



Excess α -synuclein worsens disease in mice lacking ubiquitin carboxy-terminal hydrolase L1

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Mutations in α -synuclein (α SN) and ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) have been linked to familial Parkinson's disease (PD). Physical and functional interactions between these two proteins have been described. Whether they act additively *in vivo* to influence disease has remained controversial. α SN is a presynaptic protein and the major constituent of Lewy inclusions, histopathological hallmarks of PD. UCH-L1 regulates ubiquitin stability in the nervous system and its loss results in neurodegeneration in peripheral and central neurons. Here, we used genetics to show that UCH-L1-deficiency together with excess α SN worsen disease. Double mutant mice show earlier-onset motor deficits, a shorter lifespan and forebrain astrogliosis but the additive disease-worsening effects of UCH-L1-deficiency and excess α SN are not accompanied by microgliosis, ubiquitin pathology or changes in pathological α SN protein levels and species.

Parkinson's disease (PD) is a progressive neurodegenerative disorder that affects approximately 1% of the population older than 65 years of age. Disabling clinical manifestations are caused by a selective loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc) and include tremor, bradykinesia and stiffness. DA neuronal cell loss is accompanied by an immunopathology in DA and other neurons: eosinophilic intraneuronal inclusions referred to as either Lewy bodies (LBs) or Lewy neurites (LNs). Both are composed mainly of α -synuclein (α SN) and often also ubiquitin (Ub). Like in PD, Lewy inclusions are a defined pathological hallmark of another α -synucleinopathy disease referred to as dementia with Lewy bodies (DLB; for review see^{1,2}). DLB is second after the most common neurofibrillary pathology that characterizes Alzheimer's disease (AD) and often both pathologies co-occur in the same individual. In contrast to AD pathology, Lewy pathology is not restricted to central neurons but also occurs in peripheral neurons and nerves including the gastrointestinal nervous system and cardiac nerves³.

In recent years, hope has risen to better understand the pathophysiology of α -synucleinopathies. The evidence, that the accumulation of non-physiological levels and forms of the synaptic protein α SN plays a central role in disease pathogenesis is overwhelming⁴: (i) α SN accumulates in LBs⁵⁻⁷; (ii) specific mutations in α SN causing a single amino-acid change (A30P, A53T, E46K) as well as duplication or triplication of the wildtype α SN gene cause rare familial forms of parkinsonism⁸⁻¹¹; (iii) neuronal over-expression of either wildtype or mutant human α SN in transgenic flies, rodents and non-human primates causes α -synucleinopathy¹²⁻¹⁶. Nonetheless, the molecular mechanisms causing a non-physiological accumulation of α SN and/or α SN-induced neuronal dysfunction and death have remained largely elusive.

The enzyme ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) is primarily expressed in neurons and has been implicated in neurodegenerative diseases including PD as well as cancer^{17,18}. UCH-L1 protein is abundant and widely expressed in the brain (1–2% of total protein). The enzyme has de-ubiquitylating^{19,20} as well as ubiquitylating activity²¹, and the protein functions to stabilize mono-ubiquitin²². UCH-L1 protein is found in Lewy bodies²³. A missense mutation (I93M) in UCH-L1 (UCH-L1^{I93M}) was discovered in a German family affected by autosomal dominant familial PD²⁴. Other reports linking UCH-L1 variants as risk factors for PD have remained controversial²⁵⁻²⁷. Mice naturally lacking UCH-L1 develop gracile axonal dystrophy (gad), exhibit an age-dependent sensory ataxic phenotype and motor paresis, manifest a dying-back axonal degeneration in sensory and motor nerve terminals and display β -synuclein (β SN) and γ -synuclein (γ SN) pathology but, interestingly, lack α SN pathology²⁸⁻³⁰. Evidence from two additional different UCH-L1-deficient mouse mutants showed that UCH-L1 is required for maintaining the structure and function of central and peripheral synapses^{31,32}. None of these mutants seems to show pathological changes in dopaminergic neurons and the extent of viral vector-mediated expression and α SN-induced cell loss in DA neurons is exacerbated in UCH-L1^{I93M}

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transgenic but not in wildtype UCH-L1 (UCH-L1^{wildtype}) or gad mice³³. All these findings seem consistent with a gain-of-toxic function of the UCH-L1^{193M} mutant but little or no function of UCH-L1^{wildtype} or its lack in α SN-induced DA damage.

Also in line with this hypothesis are the reports that over-expression of the UCH-L1^{193M} mutant *in vitro* causes accumulation of α SN³⁴ and in mice leads to DA neuronal cell loss³⁵. Nonetheless, other findings suggest that also UCH-L1^{wildtype} has a role in α SN-induced neurotoxicity and α SN protein homeostasis: (i) different forms of UCH-L1 de-ubiquitinate poly-ubiquitinated α SN^{36,37} or act as an α SN ubiquitin ligase²¹; (ii) UCH-L1 levels are reduced and activity is down by 40-80% because of oxidative damage in idiopathic PD, DLB and AD brains^{38,39}; (iii) reducing the membrane-bound form of UCH-L1 in cell culture models of α SN toxicity reduces α SN levels and increases cell viability⁴⁰. Here, we used mouse genetics to further elucidate a potential synergistic effect of UCH-L1-deficiency and excess α SN in disease.

