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The S18Y polymorphic variant of UCH-L1 confers an antioxidant function to neuronal cells

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A number of studies have associated the S18Y polymorphic variant of UCH-L1 with protection from sporadic Parkinson's Disease (PD). The mechanism involved in this protective function is unknown, but has generally been assumed to be linked to the ubiquitin–proteasome system (UPS). In the current study, we have investigated the effects of overexpression of UCH-L1 and its variants, including S18Y, in neuronal cells. We find that S18Y, but not WT, UCH-L1 confers a specific antioxidant protective function when expressed at physiological levels in human neuroblastoma cells and primary cortical neurons. In contrast, neither WT nor S18Y UCH-L1 appear to directly impact the proteasome, although they both lead to stabilization of free ubiquitin. Lack of WT mouse UCH-L1 in neurons derived from *gad* mice led to a decrease of free ubiquitin, but no overall decrease in UPS function or enhanced sensitivity to oxidative stress. We conclude that the S18Y variant of UCH-L1 confers a novel antioxidant function that is not present in the WT form and that this function may underlie the protective effects of this variant in certain PD populations. Our results furthermore provide indirect evidence for the importance of oxidative stress as a pathogenetic factor in certain forms of sporadic PD.

INTRODUCTION

Parkinson's Disease (PD) is characterized by the loss of nigral domapinergic neurons and the presence of Lewy Bodies (LBs) (1). Five genes, α-synuclein, Parkin, DJ-1, PINK-1 and LRRK2, are conclusively linked to PD (2). The link of yet another gene, UCH-L1, to PD is controversial. Leroy *et al*. (3) reported a missense I93M mutation in UCH-L1 associating with PD in a German family. However, studies worldwide failed to uncover another instance of this mutation, casting doubt on its pathogenicity. A polymorphism within the UCH-L1 gene, the substitution S18Y, is protective against PD in different populations, especially in early onset sporadic cases (4–8). However this finding has also been controversial (9).

UCH-L1 is an abundant neuronal protein, a member of the Ubiquitin C-terminal Hydrolases (UCHs). *In vitro* data indicate that it indeed acts as a UCH (10). The I93M mutant leads to a 50% decrease (3), and the protective S18Y variant to a slight increase of this hydrolase activity (10). Liu *et al*. (11) reported that wild-type UCH-L1 binds to alpha-synuclein and aberrantly ubiquitylates it at position K63, leading to its stabilization. The S18Y variant and the I93M mutant manifested, respectively, decreased and increased ability of K63 ubiquitylation, suggesting that this function may be critical to PD pathogenesis. In *Aplysia*, during the process of long-term facilitation, the levels of the UCH-L1 homologue are increased, and, presumably through association with, and enhancement of proteasomal activity, promote the degradation of cAMP-dependent protein kinase A (PKA) (12). UCH-L1 binds to monomeric ubiquitin and in the *gad* mice, which lack UCH-L1 expression, monomeric ubiquitin is decreased, suggesting involvement in the stabilization of monomeric ubiquitin (13). It is clear that UCH-L1 appears to be linked in some way to the ubiquitin–proteasome system (UPS). Given the evidence linking UPS dysfunction to PD (14), the general assumption is that UCH-L1 is functionally linked to PD through this pathway. However, the potential role of UCH-L1 within the UPS and its interaction with the proteasome has not been rigorously examined in mammalian systems. Furthermore, the relationship of UCH-L1 to other pathophysiological processes related to PD, such as mitochondrial impairment and oxidative stress, also remains unknown.

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We have undertaken this study in order to examine the possible functional association of UCH-L1 with the UPS in mammalian neuronal cell culture systems. We have begun with the hypothesis that, as in *Aplysia*, mammalian UCH-L1 may facilitate UPS function through an interaction with the proteasome, and that the mutants or variants identified in PD patients may alter this interaction. In the process of this work, we have confirmed that UCH-L1 acts as a stabilizer of free ubiquitin, but, unlike in *Aplysia*, it does not associate with the proteasome and does not promote its function. More importantly, we have uncovered a unique, specific ability of the S18Y UCH-L1 variant to act as a potent antioxidant in these neuronal cell culture systems, thus providing for the first time a link between this variant, which is protective in many human populations against sporadic PD, and protection from oxidative stress, linking genetic variations with environmental exposure in the pathogenesis of PD.

RESULTS

Expression of UCH-L1 and variants thereof in human SH-SY5Y cells

To assess the role of UCH-L1, we first performed transient transfections in SH-SY5Y cells, a well-characterized neuronal cell line. In addition to WT and S18Y, we also transfected C90S (a catalytically inactive mutant), I93M (the mutant identified in PD) and D30K (a mutant unable to bind ubiquitin) UCH-L1. All variants were Flag-tagged. As control, we used a vector encoding a myc-tagged inactive variant of the serine protease inhibitor PAI-2. Levels of all variants of UCH-L1 were comparable by western immunoblotting (Fig. 1A). It has been controversial whether UCH-L1 overexpression stabilizes levels of free monomeric ubiquitin in cells (13,15). We observed an increase of monomeric ubiquitin, but not polyubiquitinated proteins, with all UCH-L1 transfections, except D30K (Fig. 1A). The increase with C90S UCH-L1 just failed to reach statistical significance. We conclude that UCH-L1 and variants thereof increase levels of monomeric ubiquitin in cells in a fashion dependent on the binding of UCH-L1 to ubiquitin (13).

To assess whether transient transfection would induce physiological amounts of UCH-L1, we compared levels of the overexpressed protein, migrating higher on the gel because of the Flag-tag, with the endogenous protein. The overexpressed protein was comparable with the endogenous protein (Fig. 1B), therefore this approach does not lead to artificial gross overexpression.

We then assessed the immunostaining pattern of the variants of UCH-L1. In contrast to the diffuse staining seen with PAI-2, immunostaining of UCH-L1 and its variants with Flag occasionally showed a punctate-aggregated type of labeling. We define such labeling as focal accumulation of Flag immunostaining within the cytoplasm, usually in multiple discrete regions. Interestingly, such aggregated labeling was less apparent with the S18Y variant or the D30K mutant (Fig. 2A and B).

To examine further the issue of the effects of UCH-L1 on ubiquitin, we performed immunostaining for ubiquitin following UCH-L1 transfection. There was indeed induction of ubiquitin immunostaining in cells expressing UCH-L1, and this was the case for all expressed variants except for D30K (Fig. 2A,C,D), confirming our western immunoblotting data. UCH-L1 immunostaining within the aggregates colocalized with ubiquitin (Fig. 2A), as previously reported (15).

Because of a link between aggregation and neuronal dysfunction and death, and the idea that UCH-L1 may be toxic through a gain of function (11,16), we also assessed the effects of the overexpression of UCH-L1 or its variants on apoptosis. Although there was a trend for WT UCH-L1 to induce apoptosis relative to the control protein, this did not reach statistical significance (Fig. 2E). There was also a trend for C90S and I93M, but not D30K or S18Y, to slightly enhance apoptosis compared with the control protein.

Therefore, expression of UCH-L1 and its variants leads to aggregation, but not to significant induction of toxicity in human neuroblastoma cells. Various forms of UCH-L1 lead to a similar induction of free ubiquitin in the cells in a fashion dependent on the binding to and stabilization of ubiquitin.

Overexpression of UCH-L1 or its variants does not significantly impact the proteasome

Hedge *et al*. (12) reported that in Aplysia the functional homologue of UCH-L1 associated with the proteasome and facilitated its function. To assess this issue in mammalian systems, we performed size exclusion chromatography (SEC). We detected UCH-L1 only in low (66–68) and not high-molecular weight fractions containing the 26S proteasome (27–47) (Fig. 3). We conclude that in this cellular system UCH-L1 does not significantly associate with the proteasome, although we cannot completely exclude the possibility that a very small pool of UCH-L1, undetectable in our assays, could co-elute with the proteasome.

