within the non-Aβ component (NAC) region which is required for α-syn aggregation, and cleavage was shown to inhibit aggregation of full-length monomeric α-syn [15, 16]. Brain-specific induction of KLK6 is protective against α-syn in mouse models of DLB [21] and MSA [22], and there is evidence that CTSD has protective effects in vivo [8]. We, and others, showed previously that KLK6 and CAPN1 are reduced in human brain tissue in PD and DLB and that the reduction is most marked in regions of cerebral cortex that contain the highest levels of α-syn [23]. It seemed therefore reasonable to hypothesise that a reduction in proteolytic cleavage might contribute to α-syn accumulation in MSA.

In the present study, we have explored the hypothesis that α-syn accumulation in MSA results, at least in part, from defective proteolytic clearance. We have chosen to study CTSD, CAPN1 and KLK6 in MSA because these enzymes are expressed within neurons and oligodendrocytes [17-19]. These proteases are found within Lewy body inclusions and GCIs [11], are partly co-localised with α-syn in SH-SY5Y cells, and regulate α-syn levels *in vivo* and *in vitro* [35-39]. CTSD is the primary enzyme responsible for lysosomal α-syn degradation [9] whereas KLK6 interacts with and degrades α-syn both intracellularly [11] and extracellularly [13, 36]. A reduction in proteolytic activity of KLK6 has been proposed to play a role in the

pathogenesis of α-synucleinopathies, including MSA [21-24]. Other α-syn proteases, such as MMP-3, are active extracellularly and have been implicated in the pathogenesis rather than protection against disease and are usually upregulated in PD and DLB [40].

The present findings do not support our hypothesis. Instead, KLK6, CAPN1 and CTSD were increased in MSA in regions that have a high α-syn load and pronounced MSA pathology: KLK6 and CAPN1 were elevated in the putamen and cerebellar white matter, and CTSD in the pontine base and cerebellar white matter. CTSD has previously been shown to be induced in animal models of PD [10] and co-localises with α-syn in nigral neurons in PD [41]. These data indicate that an CTSD is induced as an early compensatory lysosomal response to elevated α-syn in PD, probably similar to the upregulation of CTSD in response to abnormal protein accumulation in Alzheimer's disease [42, 43]. CTSD, KLK6 and CAPN1 levels are all elevated in MSA, particularly in the cerebellar white matter where we noticed a strong correlation between the proteases. Our finding of elevated KLK6 and CAPN1 in MSA is in contrast to previous findings in PD and DLB [21, 23].

Despite the marked up-regulation of KLK6, CAPN1 and CTSD in MSA in regions with the highest α-syn burden, we failed to detect an association with α-syn load within individual regions (data not shown) or across regions. A recent study revealed that induction of neuronal CTSD in the pons in MSA was independent of α-syn load [41]. It is unclear why KLK6 and CAPN1 should be increased in MSA; our data suggest that induction of all three proteases could be a protective mechanism, or perhaps a response to protein accumulation that is not specific for α-syn. Whether this response affords any protection against α-syn accumulation but eventually becomes overwhelmed or even contributes to disease pathology in MSA is unknown. The findings support other studies (reviewed in [44, 45]),

indicating that α-syn-degrading proteases may have different roles in the pathogenesis of MSA compared to PD and DLB, and that there is variation in the regulation of these proteases in different brain regions.

Assessing the potential role of these enzymes in the pathogenesis of α-synucleinopathies is further complicated by their potential role in the production of intermediate partially cleaved α-syn fragments that may drive the aggregation of full-length α-syn and accelerate pathology. For instance, CAPN1 generates C-terminally truncated fragments that seed the propagation of full-length α-syn following incomplete degradation of fibrillar α-syn [14, 46, 47]. CAPN1 is also increased in PD [48-50] and CAPN1 inhibitors are protective in an experimental mouse models of PD [49, 51]. Most evidence for KLK6 suggests that this enzyme has a protective function: i.e. proteolytic cleavage reduces α-syn α-syn aggregation and toxicity and facilitates clearance. The potential for CTSD cleavage fragments to promote aggregation remains to be determined.