Results

Excess α SN worsens disease in UCH-L1-deficient mice. To test whether excess α SN and the lack of UCH-L1 have additive effects on disease worsening or outcome, we generated via breeding double transgenic mice over-expressing wildtype mouse α SN⁴¹ (Thy1-maSN) and lacking UCH-L1 [Deltagen (Uchl1^{tm1Dgen}; ID: MGI: 3604452)]. We studied UCH-L1-deficiency in the context of an

excess of murine wildtype α SN to avoid confounds that might arise because of cross-species differences in human and mouse α SN and/or UCH-L1 function. Double Uchl1^{tm1Dgen};Thy1-maSN transgenic mice (referred to as UCH-L1^{-/-}/Thy1-maSN hereafter), and their single mutant UCH-L1^{-/-} and Thy1-maSN littermates were subsequently characterized with respect to weight, muscle strength (grip strength), motor coordination (rotating beam performance) and α -synucleinopathy related changes at histopathological and biochemical levels. At 2.5 months of age, wildtype control littermates and Thy1-maSN mice showed no difference in weight (Figure 1A), grip strength (Figure 1B) or rotating beam performance (Figure 1C and 1D). In contrast, at this age UCH-L1^{-/-} mice showed already a slight weight-loss (Figure 1A) and motor deficits on the rotating beam (Figure 1C and 1D), a phenotype similar to that described for UCH-L1 deficient gad mice⁴². Muscle weakness in UCH-L1^{-/-} mice was not yet apparent at the age of 2.5 months (Figure 1B) but very prominent at the age of 4.5 months (0.62 N \pm 0.14 versus littermate controls 1.32 N \pm 0.14). Heterozygous UCH-L1 deficient mice did not show these deficits (data not shown). As compared to UCH-L1^{-/-} mice, 2.5 months old UCH-L1^{-/-}/Thy1-maSN double mutant mice showed greater deficits in both muscle strength (Figure 1B) and motor coordination (Figure 1C and 1D) whereas weight did not differ significantly (Figure 1A). At the age of 3.5 months, the general health state of double mutant mice was such, that the animals had to be

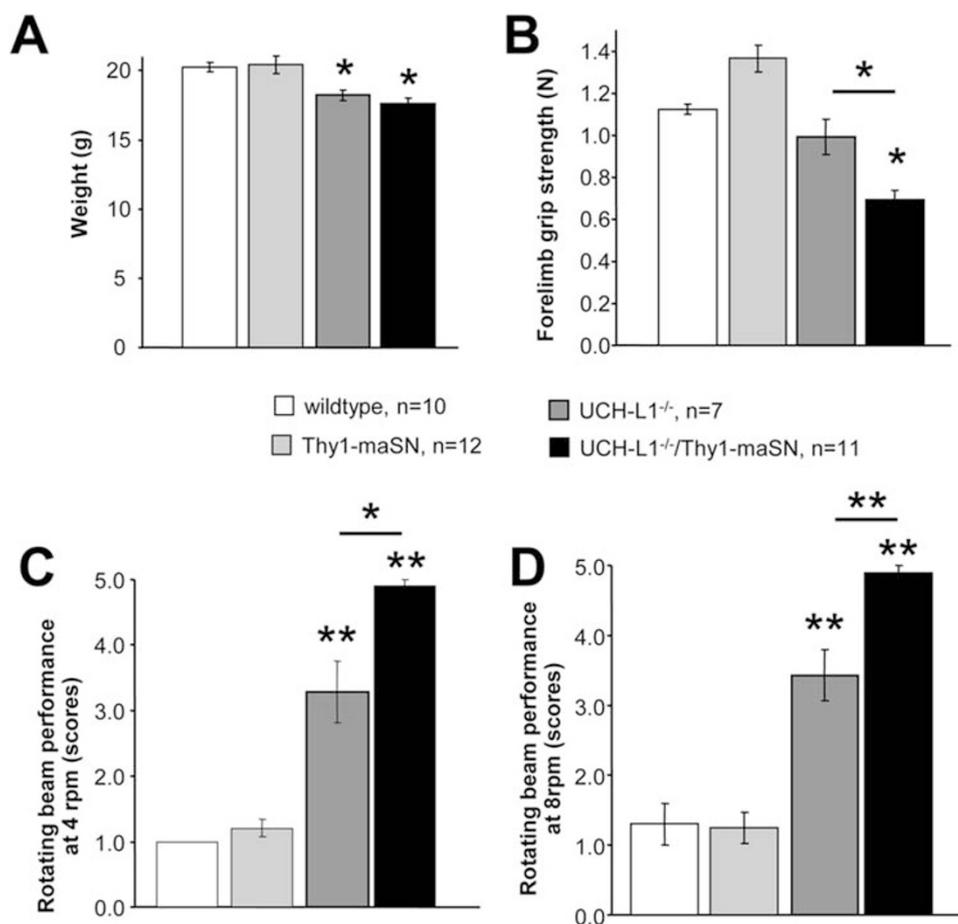


Figure 1 | Weight, muscle strength and motor behavior in UCH-L1^{-/-}/Thy1-maSN double transgenic mice. (A) Small weight-loss effect in UCH-L1^{-/-} and UCH-L1^{-/-}/Thy1-maSN mice as compared to the other genotypes and wildtype mice. (B) Significant reduction in forelimb grip strength in UCH-L1^{-/-}/Thy1-maSN mice as compared to unchanged strengths in the other genotypes. (C) Performance of motor coordination assessed on the rotating beam at 4 rpm. (D) Performance of motor coordination assessed on the rotating beam at 8 rpm. UCH-L1^{-/-}/Thy1-maSN mice are more severely impaired as compared to UCH-L1^{-/-} mice. Motor performance by the other genotypes was not impaired. Age of the mice: 2.5 months. Data are shown as mean \pm SEM. See colour code for the different genotypes. N numbers for each genotype are indicated. * $p < 0.05$, ** $p < 0.01$ (two-tailed, unequal variances Student's t-test).



sacrificed in agreement with local regulations for animal experimentation. It was clear that unlike their single UCH-L1^{-/-} and Thy1-maSN mutant littermates, double mutant mice would never reach an age of 5–6 months or older, as described for mice lacking either only UCH-L1⁴² or over-expressing mouse α SN⁴¹. For reference, UCH-L1^{+/-}/Thy1-maSN double and Thy1-maSN single mutant mice showed no significant differences in performance or health state (data not shown). Thus, disease worsening is only observed under conditions of excess murine α SN and a complete lack of UCH-L1.