Because of the purported link between UCH-L1 and the UPS (see Introduction), we wished to assess whether UCH-L1 overexpression would have a functional effect on this system. For these experiments, we used a previously characterized SH-SY5Y cell line (17), which stably expresses the UPS reporter GFPu and fluoresces upon proteasomal inhibition. We transiently expressed control or UCH-L1 constructs, and 24-h later applied low doses of the proteasomal inhibitor PSI. We used fluorescence-activated cell sorting (FACS) analysis to examine GFPu fluorescence only in transfected cells, identified by positive Myc or Flag immunostaining. The percentage of positive GFP labeling did not differ appreciably in cells transfected with WT or S18Y UCH-L1 variant compared with control (Fig. 4A). Therefore, UCH-L1 overexpression in this system did not alter proteasomal inhibition. It has to be noted that GFPu fluorescence, although specific, may not be a very sensitive indicator of proteasomal dysfunction and that therefore small differences could have been missed. On the other hand, the trend is for UCH-L1 to slightly enhance GFPu accumulation, arguing further for the lack of a beneficial effect on proteasomal function.

To further confirm these results, we examined the effects of UCH-L1 on death induced by PSI. We again failed to see any change in the rate of death among constructs (Fig. 4B).

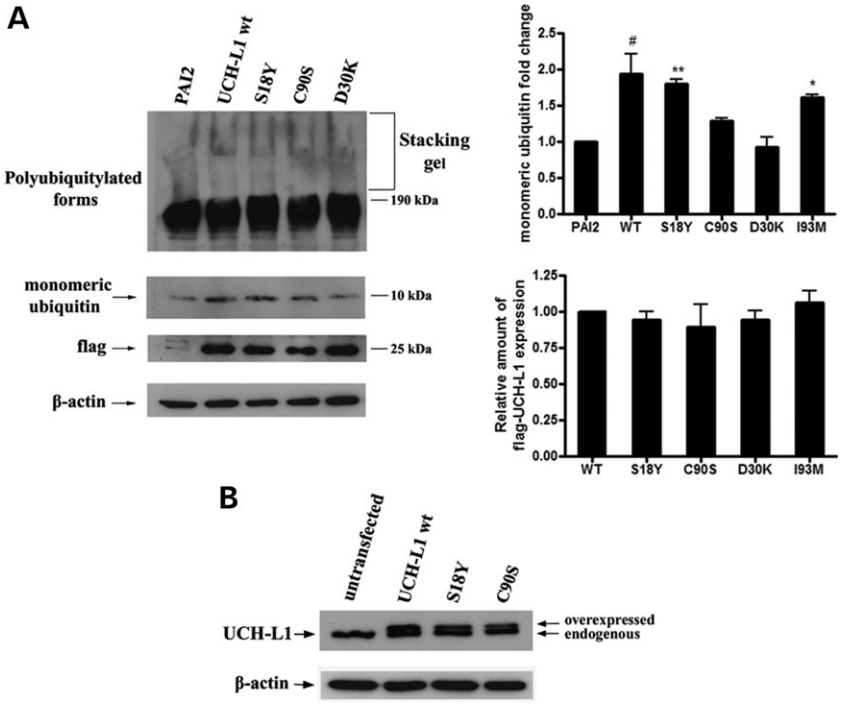

Figure 1. UCH-L1 overexpression in SH-SY5Y cells (**A** and **B**). SH-SY5Y cells were transiently transfected with Flag-tagged WT UCH-L1, variant S18Y or mutants or with mutant myc-tagged PAI-2 (PAI2M) as control. Forty-eight hours later cells were lysed and supernatants (20 μg protein) were immunoblotted with ubiquitin (top panel, molecular weights above 70 kDa and middle panel, molecular weight in the range of 10 kDa), Flag (A) or UCH-L1 (B, overexpressed flag-tagged UCH-L1 at 26 kDa and endogenous UCH-L1 at 25 kDa) and beta-actin (loading control) antibodies. The position of the stacking gel is denoted by an arrow in the top panel. These blots are representative of three independent experiments. Band densitometry was performed for the quantification of monomeric ubiquitin or flag-UCH-L1 expression levels. Fold change was evaluated compared with PAI2M levels in the case of monomeric ubiquitin, whereas in the case of UCH-L1 variants compared with WT UCH-L1. Comparisons between groups were made by one-way ANOVA with Scheffe *post-hoc* comparisons; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.001$. The graphs present the results from three independent experiments.

As a general pro-apoptotic strategy, we utilized the DNA damaging agent camptothecin (18). Again, the UCH-L1 constructs failed to differentially affect survival compared with the control protein (Fig. 4B).

In conjunction, our results suggest that overexpressed UCH-L1 does not associate with the proteasome and does not alter UPS function in the face of pharmacological proteasomal inhibition. Furthermore, UCH-L1 overexpression does not influence apoptosis in this system.

S18Y UCH-L1 acts as a potent antioxidant in SH-SY5Y cells

We then wished to assess the sensitivity of cells expressing UCH-L1 variants against other insults that are relevant to PD, such as toxicity induced by MPP^+, the active derivative of MPTP, a mitochondrial toxin that induces Parkinsonism in humans and experimental animals (19). Overexpression of WT or C90S UCH-L1 did not alter the sensitivity of SH-SY5Y cells to MPP^+, when compared with cells expressing the control PAI-2M. In contrast, a remarkable and consistent survival-promoting effect was seen with the S18Y variant (Fig. 5A). This effect was specific, because, as mentioned, it was not observed when the cells were exposed to camptothecin or PSI (Fig. 4B). Experiments performed with parallel exposure of S18Y-transfected cells to PSI, camptothecin and MPP^+ confirmed these findings (data not shown).

MPP^+ is known to exert neuronal toxicity, in part, by the release of reactive oxygen species (ROS) from the mitochondria, as a result of complex I inhibition (19). Given the specific nature of the protective effect of S18Y UCH-L1 against MPP^+, but not against proteasomal inhibition or DNA damage, we investigated whether expression of the S18Y variant could alter the induction of ROS after MPP^+ exposure. We used as a sensor of ROS the dye DCF (20), which reliably detected ROS generation after exposure to MPP^+ or H_2O_2 (Supplementary Material, Fig. S1). We found a specific reduction of ROS generation in the presence of S18Y, but not WT, UCH-L1 (Fig. 5B–D). We conclude that S18Y UCH-L1 confers a protective effect on MPP^+-treated SH-SY5Y cells, likely via reduction of ROS generation.

We wished to ensure that the presumed antioxidative effects of S18Y variant could be demonstrated using another insult. To this end, we turned to hydrogen peroxide (H_2O_2), which is a known pro-oxidant. Here, we also observed a significant protective effect and a reduction of ROS generation in cells transfected with S18Y, but not WT, UCH-L1 (Fig. 6). These results confirm a specific antioxidant capacity of S18Y in human neuroblastoma cells.