There were differences between mRNA level and protein and enzyme activities across regions, particularly in the cerebellum. For instance, KLK6 mRNA was reduced but protein level, measured by ELISA, was significantly increased in the cerebellum in MSA. CTSD mRNA and protein level, assessed by western blot, were unchanged whereas CTSD activity was significantly increased in the cerebellum in MSA. Differences were also found in other regions. The divergence may partly be an artefact of sampling, reflecting the smaller cohort size for some of the analyses but could also reflect regional differences in regulation of the mRNA, protein level and activity of the enzymes, e.g. due to mRNA processing or post-translational modification of the protein. The variability in mRNA expression was most marked in the cerebellum, a vulnerable region in MSA, particularly in patients with the

OPCA-predominant pattern of neuronal loss (Figure 1). We had selected MSA-mixed cases for this study in an effort to avoid possible bias arising from the neuropathological subtype of the disease. However, it remains possible that, even at the end stage of the disease, in which neuronal loss affects olivopontocerebellar structures and striatonigral regions equally, mRNA expression may vary between regions with differential vulnerability to the pathological process. Future studies using larger numbers of cases stratified for neuropathological subtype will be required to explore this further.

Conclusion.

We have shown that KLK6, CAPN1 and CTSD proteases are upregulated in MSA, particularly in regions with a high α-syn burden. The findings indicate that α-syn is unlikely to accumulate in MSA due to reduced activity of these enzymes. The upregulation of KLK6, CAPN1 and CTSD in MSA may be a compensatory response to elevated α-syn load. Further studies are required to determine the precise role of these enzymes in the pathogenesis of MSA. Determining whether there are cell-specific changes in the distribution and expression of these three proteases in relation to the α-syn would also be informative.

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Author contributions

SL and JH were responsible for the conception and design of experiments; JSM and AK were responsible for acquisition of data; RC and CS performed the immunohistochemistry; JSM analysed and interpreted the data; all authors contributed to drafting the manuscript; SL and JH revised and reviewed the final article for intellectual content and final approval.

Ethical approval

We used brain tissue from cases donated to the Queen Square Brain Bank for Neurological Disorders, UCL Institute of Neurology. The brain donation programme and protocols have received ethical approval for research by the NRES Committee London – Central and tissue is stored for research under a license issued by the Human Tissue Authority (No. 12198).

Conflict of interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Figure Legends:

Figure 1. Graphical representation of fold change in mRNA expression in MSA (n= 6) compared to control (n = 6). Significantly greater KLK6 mRNA is detected in MSA frontal lobe compared to control (A). No significant difference in CTSD (B) or SNCA (C) mRNA expression is detected in MSA frontal lobe. No significant difference in detected in the mRNA expression of KLK6 (D), CTSD (E) or SNCA (F) in the cerebellar white matter of MSA cases compared to control. Bars indicate the mean and standard deviation.

Figure 2. Graphical representation of levels of kallikrein-6 (KLK6) in control (C) (n = 7) and MSA (M) (n = 7) following western blot analysis. Densitometry analysis of immunoblots were performed in triplicate and normalised to β-actin for frontal lobe, putamen, caudate nucleus, pontine base, cerebellar white matter and occipital lobe. Bars indicate the mean and standard deviation

Figure 3. Graphical representation of levels of calpain-1 (CAPN1) in control (C) (n = 7) and MSA (M) (n = 7) following western blot analysis. Densitometry analysis of immunoblots were performed in triplicate and normalised to β-actin for frontal lobe, putamen, caudate nucleus, pontine base, cerebellar white matter and occipital lobe. Bars indicate the mean and standard deviation

Figure 4. Graphical representation of levels of cathepsin-D (CTSD) (n = 6) in control (C) (n = 7) and MSA (M) (n = 7) following western blot analysis. Densitometry analysis of immunoblots were performed in triplicate and normalised to β-actin for frontal lobe, putamen, caudate nucleus, pontine base, cerebellar white matter and occipital lobe. Bars indicate the mean and standard deviation

Figure 5. Bar chart showing regional α-syn load in multiple system atrophy (n = 20). α-syn load represents the percentage section area immunopositive for α-syn determined in putamen (PUT) caudate nucleus (caudate), pontine base (PNS), cerebellar white matter (CBM), occipital lobe (OCC) and frontal lobe (FCX)). Bars indicate the mean and SEM. **P < 0.01 ***P < 0.001 ****P < 0.0001.