UCH-L1 deficiency promotes early-onset astrogliosis in Thy1-maSN mice. Next, we determined whether UCH-L1-deficiency combined with excess wildtype α SN affected histopathological hallmarks of disease and/or pathological α SN protein levels and species. Over-expression of murine α SN resulted in increased perikaryal α SN accumulation in the cell soma as illustrated in hippocampal neurons (Figure 2). The same phenomenon occurs also in mice expressing human wildtype or A53T mutant α SN¹⁶ but with one distinction: mice expressing murine wildtype α SN appear to have less and a more diffuse neuritic α SN staining as opposed to the overall increased intensity and often focal accumulation type α SN staining patterns observed in neurites of mice expressing human α SN forms. Stainings of littermate brains with different genotypes were always done in parallel and at the same time using identical conditions so it remains to be determined whether epitope masking or other reasons account for this phenomenon in Thy1-maSN mice. Control littermates and age-matched UCH-L1^{-/-} mice did not show this transgene-specific pattern of perikaryal α SN immunolabeling in stratum pyramidale neurons. Instead, UCH-L1^{-/-} mice showed a for wildtype mice typical strong α SN immunolabeling in stratum radiatum and stratum oriens and virtually no staining in cell soma (Figure 2). At age 3.5 months, Thy1-maSN single and UCH-L1^{-/-}/Thy1-maSN double mutants showed similar patterns of perikaryal murine α SN accumulation in hippocampal neurons (Figure 2). Therefore, the lack of UCH-L1 by itself does not provoke a perikaryal accumulation of endogenous murine α SN and in double mutants, it does not significantly alter the pattern of perikaryal α SN accumulation seen in hippocampal neurons and other brain areas including the motor cortex, the striatum and the brainstem (Figure 2).

The most prominent finding that distinguished double from single mutant mice at the age of 3.5 months was a substantial increase in astrogliosis. 4/7 double mutant mice as compared to their single mutant littermates showed massive astrogliosis, especially in the cortex and to a lesser extent in the hippocampus and the striatum (Figure 3). This was verified in immunoblots of anterior brain extracts detecting increased levels of GFAP in 3/6 UCH-L1^{-/-}/Thy1-maSN double mutant mice (see Supplementary Fig. S1 online). The fact that not all double mutants showed these histopathological changes at age 3.5 months is not surprising because we also observed significant heterogeneity in (i) the age at which disease-onset is phenotypically manifest in individual Thy1-maSN mice, (ii) the age-at-onset at which astro- and microgliosis occurs in the most profoundly affected brain regions such as brainstem in Thy1-maSN mice, and (iii) the age at which end-stage pathology and disease is reached (varies between 6 and 10 months). Puzzling is that other brain regions including e.g. cerebellum, brainstem, and thalamus showed no signs of astrogliosis (Figure 3 and data not shown). Also, all brain regions examined in 3.5 month old mice were devoid of enhanced ubiquitin staining, microgliosis and axon degeneration (data not shown), which all are histopathological changes present in end-stage Thy1-maSN mice⁴¹. In summary, excess α SN in UCH-L1-deficient mice specifically leads to an early-onset astrogliosis in forebrain regions where neurons express high levels of transgene-derived α SN.

UCH-L1 deficiency in Thy1-maSN mice does not alter the levels of monomeric, oligomeric or S129-phosphorylated α SN forms. Immunoblot analysis of the soluble protein fraction extracted from forebrain and brainstem regions of 3.5-month-old Thy1-maSN mice revealed, besides increased levels of monomeric α SN, high-molecular weight α SN forms as well as truncated species and increased levels of S129-phosphorylated α SN (Figure 4A and 4B). We have described similar soluble α SN species in mice over-expressing the human α SN(A53T) mutant⁴³. The lack of UCH-L1 in the double mutant mice did not significantly change the pattern and level of α SN species seen in single Thy1-maSN mutants (Figure 4A, 4B and 4C). In addition, UCH-L1^{-/-} and wildtype littermates showed indistinguishable levels and patterns of soluble α SN species (Figure 4A, 4B and 4C). Finally, genotype-specific differences were also not observed following immunoblotting the insoluble α SN (pellet) fractions (data not shown).