In order to examine if the ubiquitin-binding ability or hydrolase activity contribute to the antioxidative effects of the S18Y

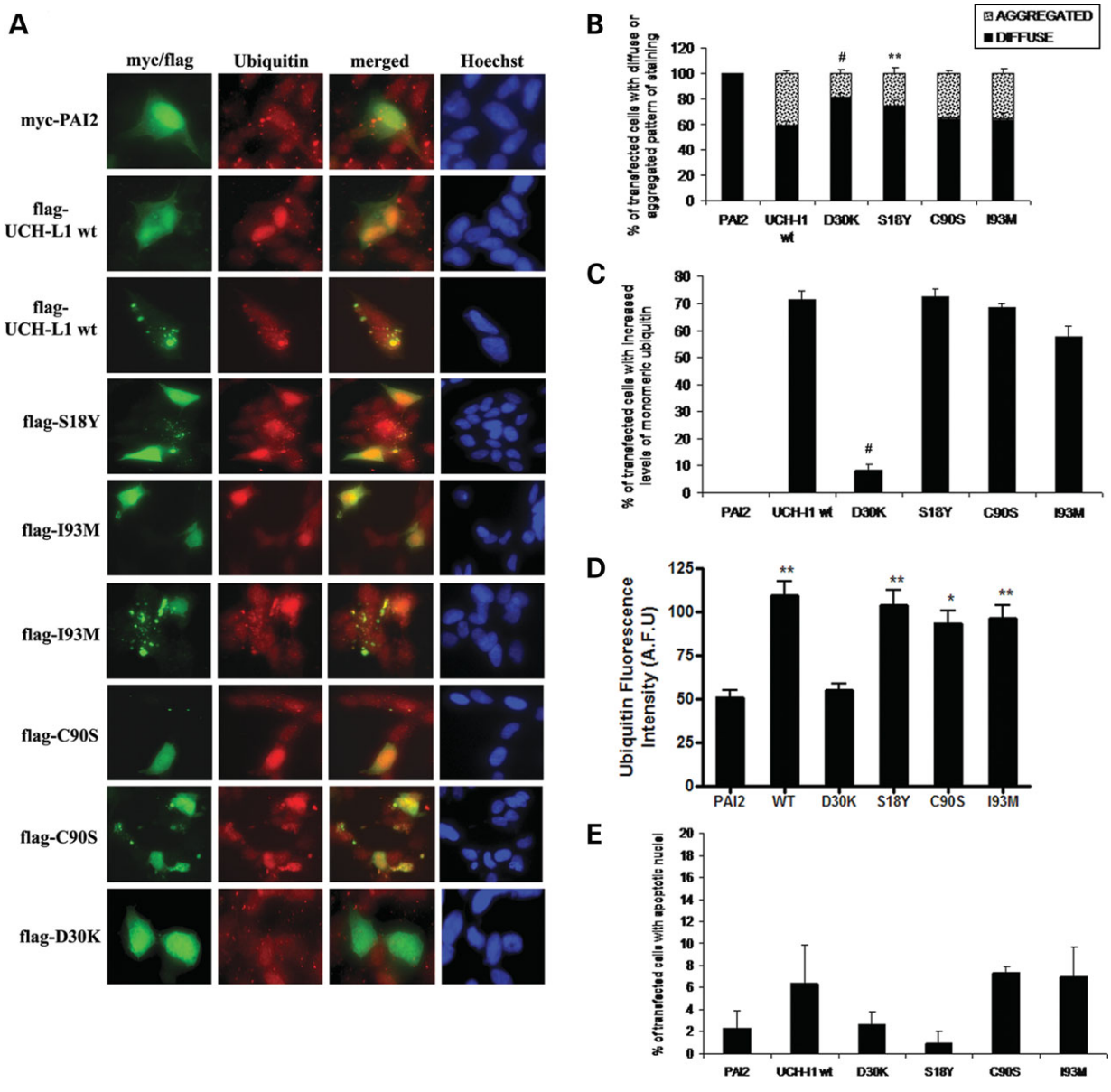

Figure 2. Overexpression of UCH-L1 leads to protein aggregation, but not apoptosis. (**A**) SH-SY5Y cells were transfected with flag-tagged forms of UCH-L1 or PAI2M as control. Forty-eight hours later, cultures were fixed, immunostained for myc or flag (green) and ubiquitin (red) and stained with Hoechst for apoptotic nuclei. Representative photos are shown. (**B**–**E**) The presence of aggregated Myc (PAI2) or Flag (UCH-L1), ubiquitin levels and the presence of apoptosis in myc/flag positive cells were examined by fluorescent microscopy. The graphs show the percentage of transfected cells that displayed myc (PAI2) or flag (UCH-L1) aggregation (B), increased levels of ubiquitin compared with surrounding non-transfected cells (C) and apoptotic nuclei (E), as mean ± SD ($n = 3$ of 100 cells each in independent wells). In (D) measurement of ubiquitin fluorescence intensity in transfected cells was performed with the use of Image J software, as described in Materials and Methods. Comparisons between groups were made by one-way ANOVA with Scheffe *post-hoc* comparisons; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.001$ compared with control PAI2M; in (B and C) comparisons were made using WT UCH-L1 as the control. These results were verified in three independent experiments.

variant, we introduced the S18Y polymorphism in a catalytically inactive or ubiquitin-binding deficient background constructing the hybrid variant mutants S18Y/C90S and S18Y/D30K, respectively. As shown in Figure 7, protection against MPP^{+} or H_2O_2 treatments remained unaffected. Therefore, the previously identified UCH-L1 properties of ubiquitin hydrolysis and ubiquitin binding are not required for the protective effect of S18Y UCH-L1 against oxidative stress insults, further arguing for a novel antioxidant function conferred to UCH-L1 by the S18Y polymorphism.

Effects of the lack of UCH-L1 in mouse cortical neurons on ubiquitin, the UPS and differential sensitivity to stress stimuli

We wished to also address the normal function of UCH-L1. We therefore turned to primary cortical neuron cultures derived from *gad* mice, which lack UCH-L1 expression (21). These mice demonstrate progressive ataxia and paralysis, due to involvement of the gracile nucleus and tract. Lysates of embryonic cortical neurons derived from these mice, as

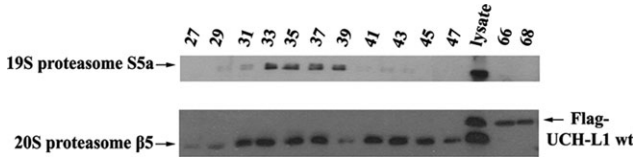

Figure 3. Overexpressed WT UCH-L1 does not co-elute with the 26S proteasomal complex. SH-SY5Y cells were transfected with flag-tagged WT UCH-L1 and 48 h later the protein lysate was analyzed by SEC. All fractions where the proteasome elutes, as well as lower molecular weight fractions where UCH-L1 would be expected to elute, were analyzed by western blot for UCH-L1 co-elution. Fraction numbers are shown on top. This is representative of two independent experiments.

previously reported in brain tissue (13), showed reduction of monomeric ubiquitin compared with littermate controls, whereas polyubiquitinated proteins did not differ appreciably (Fig. 8A). We wished to investigate first whether an impact on proteasomal function could be observed in these cultures. Surprisingly, lack of UCH-L1 led to a small, but significant, increase of enzymatic proteasomal activity (Fig. 8B). We also assessed the levels of a known substrate of the UPS, c-jun (22,23), and failed to find significant accumulation in neurons derived from *gad* mice (Fig. 8C). Furthermore, there was no difference in the rate of death in response to proteasomal inhibition (Fig. 8D). Given the antioxidant effects of the S18Y variant in neuroblastoma cells, the question arose whether such functions were latent in the endogenous wild-type protein, and would be uncovered when this was deleted. We therefore assessed sensitivity of these neurons to oxidative stress induced by hydrogen peroxide. There was no difference compared with neurons derived from littermate controls. Similar results were achieved after exposure to camptothecin or PSI (Fig. 8D).

We conclude that although the absence of UCH-L1 in cultured neurons leads to a reduction of free ubiquitin, this does not appear to have a functional impact on proteasomal degradation *per se*, perhaps because of a compensatory induction of 26S enzymatic proteasomal activity, and that a latent antioxidant capacity of WT mouse UCH-L1 could not be demonstrated.