Figure 6. Bar charts showing differences in (A) KLK6 (B) CAPN1 and (C) CTSD in MSA (n = 20) and controls (n = 20) across regions with different predilection for pathology. KLK6 protein levels, measured by sandwich ELISA and CAPN1 and CTSD enzyme activities using fluorogenic peptide substrate activity assays were measured in MSA (n=20) and controls (n=20) in putamen (PUT) caudate nucleus (caudate), pontine base (PNS), cerebellar white matter (CBM), occipital lobe (OCC) and frontal lobe (FCX)). Bars indicate the mean and SEM. *P < 0.05 ***P < 0.001 ****P < 0.0001.

Figure 7. Regional association between (A) KLK6, (B) CAPN1, (C) CTSD and α-syn load in MSA (n = 20) and controls (n = 20). The protein level or activity of each protease was plotted against α-syn load in putamen (PUT) caudate nucleus (caudate), pontine base (PNS), cerebellar white matter (CBM), occipital lobe (OCC) and frontal lobe (FCX)) in MSA cases. The solid circles and thin bars indicate the mean values and SEM for α-syn load (horizontal bars) and protease (vertical bars). The thick solid and dotted lines indicate the best-fit linear regression and 95% confidence intervals.

Figure 8. Relationship between KLK6, CAPN1 and CTSD in cerebellum in a combined cohort of MSA (n = 20) and controls (n = 20). (A) KLK6 protein level (ng/ml) plotted against CTSD activity (relative fluorescence units (rfu)). (B) KLK6 protein level (ng/ml) plotted against CAPN1 enzyme activity (rfu). (C) CAPN1 enzyme activity plotted against CTSD enzyme activity (rfu). The solid circles represent the mean level from each individual. The thick inner line and outer lines indicate the best-fit linear regression and 95% confidence intervals respectively.

Patient	Application used for	Gender	Age	Post Mortem delay (h:m)
Control 1	WB, IHC, A	F	83	**99:00**
Control 2	WB, IHC, A	F	94	**89.25**
Control 3	WB, IHC, A	F	79	**88:50**
Control 4	NS, CA, WB, IHC, A	F	80	**49:10**
Control 5	WB, IHC, A	F	86	**120:00**
Control 6	NS, CA, WB, IHC, A	F	93	**29:40**
Control 7	WB, IHC, A	M	69	**168.00**
Control 8	IHC, A	F	87	**51.45**
Control 9	NS, CA, IHC, A	M	87	**57.00**
Control 10	IHC, A	F	92	**63.5**
Control 11	IHC, A	F	87	**84.15**
Control 12	IHC, A	F	91	**53:25**
Control 13	IHC, A	F	86	**119:05**
Control 14	IHC, A	M	88	**97:30**
Control 15	IHC, A	M	84	**76.5**
Control 16	IHC, A	M	95	**89.2**
Control 17	NS, CA, IHC, A	M	88	**16:15**
Control 18	IHC, A	M	83	**60:00**
Control 19	CA, IHC, A	M	38	**80.35**
Control 20	CA, IHC, A	M	89	**38:30**
MSA 1	WB, IHC, A	F	68	**36:35**
MSA 2	NS, CA, WB, IHC, A	F	66	**23:20**
MSA 3	WB, IHC, A	F	62	**118:35**
MSA 4	WB, IHC, A	F	59	**48:15**
MSA 5	WB, IHC, A	M	69	**43:15**
MSA 6	WB, IHC, A	M	78	**100:15**
MSA 7	WB, IHC, A	F	71	**80:00**
MSA 8	IHC, A	F	70	**83:15**
MSA 9	IHC, A	F	70	**64:45**
MSA 10	IHC, A	M	72	**89:49**
MSA 11	NS, CA, IHC, A	F	60	**35:30**
MSA 12	IHC, A	M	66	**107:20**
MSA 13	IHC, A	M	51	**56:10**
MSA 14	IHC, A	F	52	**72:30**
MSA 15	IHC, A	F	68	**107:00**
MSA 16	IHC, A	M	54	**25:35**
MSA 17	NS, CA, IHC, A	M	63	**20:40**
MSA 18	IHC, A	F	55	**29:02**
MSA 19	IHC, A	M	63	**26:55**
MSA 20	CA, IHC, A	F	85	**89:00**