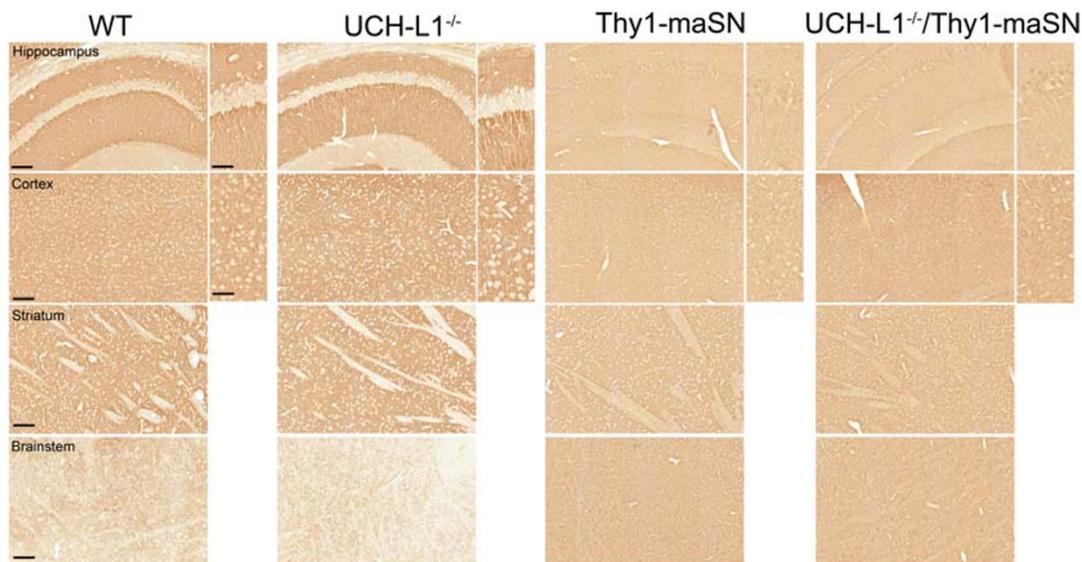


Figure 2 | α SN staining in UCH-L1^{-/-}/Thy1-maSN double as compared to Thy1-maSN single transgenic mice. Paraffin-embedded sagittal brain slices were stained for α SN. Hippocampus, motor cortex, striatum and brainstem for wildtype (WT), UCH-L1^{-/-}, Thy1-maSN and UCH-L1^{-/-}/Thy1-maSN mice are displayed. Brain regions and genotypes are indicated. Age of the animals: 3.5 months. Scale bars: 100 μ m and for higher magnification pictures 50 μ m.

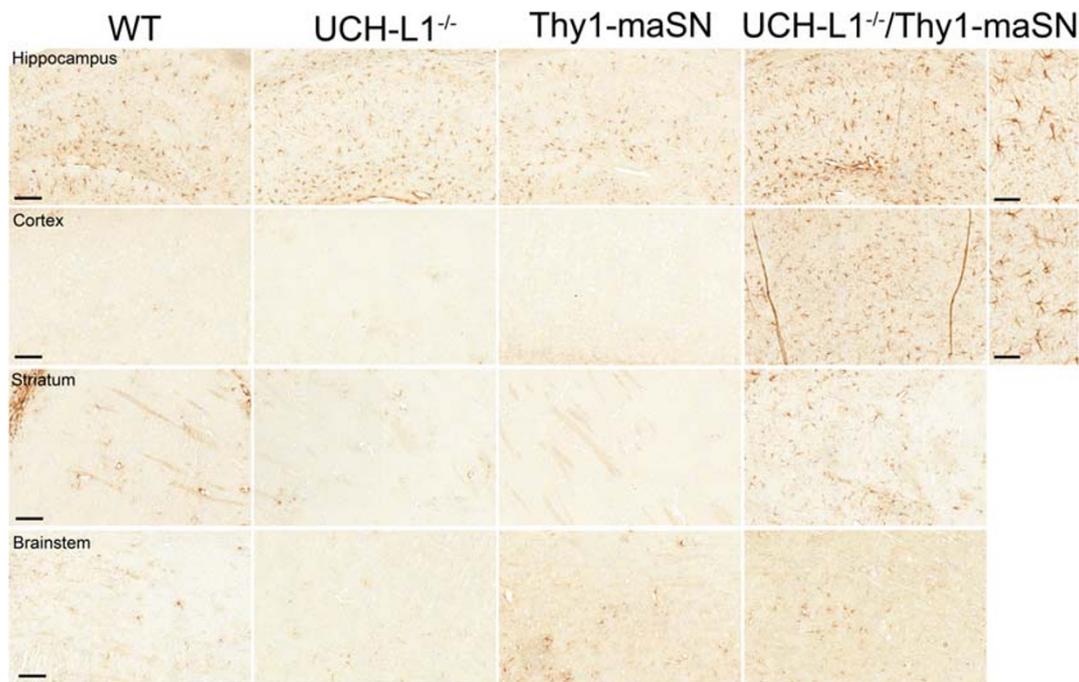


Figure 3 | Astrogliosis in 3.5-month-old UCH-L1^{-/-}/Thy1-maSN mutant mice. Paraffin-embedded sagittal brain slices were stained for GFAP. Hippocampus, motor cortex, striatum and brainstem for wildtype (WT), UCH-L1^{-/-}, Thy1-maSN and UCH-L1^{-/-}/Thy1-maSN mice are displayed. Brain regions and genotypes are indicated. Scale bars: 100 μ m and for higher magnification pictures 50 μ m.

In gad mice lacking UCH-L1, β SN and γ SN but no α SN pathology has been reported³⁰ and these histopathological changes seem confined to the gracile nucleus in the brainstem. We analyzed γ SN levels by Western blot in brainstem and forebrain extracts from UCH-L1^{-/-} and double mutant mice but these proved similar (Figure 5) suggesting that the lack of UCH-L1 did not significantly affect α SN or γ SN levels. For reference, the levels of endogenous huntingtin protein, which are regulated by proteasomal degradation, were also unaffected by UCH-L1 ablation (Figure 5).