The antioxidant protective effects of S18Y UCH-L1 are recapitulated in primary cortical neurons

To assess whether the antioxidant effects of the S18Y variant could be replicated in primary cortical neurons, we overexpressed in these cultures this variant or WT UCH-L1 or the control protein, and exposed the cultures to hydrogen peroxide. A significant and specific effect on survival and on ROS generation was seen again for S18Y, but not WT UCH-L1 (Fig. 9 and Supplementary Material, Fig. S2).

DISCUSSION

In this work, we report the novel finding that the S18Y variant of UCH-L1, which is linked to protection from sporadic PD in many human populations, confers a significant antioxidant capacity that protects neuronal cells against oxidative insults. Our findings therefore are consistent with the idea that the S18Y variant of UCH-L1 confers a protective effect in humans through an antioxidant action and strengthen the notion that oxidative stress is a factor involved in certain forms of sporadic PD. To our knowledge, our present study represents the first in which a common polymorphism in a neuronal-specific gene is proved to confer a specific antioxidant capacity.

We do not believe that our results are due to non-specific effects of gross overexpression of the S18Y variant, as: (i) the levels of overexpression were comparable with endogenous UCH-L1 (Fig. 1), likely because of the abundance of UCH-L1 in neuronal cells (10,24), (ii) the effects were very specific, as no protection was seen against other insults or with the WT protein. Therefore, our results suggest that the S18Y variant, when co-expressed with the WT protein in a heterozygote human carrier, will confer a significant, specific antioxidant capacity to the nervous system of that individual. Unfortunately, this idea could not be directly tested in specimens derived from human carriers, because of the specificity of expression of UCH-L1 in the nervous system and the testes.

Human UCH-L1 is 96% identical to its rodent homologue, however position 18 in rat and mouse UCH-L1 is alanine instead of serine. The substitution of serine with tyrosine, another aminoacid with uncharged polar side chains, is not expected to grossly alter the structure of the protein. The fact that overexpression of human UCH-L1 leads to upregulation of monoubiquitin, whereas lack of UCH-L1 in the *gad* mice leads to a reduction of free ubiquitin (13) and our own data presented here) suggests that the human and mouse UCH-L1 are functionally equivalent, at least regarding this function. As mentioned, overexpression of human WT UCH-L1 failed to demonstrate antioxidant capacity and lack of mouse UCH-L1 did not render neurons more susceptible to oxidative stress. Therefore, the S18Y polymorphism confers a novel antioxidant function to UCH-L1, which is not latent in the WT protein.

Our results with hydrogen peroxide and, especially, MPP^+, raise the interesting issue of the interaction between genetic susceptibility and exposure to environmental factors. Our data suggest that individuals exposed to environmental stimuli that induce oxidative stress may develop or not the disease depending on their genetic background regarding the UCH-L1 gene. Pesticide use has been linked to mitochondrial dysfunction and resultant oxidative stress, and in most studies confers a risk to develop PD (25). In one study however by Elbaz *et al*. (5), no significant interaction was found between the protective effect of S18Y in young-onset cases and pesticide use. More such studies are needed. In any case, other unknown pro-oxidant environmental factors may be crucial in this regard.

The mechanisms through which S18Y UCH-L1 exerts its survival-promoting effects against MPP^+ and hydrogen peroxide are unclear. These effects are commensurate with the effects on ROS induction, but how this antioxidant capacity is conferred is unknown. Using a double mutant strategy, we have clarified that the known functions of ubiquitin hydrolysis and ubiquitin binding are not required for these effects. It is unlikely that the ligase function that has been reported for UCH-L1 is relevant, as S18Y exhibits this activity to a significantly lower extent compared with the WT protein (11).

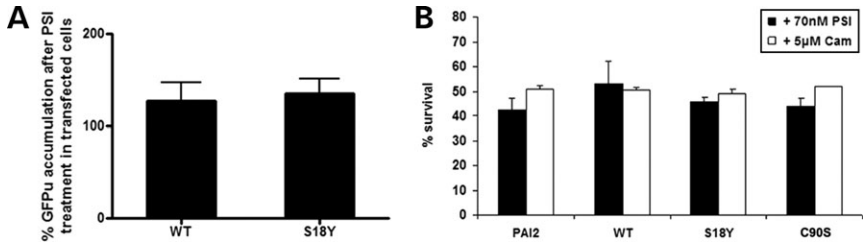

Figure 4. Overexpressed UCH-L1 does not influence sensitivity to proteasomal dysfunction. (**A**) SH-SY5Y cells stably expressing GFPu were transfected with WT, S18Y UCH-L1 or with PAI2M as a control. Cells were treated for 18 h with 50 nM PSI. Forty-eight hours after transfection, cells were collected, fixed in suspension and immunostained with myc or flag. Fixed cells were analyzed for green (GFPu) and red (myc or flag) fluorescence by FACS analysis. The graph shows the fold change (percentage) of transfected cells that displayed GFPu accumulation after PSI treatment, relative to control PAI-2-transfected cells. (**B**) SH-SY5Y cells were co-transfected with EGFP and WT, S18Y, C90S UCH-L1 or PAI-2M. Twenty-four hours after transfection, cells were treated with PSI (70 nM) or camptothecin (5 μM). Survival was evaluated by strip counts of EGFP positive cells at 0 and 24 h after PSI or camptothecin addition. The results are reported as mean ± SD ($n = 3$).

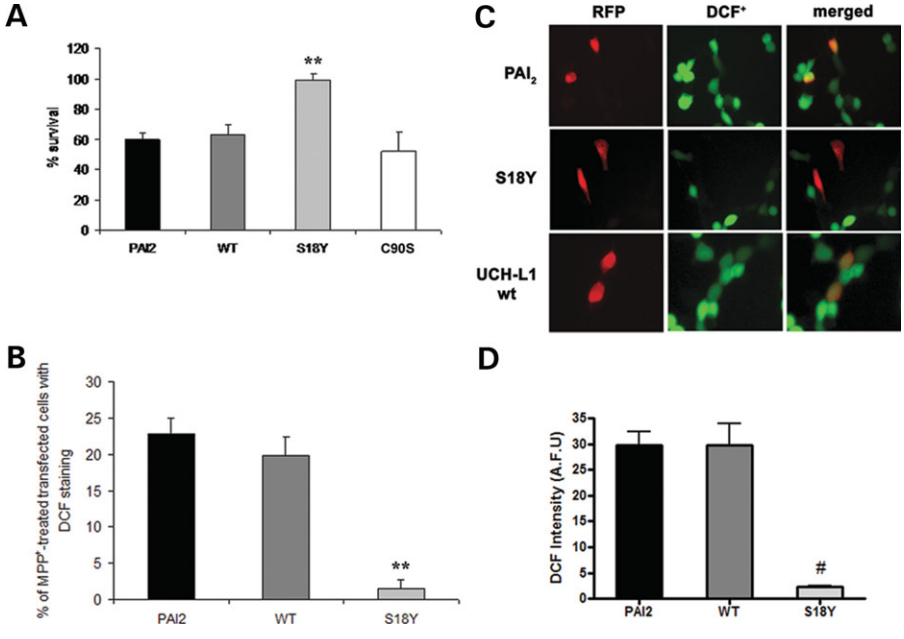

Figure 5. S18Y UCH-L1 is protective against MPP^+-induced death and ROS generation. SH-SY5Y cells were co-transfected with EGFP (**A**) or RFP (**B**) and WT UCH-L1, variant S18Y, C90S mutant or with control PAI-2M. Twenty-four hours (**A**) or 40 h (**B**) after transfection, cells were treated with 2 mM (A) or 4 mM (B) MPP^+. Survival in (A) was evaluated by strip counts of EGFP positive cells at 0 and 16 h after MPP^+ addition. In (B), cultures were labeled with DCF 6 h after MPP^+ addition. The graphs show the percentage of surviving EGFP-positive cells (A), the percentage of RFP/DCF-positive cells (B) or quantification of DCF fluorescence intensity (A.F.U., arbitrary fluorescence units) (**D**) in transfected cells by Image J analysis, as mean ± SD from triplicate wells each. Comparisons between groups were made by one-way ANOVA with Scheffe *post-hoc* comparisons; $^{**}P < 0.01$, $^{\#}P < 0.001$ compared with control. In (**C**), representative photos are shown.