Figure 1

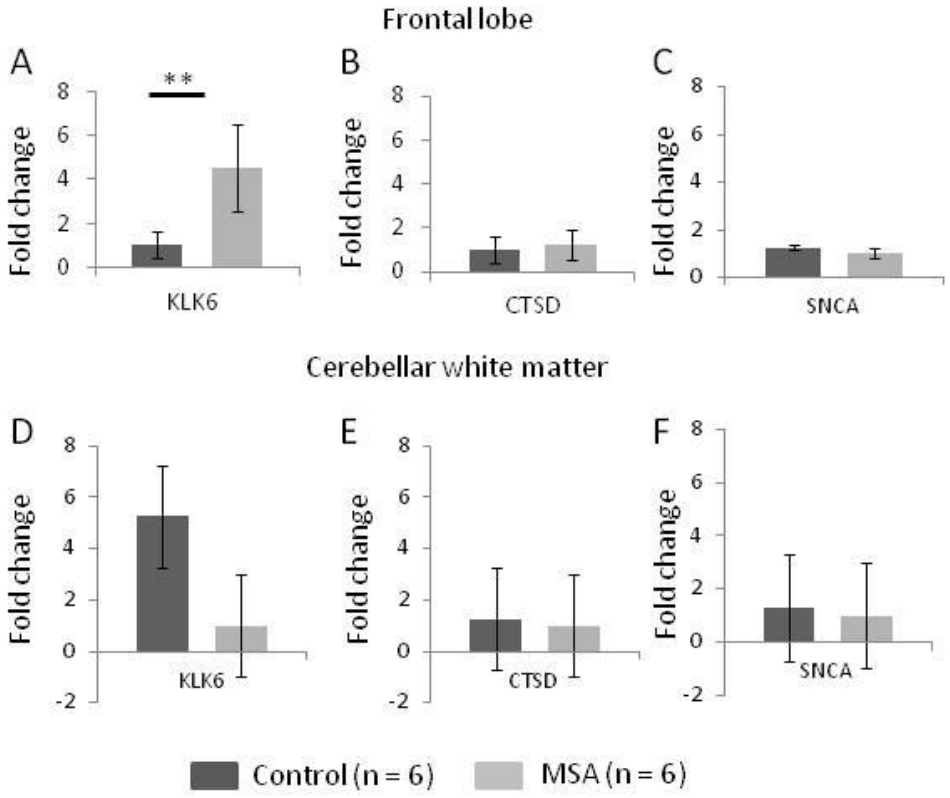

Figure 2