Discussion

Collectively, our data show that UCH-L1-deficiency and excess murine wildtype α SN in mice act additively to aggravate disease. The disease process is accompanied by a massive astrogliosis in brain areas such as cortex, a phenomenon that is not seen in either of the single mutant mice. We found no evidence for UCH-L1-deficiency causing any significant changes in pathological α SN protein levels or species including S129-phosphorylated α SN and oligomers. Nor did we detect an earlier onset of immunohistopathological changes that are typically seen in end-stage α SN mice. It is therefore tempting to speculate that the observed disease-worsening effects occur by a mechanism that involves excess α SN exacerbating UCH-L1 deficiency and not *vice versa*. A perhaps provocative idea that will need further experimental support is that excess α SN may compromise UCH-L1 activity as part of α SN's pathophysiological effects in central and peripheral synapses.

UCH-L1 is widely expressed in brain and an abundant neuronal enzyme associated with several neurodegenerative diseases and cancer^{17,18}. UCH-L1 protein is also found in neuronal inclusions of human PD and DLB brains²³, which perhaps in part explains the decreases in UCH-L1 activity that have been observed in such brain tissue^{38,39}. UCH-L1 belongs to a family of enzymes that disassembles polyubiquitin chains to increase availability of free monomeric ubiquitin^{19,44}. In addition to its hydrolase activity, UCH-L1 has been shown to have a dimerization-dependent ubiquityl ligase activity²¹. This latter activity was proposed to be in part pathogenic by causing an accumulation of α SN-ubiquitin conjugates and inhibiting α SN degradation, perhaps

impairing also ubiquitin-proteasome activity²¹. Scenarios independent of UCH-L1 that impair ubiquitin-proteasome activity have also been postulated to explain α SN accumulation and the formation of Lewy inclusions in PD and other α -synucleinopathies^{45–47}.

In gad mice, loss of UCH-L1 leads to β SN and γ SN pathology in specific brain nuclei and it gives rise to a dramatic phenotype that includes sensory ataxia, motor paresis and eventually premature death^{29,30,48}. α SN pathology, however, has not been detected in gad and other UCH-L1-deficient mice. The observed increase in β SN in gad mice has been proposed as one possible explanation why α SN fails to accumulate in these mice because others have shown that β SN can counteract α SN aggregation⁴⁹. Alternatively, UCH-L1 deficiency itself might suppress α SN aggregation.

Excessive amounts of murine wildtype α SN likely challenge the ubiquitin-proteasome protein-degradation machinery. UCH-L1 deficiency may compromise this system even further although direct evidence is lacking that the catalytic activity of the proteasome *per se* is significantly reduced when UCH-L1 is decreased⁵⁰. On the other hand, UCH-L1 deficiency does reduce ubiquitin levels in the nervous system and this is accompanied by an up-regulation of lysosomal components. Therefore, Walters and colleagues put forward the hypothesis that UCH-L1 deficiency might affect a pool of ubiquitin that is utilized for lysosomal trafficking³¹. It is tempting to speculate whether excess α SN further compromises a lysosomal route in UCH-L1 mice because synuclein can not only impair chaperone-mediated autophagy but the protein itself seems to undergo intra-lysosomal degradation involving cathepsin D⁵¹. Further evidence is needed to strengthen or refute such a mechanism. Finally, we can not fully rule out the possibility that excess α SN and UCH-L1 deficiency act via two entirely independent pathways acting nonetheless additively to worsen disease.

Functional abnormalities in double mutants (earlier-onset motor deficits and increased muscle weakness) were already obvious at the age of 2.5 months but did not occur in mice that either over-express murine α SN in a wildtype UCH-L1 background or lack UCH-L1 but had wildtype α SN levels. At the age of 3.5 months, double mutants displayed one major histopathological change pointing at specific