The antioxidant function of S18Y UCH-L1 may be direct or indirect. The presence of tyrosine may act as a sink for ROS (although tyrosine residues are not thought to be very active in this regard), or it may modulate the interaction with endogenous metals, such as iron, involved in ROS generation, or it may impact endogenous antioxidant responses. These mechanistic issues represent the focus of future investigations.

Regarding the impact of UCH-L1 on ubiquitin, our studies are consistent with those performed by Osaka *et al*. (13), showing a reduction of monomeric ubiquitin in primary cortical neurons derived from *gad* mice, and an increase of free ubiquitin with overexpression of UCH-L1 in neuroblastoma cells, in a fashion dependent on an interaction with ubiquitin. These results contrast with those of Ardley *et al.* (15). The reasons for this discrepancy are unclear, but may involve cellular context. In any case, our data, together with those of Osaka *et al*. (13) and others (26), further establish the fact that UCH-L1 acts as a stabilizer of monomeric ubiquitin in neuronal cells. The function of S18Y UCH-L1 as a stabilizer of ubiquitin does not appear sufficient or necessary for its antioxidant effect, as WT UCH-L1, which also stabilizes free ubiquitin to the same extent, is not protective against oxidative stress, and the double mutant S18Y/D30K is still protective, as mentioned earlier.

Our starting point for this study was the interaction of UCH-L1 with the proteasome in Aplysia, resulting in the facilitation of proteasomal function (12). We however did not detect co-elution of UCH-L1 with the 26S proteasome.

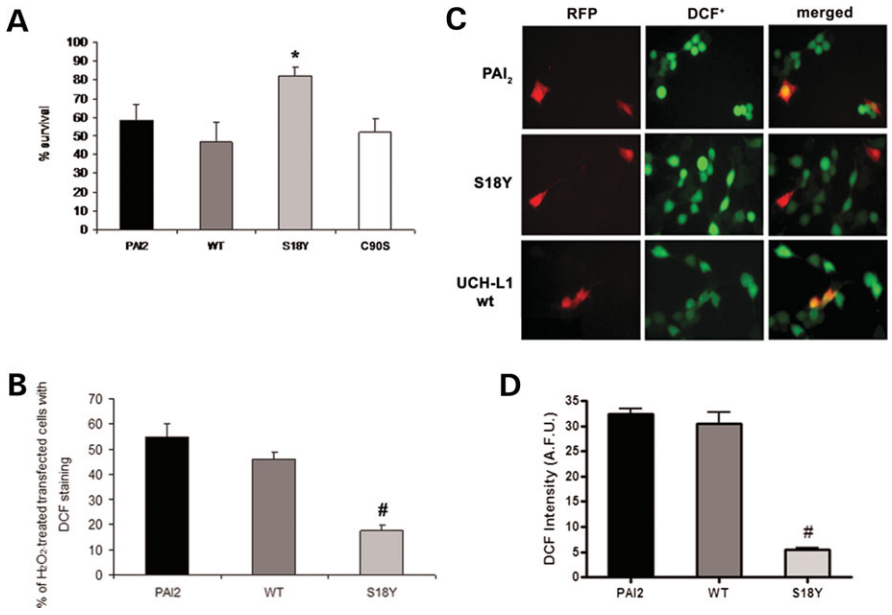

Figure 6. S18Y UCH-L1 is protective against H_2O_2-induced death and ROS generation. SH-SY5Y cells were co-transfected with EGFP (**A**) or RFP (**B**) and WT UCH-L1, variant S18Y, C90S mutant or with control PAI-2M. Twenty-four hours (A) or 40 h (B) after transfection, cells were treated with 20 μM (A) or 40 μM (B) H_2O_2. Survival in (A) was evaluated by strip counts of EGFP positive cells at 0 and 16 h after H_2O_2 addition. In (B), cultures were labeled with DCF 6 h after H_2O_2 addition. The graphs show the percentage of surviving EGFP-positive cells (A), the percentage of RFP/DCF-positive cells (B) or quantification of DCF fluorescence intensity (A.F.U., arbitrary fluorescence units) (**D**) in transfected cells by Image J analysis, as mean ± SD from triplicate wells each. Comparisons between groups were made by one-way ANOVA with Scheffe *post-hoc* comparisons; $^{*}P < 0.05$, $^{\#}P < 0.001$ compared with control. In (**C**), representative photos are shown.

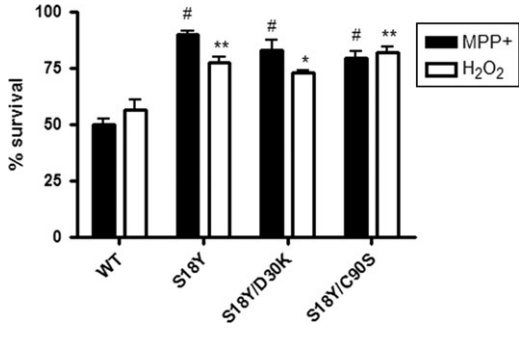

Figure 7. S18Y UCH-L1 protection against oxidative insults is independent of its ubiquitin-binding competency and hydrolase activity. SH-SY5Y cells were co-transfected with EGFP and WT UCH-L1, variant S18Y, mutant variants S18Y/C90S, S18Y/D30K or with control PAI-2M. Twenty-four hours after transfection, cells were treated with 2 mM MPP^+ or 20 μM H_2O_2. Survival was evaluated by strip counts of EGFP positive cells at 0 and 16 h after MPP^+ or H_2O_2 addition or exposure to no additives. The graph shows the percentage of surviving EGFP-positive cells as mean ± SD from triplicate wells each. Comparisons between groups were made by one-way ANOVA with Scheffe *post-hoc* comparisons by using S18Y UCH-L1 as a control. Survival in case of mutant variants S18Y/C90S and S18Y/D30K did not differ significantly compared with S18Y variant, whereas it was significantly increased compared with WT UCH-L1; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.001$.

Furthermore, overexpression of UCH-L1 did not alter sensitivity to proteasomal inhibition-induced death or UPS substrate accumulation. Interestingly, lack of UCH-L1 in cortical neurons was associated with a slight induction of enzymatic proteasomal activity, raising the possibility that compensatory changes at the level of the 26S proteasome may account, in part, for the paucity of functional effects on the UPS by the lack of UCH-L1 and the resultant decrease of monomeric ubiquitin. Alternatively, in primary neuronal cells in culture the UPS may function quite normally in the absence of UCH-L1, even though levels of monomeric ubiquitin are lower, because these levels are not limiting under non-stressed conditions.

A number of studies have reported a pro-apoptotic or anti-apoptotic role of UCH-L1 in certain situations in non-neuronal cells and tissues (27,28). In our studies, UCH-L1 did not exert such effects in neuronal cells. Overexpression of WT UCH-L1 alone tended to increase baseline apoptosis, but this result was not statistically significant. The I93M mutant, linked to PD in one family, also failed to induce significant detrimental effects in our assays. Recently, a transgenic mouse expressing I93M UCH-L1 was found to exhibit increased neurodegeneration within the substantia nigra, raising the possibility of a toxic gain of function (27). We have not detected such a function, but more prolonged periods of exposure may be needed for this to become manifest.