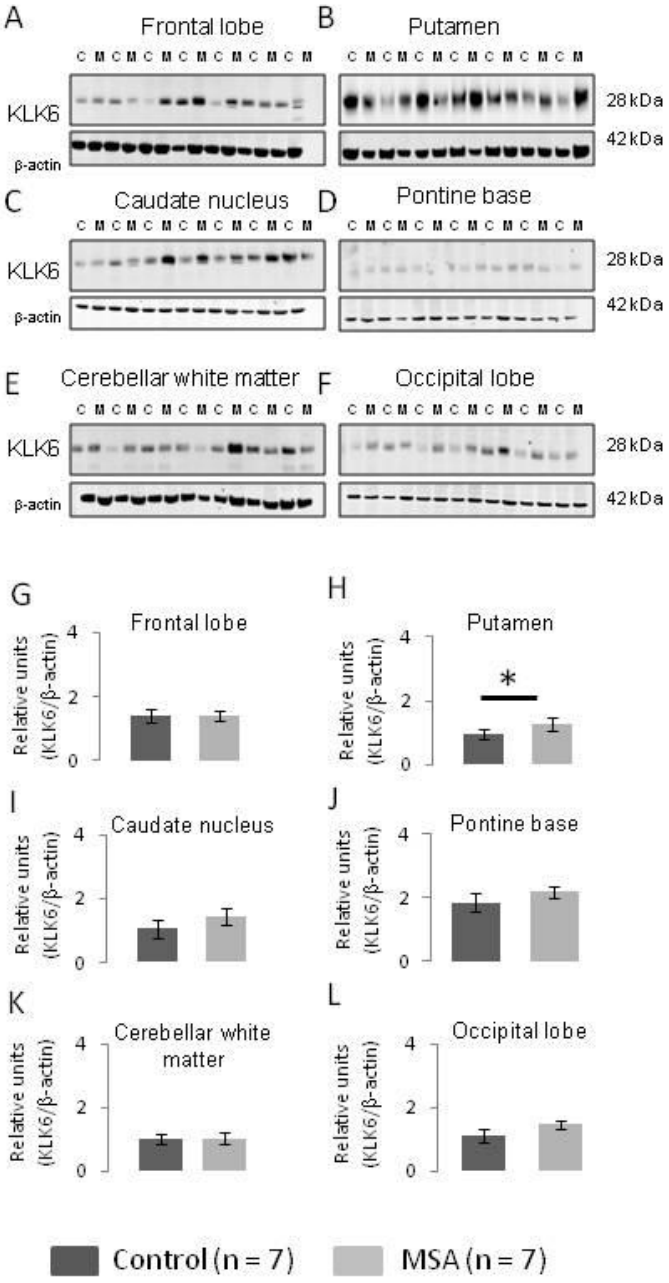

Figure 3

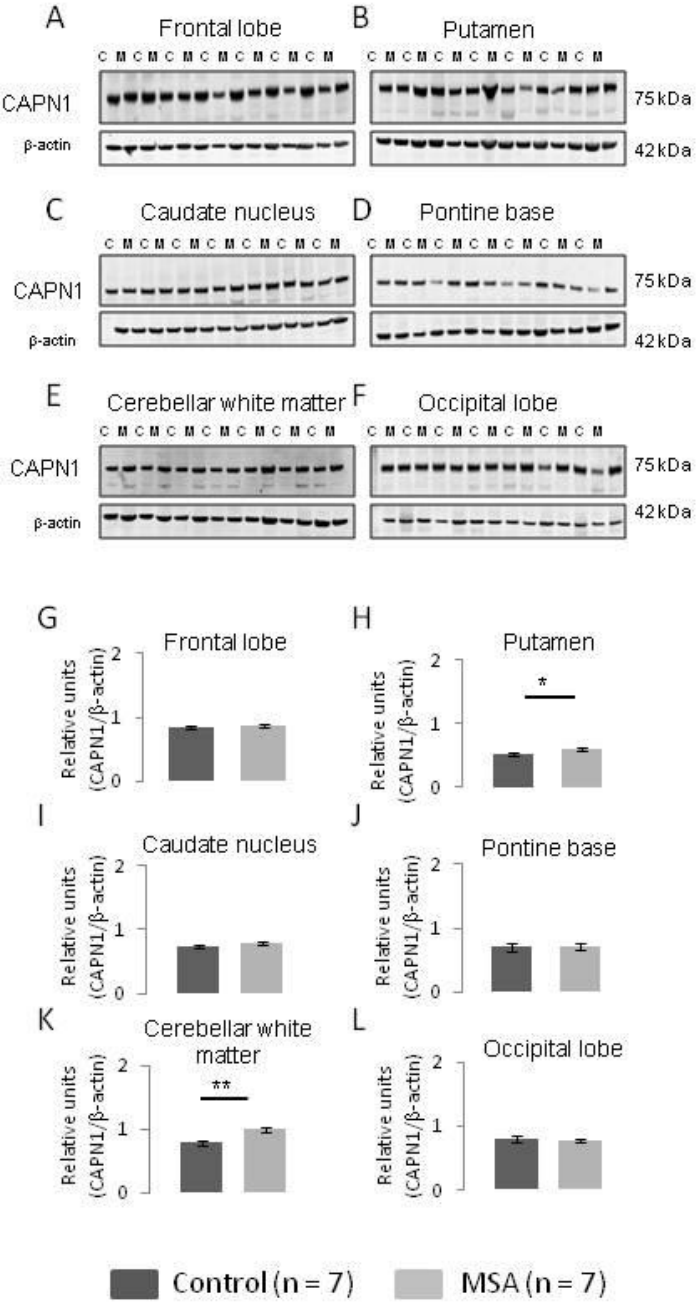

Figure 4