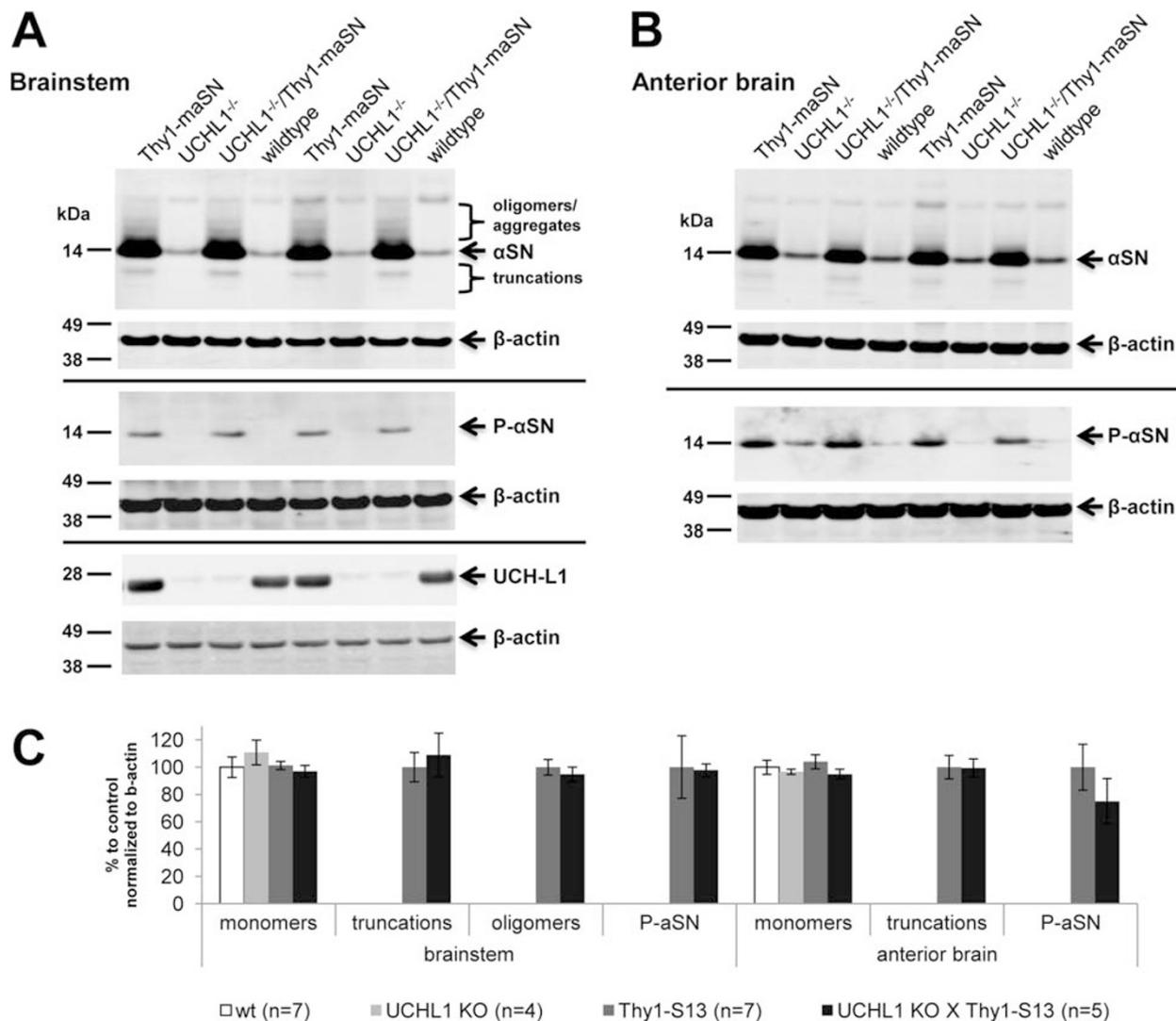


Figure 4 | Brain soluble α SN forms in UCH-L1^{-/-}/Thy1-maSN and Thy1-maSN mice. (A) Immunoblot analysis of brainstem extracts (supernatant/soluble fraction) from wildtype, UCH-L1^{-/-}, Thy1-maSN and UCH-L1^{-/-}/Thy1-maSN mice detecting α SN, S129-phosphorylated α SN and UCH-L1. β -actin was used as loading control. Indicated are the oligomers and truncations in mice over-expressing murine α SN. (B) Immunoblot analysis of anterior brain extracts (supernatant/soluble fraction) from wildtype, UCH-L1^{-/-}, Thy1-maSN and UCH-L1^{-/-}/Thy1-maSN mice detecting α SN and S129-phosphorylated α SN. β -actin is used for loading control. (C) Bar graph depicting quantification of the immunoblots shown in A. and B. for the different α SN species. See colour code for the different genotypes. Age of the animals: 3.5 months. N numbers for each genotype are indicated. Statistical analysis: two-tailed, unequal variances Student's t-test.

disease-associated changes: a massive astrogliosis restricted to fore-brain areas including cortex, hippocampus and striatum. Neurons in these brain areas express high levels of the α SN transgene but nonetheless remain largely devoid of hallmark histopathological cellular changes such as synuclein and ubiquitin pathology and axonal degeneration seen in brainstem and spinal neurons of end-stage Thy1-m α SN mice⁴¹. Interestingly, the brainstem in our double mutants showed no astrogliosis or microgliosis unlike in end-stage Thy1-m α SN mice. Why the forebrain-selective astrogliosis occurs in the double mutants is currently unknown. α SN seems to play a key role in the maintenance of SNARE-complex assembly and vesicle turnover in presynaptic terminals⁵²⁻⁵⁴ and it seems likely that excessive amounts of α SN can perturb synaptic function that might trigger adaptive changes in astrocytes^{55,56}. Astrogliosis is for example detected in mice showing enhanced synaptic excitability because of an editing-deficient GluR-B (GluA2) glutamate receptor subunit⁵⁷. The combination of excess α SN together with UCH-L1 deficiency, which by itself is known to cause defects in central and peripheral

synapses, perhaps suffices to pass a synaptic insult threshold that triggers astrocyte activation. Why this would occur mainly in fore-brain and not in brain areas that otherwise seems most sensitive to α SN-induced pathology such as brainstem remains speculative. UCH-L1 is strongly expressed in both forebrain and brainstem^{48,58}. Perhaps forebrain neurons are more responsive to a loss of UCH-L1 when α SN is in excess. UCH-L1 inhibition is known to preferentially affect the stability of certain synaptic proteins and their role is perhaps more relevant to forebrain neurons⁵⁰. On the other hand, fore-brain neurons seem normally quite capable of coping with an excess of α SN because in Thy1-m α SN mice, cortical and hippocampal neurons lack the dramatic histopathological changes seen in brainstem and spinal cord neurons. Possibly, forebrain neurons can better call upon compensatory mechanisms that reduce synaptic damage that at the same time trigger astrogliosis which might serve a neuroprotective role^{59,60}. To conclude, further experiments are needed to better understand if and what kind of molecular interplay between α SN and UCH-L1 underlies the disease-worsening effect seen in double