Apart from directly inducing apoptosis, a possible toxic gain of function of UCH-L1 could be due to protein aggregation. Consistent with the results of Ardley (15), we found that WT UCH-L1 induces smallish aggregates upon its overexpression in SH-SY5Y cells. The nature of these aggregates is unclear. We have not detected any clear induction of detergent-insoluble species on western immunoblotting of lysates of cells transfected with UCH-L1 variants using Flag antibodies (data not shown). Interestingly, the S18Y variant demonstrated less of a propensity to induce such aggregates; it may therefore be more available to exert effects in soluble cell compartments, and this may relate to its protective

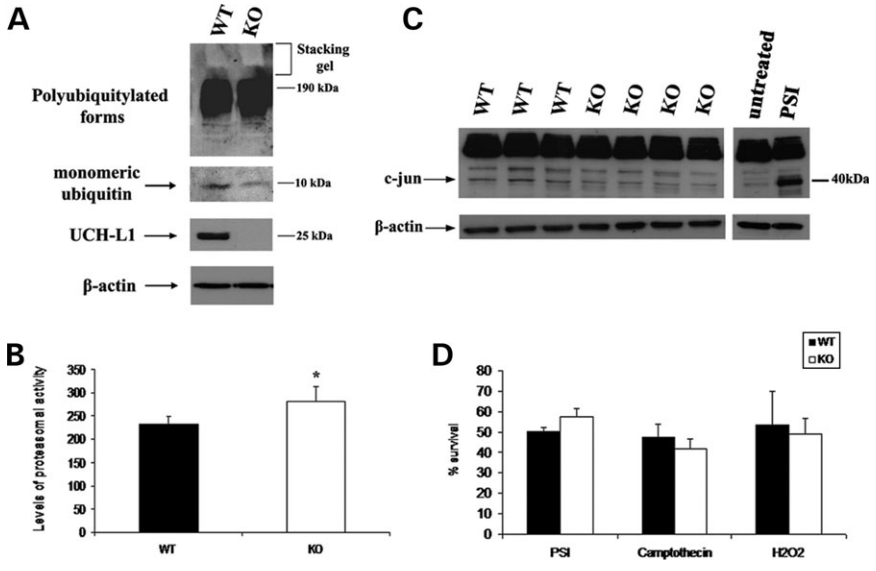

Figure 8. Cortical neurons derived from gad mice show no overall differential effects on the UPS. (**A**) Cortical neuron cultures were generated from UCH-L1+/+ (WT) and −/− (KO, *gad*) E16 mouse embryos. Seven days later, cortical neurons were lysed and cell lysates (13 μg protein) were resolved by SDS/PAGE and immunoblotted for ubiquitin (top panel, molecular weights above 70 kDa and middle panel, molecular weight in the range of 10 kDa), UCH-L1 and beta-actin (loading control). This blot is representative of two independent experiments. (**B**) Cortical neurons from WT and KO embryos-derived cultures were lysed and the supernatants were used to measure the enzymatic chymotrypsin-like proteasomal activity. The graph shows the levels of proteasomal activity in cortical neuron extracts from WT and KO mouse embryos. The results are reported as mean ± SD ($n = 3$). Comparisons between groups were made by Student's *t*-test; $^{*}P < 0.05$ compared with WT control. (**C**) Cortical neuron cultures were generated as in (A). Cell lysates (37 μg) were prepared as in (A) and immunoblotted for c-jun and beta-actin (loading control). On the right, c-jun accumulation in cell lysates of PSI-treated cortical neuron cultures from WT mice are depicted as a positive control. (**D**) Seven days old cortical neuron cultures were either left untreated or were treated with 150 μM H_2O_2 for 24 h, PSI (5 μM for 30 h) or camptothecin (5 μM for 16 h). The graph shows the percentage of intact nuclei in the H_2O_2-, PSI- or camptothecin-treated cultures compared with untreated cells, as mean ± SD ($n = 4$ each in independent wells).

properties, although one would still have to account for the specificity of the effect against oxidative stress. In fact, the less propensity of this variant to induce protein aggregation and cell death, and its antioxidant effect confer unique attractive therapeutic properties to this molecule. Based on the fact that WT UCH-L1 is downregulated or inactivated in various neurodegenerative conditions (29), and on the restoration of neurophysiological function in an animal model of Alzheimer's disease upon its overexpression (30), it has been proposed that strategies increasing WT UCH-L1 levels may have therapeutic value in neurodegenerative conditions (31). Our results argue that overexpression of the S18Y variant may provide a better alternative.

Our results have implications for the pathogenesis and treatment of PD and other neurodegenerative diseases where oxidative stress is thought to play a role. In PD, the significant protective effects of the S18Y polymorphism in young-onset sporadic cases suggest that in these patients oxidative stress may play an important role. In such patients, examination of the existence of this polymorphism may be useful in designing clinical trials with antioxidant therapies or in implementing such therapies only in those who do not harbor the polymorphism. Some studies suggest that the S18Y polymorphism may be protective in Huntington's disease and in Alzheimer's disease (32,33). This may signify that S18Y-modulated oxidative stress may also play a role in these diseases and, consequently, may be amenable to therapeutic intervention.

MATERIALS AND METHODS

cDNAs

Full-length human flag-UCH-L1 WT, C90S and I93M cDNA cloned into pCI-Neo vector between *Nhe*I and *Not*I sites, were generously provided by Dr K. Wada, National Institute of Neuroscience, Tokyo, Japan. The UCH-L1 point mutant pCI-flag-UCH-L1Asp30Lys, the polymorphic variant pCI-flag-UCH-L1Ser18Tyr as well as the polymorphic variant mutants pCI-flag-UCH-L1 Ser18Tyr/Asp30Lys and pCI-flag-UCH-L1 Ser18Tyr/Cys90Ser were generated by PCR-mediated site-directed mutagenesis, as described (34) and reintroduced into the same pCI-Neo vector. Sequencing verified the presence of the intended mutations and the absence of other nucleotide changes. The control PAI-2M cDNA cloned into pCDNA3-myc vector has been previously described (35).

Culture of cell lines

SH-SY5Y human neuroblastoma cells and SH-SY5Y cells stably expressing a short degron, CL1, fused to the C-terminus of green fluorescent protein (GFPu), as a reporter for proteasomal function (17) were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin–streptomycin (P/S; Invitrogen) in a 5% CO_2 atmosphere at 37°C.

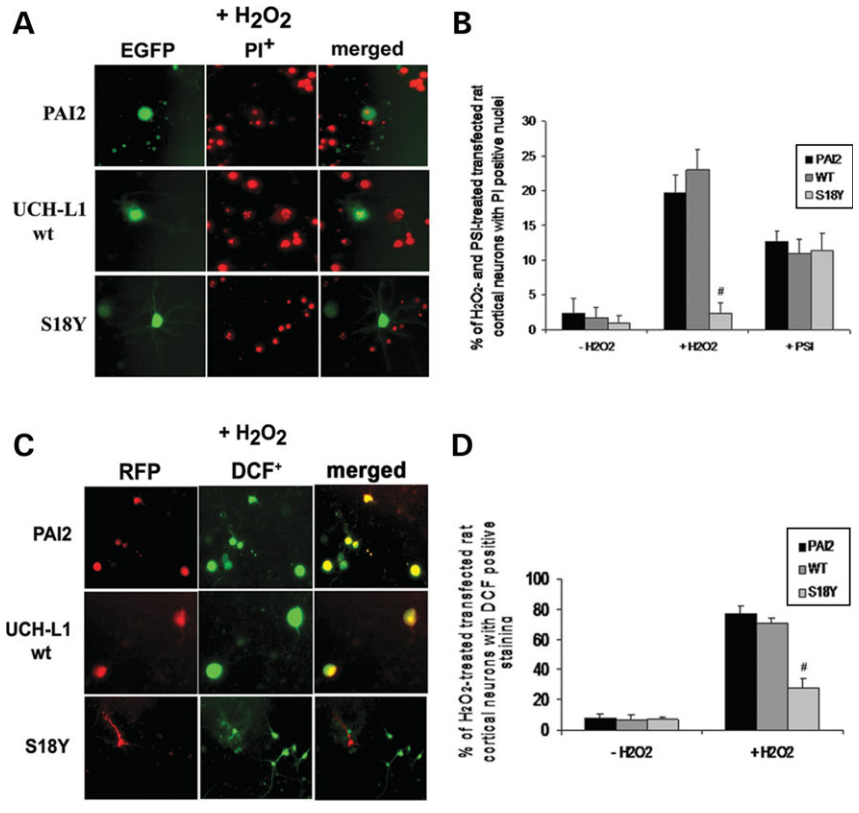

Figure 9. S18Y UCH-L1 overexpression in rat primary cortical neurons is protective against H_2O_2-induced death and ROS generation. (**A** and **B**) Rat primary cortical neurons from E16 cultures were co-transfected with EGFP and WT, S18Y UCH-L1 or with control PAI-2M. Twenty-four hours after transfection cultures were exposed to no additives (Supplementary Material, Fig. S2A) or to 40 μM H_2O_2 (for 16 h) or to 10 μM PSI (for 24 h). The cells were then stained with propidium iodide (PI). Representative photos from H_2O_2-treated cultures are shown in (A). In (B), the percentage of H_2O_2- or PSI-treated transfected cells with PI-positive nuclei is depicted. The results are reported as mean ± SD ($n = 3$). Comparisons between groups were made by one-way ANOVA with Scheffe *post-hoc* comparisons; $^{\#}P < 0.001$ compared with control PAI-2M-transfected. Similar results were achieved in three independent experiments. (**C** and **D**) Rat primary cortical neurons from E16 cultures were co-transfected with RFP and WT, S18Y UCH-L1 or with control PAI-2M. Forty hours after transfection, cultures were exposed to no additives (Supplementary Material, Fig. S2B) or to 70 μM H_2O_2 for 6 h. The cultures were then labeled with DCF. In (C), representative photos are shown. In (D), the percentage of H_2O_2-treated transfected cells with DCF positivity is depicted. The results are reported as mean ± SD ($n = 3$). Comparisons between groups were made by one-way ANOVA with Scheffe *post-hoc* comparisons; $^{\#}P < 0.001$ compared with PAI-2M control. Similar results were achieved in two independent experiments.

Knockout mice and genotyping

Heterozygote breeding pairs of +/− *gad* mice were generously provided to us by Dr K. Wada, National Institute of Neuroscience, Tokyo, Japan. +/+ (wt) and −/− (gad/gad, KO) UCH-L1 neurons were obtained from Embryonic Day 16 (E16) mouse embryos resulting from heterozygous matings. Genotyping was performed on each individual embryo by polymerase chain reaction (PCR) with genomic DNA extracted from tails, as described (21), with minor modifications.

Cortical neuronal cultures

Cultures of rat E18 cortical neurons or embryonic mouse E16 cortical neuronal cultures were prepared as described previously (18,36,37). Neurons were cultured in serum-free medium [SFM, B27-supplemented Neurobasal medium (NB) (Gibco) containing 0.5 mM L-glutamine and 1% penicillin/streptomycin) and plated on poly-D-lysine-coated tissue cultures dishes. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Such cultures consist of at least 97% of neurons (18,37).

Transfection

SH-SY5Y cells were transfected with UCH-L1 or PAI2M constructs alone or co-transfected with the gene of interest (UCH-L1 or PAI2M constructs) and fluorescent protein, either EGFP or RFP, in 3:1 plasmid DNA ratio, respectively, using Lipofectamine 2000 (Invitrogen)-DNA ratios as suggested by the manufacturer. Rat primary cortical neurons from E18 cultures were similarly transfected using Lipofectamine 2000, with some modifications. Briefly, a total amount of 1.5 μg DNA with a ratio DNA:Lipofectamine of 1:2 were added in Neurobasal medium (NB) (Gibco) without serum and supplements. Using the afore-mentioned ratio of plasmid DNA in the case of co-transfection, it was evaluated by immunohistochemistry that 95% of fluorescent protein positive cells co-expressed also the gene of interest. This percentage did not differ among the various constructs utilized. In the case of survival assessment by strip counts, the plasmid ratio of the gene

of interest (UCH-L1 or PAI2M constructs) to fluorescent protein EGFP was increased to 8:1. With this ratio, 100% of fluorescent protein positive cells transfected with any one of the constructs co-expressed the gene of interest.

Cell treatments

Cells were treated with the mitochondrial toxin MPP^+ (Sigma) or the ROS generator H_2O_2 (H_2O_2 30%, Merck) or the proteasomal inhibitor PSI (Benzyloxycarbonyl-L-Isoleucyl-Gamma-*t*-Butyl-L-Glutamyl-L-Alanyl-L-Leucinal) (Calbiochem) or the DNA damaging agent camptothecin (Sigma).

Assessment of survival

After application of the above reagents, survival in SH-SY5Y cells was evaluated by strip counts of EGFP-positive cells at 0 and 16 or 24 h of application. In this method, we count the number of transfected cells in a defined area (the strip), and then go back and count the number of transfected cells in the same area some time later. We express viability as the percentage of transfected cells that remain at this second time point relative to time 0, and then normalize it for control cultures which have not been exposed to an insult. Alternatively, Hoechst staining was utilized; nuclei were considered apoptotic if they showed condensation, chromatin margination or clumping or fragmentation. Survival of cortical neurons was evaluated by propidium iodide (PI) staining (Fluka), which permeabilizes only dying neurons, or by cell lysis and counting of intact nuclei in a hemacytometer as described previously (18,38). The data are expressed as the percentage of the number of cells in the control cultures (i.e. cultures that were treated with no additives) at each time point. All counts were performed in a blinded manner. Cell counts were performed in triplicate and are reported as means $\pm$ SD. All data shown are representative of at least three independent experiments.

Cell lysis

Cell lysates from SH-SY5Y cells or cortical neurons were prepared using a Dounse (Teflon, FALC, Treviglo, BG, Italy) homogenizer (30 s/sample) in lysis buffer containing 25 mmol/l Tris–HCL pH 7.6, 2 mmol/l ATP, 1 mmol/l dithiothreitol and 2 mmol/l $MgCl_2$. The cell lysate was centrifuged at 10 000*g* for 5 min at 4°C. Protein concentrations in supernatants (soluble fractions) were determined by using Bradford protein assay.

Western immunoblotting

Soluble material from cell lysates in sample buffer was used for western immunoblotting, as described (18). Primary antibodies were: mouse monoclonal anti-ubiquitin [(1: 750; Chemicon, Temecula, CA, USA), anti-b-actin (1: 20 000; Sigma), rabbit polyclonal anti-20S proteasome b5 subunit (1: 1000; Affinity)], rabbit polyclonal anti-19S proteasome S5a subunit (1:1000; Affinity), mouse monoclonal anti-M_2Flag (1:500; Sigma), rabbit polyclonal anti-UCH(PGP 9.5) (1: 2000; Affinity), rabbit polyclonal anti-c-jun (1:500; Santa Cruz).

Immunocytochemistry and fluorescent microscopy

Immunostaining and Hoechst labeling of SH-SY5Y cells and cortical neurons was performed as described (17,18). Primary antibodies were the following: rabbit polyclonal anti-ubiquitin (1:100; Dako, Glostrup, Denmark), anti-myc (clone 9E10, medium from hybridoma cell line; 1:4), mouse monoclonal anti-M_2Flag (1:400; Sigma). Cells were finally rinsed twice with 1 × PBS and imaged with a Leica DMIRB inverted fluorescent microscope (Leica Microsystems, Bannockburn, IL, USA). Representative digital images were captured with SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI, USA) and saved with SPOT software. In cases where comparisons were done across different conditions, care was taken to obtain images at identical exposure times.