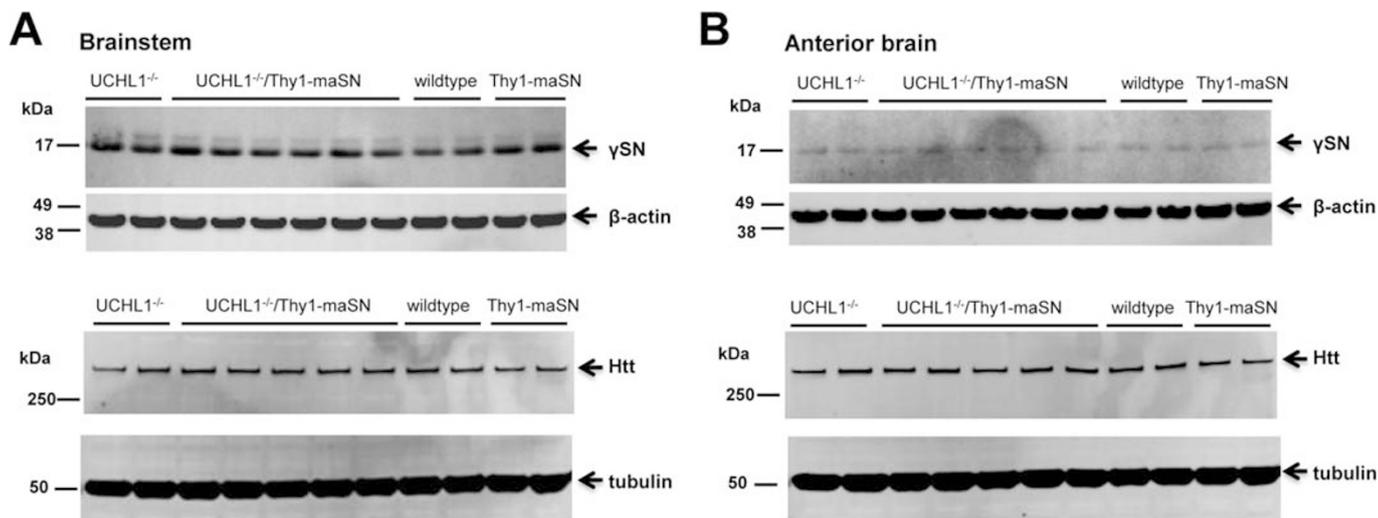


Figure 5 | γ SN and huntingtin levels in the UCHL1^{-/-}/Thy1-maSN mouse brain. (A) Immunoblot analysis of brainstem extracts from wildtype, UCHL1^{-/-}, Thy1-maSN and UCHL1^{-/-}/Thy1-maSN mice detecting soluble γ SN. (B) Immunoblot analysis of anterior brain extracts from wildtype, UCHL1^{-/-}, Thy1-maSN and UCHL1^{-/-}/Thy1-maSN mice detecting soluble huntingtin (Htt). β -actin and tubulin were used as loading controls. Age of the animals: 3.5 months.

mutants as our data do not support a clear role for UCH-L1 in regulating monomeric, oligomeric, truncated and phosphorylated α SN levels. In addition, it has been reported that no alterations of dopaminergic cell loss in the absence of UCH-L1 in a viral α -synucleinopathy model could be observed³³. We can of course not exclude that compensatory changes in UCH-L1 deficient mice such as the reported upregulation of lysosomal enzyme activities including cathepsin D³¹, mask a role for UCH-L1 in the clearance of α SN. Future experiments using conditional UCH-L1 knockouts and/or determining whether perhaps more subtle effects can be detected in Thy1-maSN mice treated with UCH-L1 inhibitors or lacking only one UCH-L1 allele seem worth pursuing.

Methods

Statement on Animal Health. All experiments were carried out in accordance with authorization guidelines of the Swiss Federal and Cantonal veterinary offices for care and use of laboratory animals. Studies described in this report were approved by the Swiss Cantonal veterinary office and performed according to Novartis animal licence number 2063.

Transgenic mice. Thy1-maSN mice were generated and genotyped as described⁴¹. α SN knockout mice were obtained from Jackson laboratory (JolaHSD) and were kept as a homozygous line. UCH-L1 knockout (complete name: T1320UCHL1) mice were obtained from Deltagen (San Mateo, CA). Genotyping was performed with standard PCR protocol with the following primers: Oligo 56474 (5'-CTCTCCCCAGACTTAAGCTGCTTTG-3') and Oligo 3196 (5'-GGGTGGGATTAGATAAATGCCTGCTCT-3'), expected band size of the targeted allele: 496 bp. Oligo 67696 (5'-CCTTGCCTCCGTCCTCTATTAAGC-3') and Oligo 56474, expected band size of the endogenous allele: 219 bp. All animals are kept in the C57BL/6 background and littermates were used as controls.

Immunoblot analysis. For infrared Licor western blot analysis brains were homogenized in homogenization buffer (10 v/w; 0.25 M Sucrose, 20 mM Tris 20 mM pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.5 mM PMSF, 5 μ g/mL Pepstatin A, 5 μ g/mL Leupeptin, 1:1000 Okadaic acid 100 ng/ μ l in ethanol, 1:1000 Calyculin A 100 ng/ μ l in ethanol, 1:100 Pierce phosphatase inhibitor cocktail) with Precellys24 (Bertin Technologies, Rotation speed 5000 rpm, number of cycles: 2 \times 30 sec, pause: 10 sec), then incubated for 30 min on ice and subsequently centrifuged at 13 krpm at 4°C for 20 min. Supernatant was used and protein concentration was determined by Bradford (Bio-Rad). 10 μ g protein was loaded (LDS Sample Buffer, Sample Reducing Agent (Invitrogen), heated for 10 min at 95°C) on NUPAGE 4–12% Bis-tris gel 1.0 mm (Invitrogen) and ran at 180 V for 40–50 min with NuPAGE MES SDS Running Buffer (Invitrogen). Gels were blotted by semi wet transfer (Invitrogen X Cell II Blot Module) with NuPAGE Transfer Buffer, 20% methanol on invitrolon PVDF membrane (Invitrogen) at constant 30 V for 1 h. After transfer proteins were fixed on membrane (1 min in PBS full power in microwave). For Licor Odyssey detection membrane was blocked for 1 hr at room temperature in Odyssey Blocking Buffer (Odyssey, 1:1 diluted with PBS). Primary Antibodies (anti-phosphorylated

α -synuclein, WAKO, 1:5000; mouse anti- α -synuclein, BD Transduction Laboratories, 1:5000; mouse anti- β -actin, SIGMA, 1:15000; mouse anti-tubulin, AA13, abcam, 1:1000; rabbit anti-UCH-L1, #3524, Cell Signaling, 1:1000; rabbit anti-GFAP, Sigma, G9269, 1:500; mouse anti-Huntingtin, 2B7, 1 μ g/ml; rabbit anti- γ -synuclein, #158, 1:1000) in Odyssey Blocking Buffer (0.2% Tween20). Membranes were washed 4 times for 5 min at room temperature in PBS containing 0.1% Tween20 and then incubated for 45 min (light protected) with secondary antibodies (Alexa Fluor 680, F(ab')₂ fragment of goat anti-mouse 2 mg/mL, 1:5000; IRDye 800CW anti-rabbit IgG, LI-COR, 1:5000) in Odyssey Blocking Buffer. Membranes were again washed 4 times for 5 min at room temperature in PBS containing 0.1% Tween20, then washed 2 times for 10 min at room temperature in PBS only and finally scanned and quantified on the Odyssey LI-COR System.

Immunohistochemistry. PBS-perfused brains were postfixed overnight in 4% formalin in PBS at 4°C. Then, the brains were dehydrated by different ethanol treatments using a Tissue-Tek VIP system (GMI Inc). The ethanol treatment series consisted of 70%, 80%, 90%, 2 \times 94%, 3 \times 100% ethanol each for 1 h. Brains were then washed 2 times in xylol for 30 min followed by 2 times in paraffin (30 min, 60 min). Subsequent, the dehydrated brains were embedded at 55°C in paraffin using a Tissue Block System TBS 88 (Geneq Inc). Slices were cut on a Microm HM 355 device (Microm International GmbH) at a thickness of 4–5 μ m and mounted on superfrost glass slides (Microm International GmbH) and air-dried. Slides were kept at room temperature until use.

Immunohistochemistry was performed as following: Slides were incubated 3 \times 10 min in day-1 buffer (0.01 M PBS, 1% BSA, 0.3% Triton X-100) and blocked by addition of 4% normal goat serum for 20 min. Paraffin-embedded slides were incubated in day-1 buffer with 1% normal goat serum and subsequently incubated overnight with a mouse monoclonal α SN antibody (1:800, 4B12, Abcam) and rabbit polyclonal anti-Iba1 (1:2000, Wako), anti-GFAP (1:2000, Z0334, Dako) and anti-ubiquitin (1:200, Z458, Dako) in a humidity chamber at room temperature. Slides were then rinsed 3 \times 10 min in day-2 buffer (day-1 buffer diluted 1:3 in 0.01 M PBS). Slides were incubated for 1 h with secondary antibody (Biotin-conjugated goat anti-mouse IgG (1:200, Vectorlabs) and then with ABC reagent (Vectorlabs) for 1 h at room temperature. Slides were then rinsed in day-2 buffer 2 \times 10 min, in 0.01 M PBS 2 \times 20 min and were then desalted in 0.01 M Tris (pH 7.8). Slides were air-dried at room temperature and coverslipped with eukitt mounting medium and analysed on a Nikon microscope.

Forelimb grip strength and rotating beam. To measure forelimb grip strength, mice are allowed to grasp a handle connected to a force-measuring device (San Diego Instruments, USA) and then pulled back with their tails until they release the handle. The best out of four consecutive trials is evaluated.

To measure motor coordination mice were placed on a rotating beam. The rotating beam was build in-house and is connected to a rotarod (Lugo Basile). It consists of a metal beam (\varnothing 1 cm, length 122 cm) coated with rubber attached to a rotarod (gradient angle upwards of 10°) which controls the constant rotating speed (4 rpm and 8 rpm). The beam is divided into four equal sections which are used for scoring the performance of the mice on the rotating beam (scores 1–5). The mouse is placed at the beginning on the already rotating beam facing upwards. If the mouse reaches the end of the beam without falling down or being head first hanging on the beam it gets a score of 1, if it falls down or is head first hanging in the last section (4th) it scores a 2, in



the 3rd section 3, in the 2nd section 4 and in the 1st section a score of 5. There is no time limit as the mice readily go upwards the rotating beam. One session consist out of two trials of 4 rpm and two trials of 8 rpm (all trials were included in the measurements), whereas the sequence is one trial of 4 rpm followed by on trial of 8 rpm in the morning and in the afternoon.

Maintenance. The animals were housed in a temperature-controlled room that was maintained on a 12 h light/dark cycle. Food and water were available *ad libitum*.

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Authors contributions

DRS and PHvdP wrote the main manuscript text. DRS and PHvdP conceived and designed and DRS, TS and PS performed the experiments. DRS and TS prepared figures. All authors reviewed the manuscript.

Additional information

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