Proteasomal activity assay

Primary cortical neurons were lysed as earlier in lysis buffer. The supernatants were collected for the measurement of the enzymatic chymotrypsin-like activity of the proteasome, essentially as described (34), based on cleavage of proteasomal substrate III (Calbiochem), using a Perkin Elmer fluorimeter LC-55 with excitation at 380 nm and emission at 438 nm. Three independent reactions were performed for each sample, in the presence or absence of the selective proteasomal inhibitor epoxomicin (1 μM), and the mean of the difference between these measurements was recorded. Three independent samples from three different mice were assessed for each group. The results are reported as mean $\pm$ SD.

Fluorescence-activated cell sorting

Cells fixed in suspension [transfected SH-SY5Y cells stably expressing GFPu, untransfected cells and cells treated with the specific proteasomal inhibitor PSI (50 nM for 18 h)] were used for FACS analysis, as described (39). Primary antibodies were: mouse monoclonal anti-myc (1:400; Sigma) and mouse monoclonal anti-M_2Flag (1:400; Sigma). Secondary antibody was goat anti-mouse IgG(H+L) conjugated with human PE ($\leq$0.1 μg/10^6 cells; Beckman Coulter). Fixed cells (at least 13 000 events per sample) were analyzed for green (GFPu) and red (myc or flag) fluorescence in FACScan cytometer (Beckman Coulter) equipped with a single 488 nm Argon laser, recorded in list mode and registered on logarithmic scales. Green (GFPu) fluorescence was detected at FL1 channel and red (PE) fluorescence at FL2. GFPu background fluorescence, corresponding to cells transiently transfected with each plasmid in the absence of the inhibitor PSI, was subtracted from samples. Analysis was performed with CXP software (Beckman Coulter).

Assessment of oxidative stress

The determination of intracellular oxidant production was based on the oxidation of carboxy-H_2DCFDA (DCFH) (Molecular Probes, Inc., Eugene, OR) to the fluorescent product,

carboxy-2′,7′-dichlorofluorescein (DCF) (40). Cells were rinsed once with culture medium and cultures were exposed to culture medium without FBS containing 2 μM DCFH for 20 min at 37°C. After DCFH labeling, the cells were rinsed twice, replenished with fresh medium and, after 30 min incubation at 37°C, visualized under a fluorescent microscope (Leica DMIRB inverted fluorescent microscope, Leica Microsystems, Bannockburn, IL, USA). The percentage of RFP-transfected cells that showed green fluorescence (DCF positive staining) was assessed in each condition. Counts were performed in triplicate and are reported as means ± SD. DCF labeling, under the conditions we have employed, gives extremely low background fluorescence in control cultures and extremely robust induction following oxidative insults. It is therefore quite straightforward to assign a particular cell a positive or negative fluorescence signal.

Quantitative analysis of fluorescence

To further solidify the binary assessment of cells as ubiquitin-positive or -negative and DCF-positive or -negative, we also, in select experiments, performed quantitative fluorescence analysis, following either DCF incubation or ubiquitin immunostaining. Following fluorescence image acquisition at identical exposure times across conditions, we selected at random 100 transfected cells per well, in three separate wells per condition, and acquired fluorescence signal intensity measurements in the green (DCF) or red (ubiquitin) spectra from individual whole cells using Image J software. Results are presented as means ± SD.

Size exclusion chromatography

For SEC, cells were treated as described previously (41,42) with some minor modifications. Lysates were injected into a Superose 6 10/300 GL column (Amersham Pharmacia) and fractions of 250 μl were collected.

Statistical analysis

Statistical analysis between groups was made by one-way ANOVA with Scheffe *post-hoc*comparisons for multiple comparisons or unpaired two-tailed Student's *t*-test for single comparisons with a probability value of <0.05.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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REFERENCES

1. Dauer, W. and Przedborski, S. (2003) Parkinson's disease: mechanisms and models. *Neuron*, **39**, 889–909.
2. Klein, C. and Schlossmacher, M.G. (2007) Parkinson disease, 10 years after its genetic revolution: multiple clues to a complex disorder. *Neurology*, **69**, 2093–2104.
3. Leroy, E., Boyer, R., Auburger, G., Leube, B., Ulm, G., Mezey, E., Harta, G., Brownstein, M.J., Jonnalagada, S., Chernova, T. *et al.* (1998) The ubiquitin pathway in Parkinson's disease. *Nature*, **395**, 451–452.
4. Maraganore, D.M., Lesnick, T.G., Elbaz, A., Chartier-Harlin, M.C., Gasser, T., Kruger, R., Hattori, N., Mellick, G.D., Quattrone, A., Satoh, J. *et al.* (2004) UCHL1 is a Parkinson's disease susceptibility gene. *Ann. Neurol.*, **55**, 512–521.
5. Elbaz, A., Levecque, C., Clavel, J., Vidal, J.S., Richard, F., Correze, J.R., Delemotte, B., Amouyel, P., Alperovitch, A., Chartier-Harlin, M.C. *et al.* (2003) S18Y polymorphism in the UCH-L1 gene and Parkinson's disease: evidence for an age-dependent relationship. *Mov. Disord.*, **18**, 130–137.
6. Maraganore, D.M., Farrer, M.J., Hardy, J.A., Lincoln, S.J., McDonnell, S.K., Rocca, W.A. *et al.* (1999) Case-control study of the ubiquitin carboxy-terminal hydrolase L1 gene in Parkinson's disease. *Neurology*, **53**, 1858–1860.
7. Satoh, J. and Kuroda, Y. (2001) A polymorphic variation of serine to tyrosine at codon 18 in the ubiquitin C-terminal hydrolase-L1 gene is associated with a reduced risk of sporadic Parkinson's disease in a Japanese population. *J. Neurol. Sci.*, **189**, 113–117.
8. Carmine Belin, A., Westerlund, M., Bergman, O., Nissbrandt, H., Lind, C., Sydow, O., Galter, D. *et al.* (2007) S18Y in ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) associated with decreased risk of Parkinson's disease in Sweden. *Parkinsonism Relat. Disord.*, **13**, 295–298.
9. Healy, D.G., Abou-Sleiman, P.M., Casas, J.P., Ahmadi, K.R., Lynch, T., Gandhi, S., Muqit, M.M., Foltynie, T., Barker, R., Bhatia, K.P. *et al.* (2006) UCHL-1 is not a Parkinson's disease susceptibility gene. *Ann. Neurol.*, **59**, 627–633.
10. Setsuie, R. and Wada, K. (2007) The functions of UCH-L1 and its relation to neurodegenerative diseases. *Neurochem. Int.*, **51**, 105–111.
11. Liu, Y., Fallon, L., Lashuel, H.A., Liu, Z. and Lansbury, P.T., Jr (2002) The UCH-L1 gene encodes two opposing enzymatic activities that affect alpha-synuclein degradation and Parkinson's disease susceptibility. *Cell*, **111**, 209–218.
12. Hegde, A.N., Inokuchi, K., Pei, W., Casadio, A., Ghirardi, M., Chain, D.G., Martin, K.C., Kandel, E.R., Schwartz, J.H. *et al.* (1997) Ubiquitin C-terminal hydrolase is an immediate-early gene essential for long-term facilitation in Aplysia. *Cell*, **89**, 115–126.
13. Osaka, H., Wang, Y.L., Takada, K., Takizawa, S., Setsuie, R., Li, H., Sato, Y., Nishikawa, K., Sun, Y.J., Sakurai, M. *et al.* (2003) Ubiquitin carboxy-terminal hydrolase L1 binds to and stabilizes monoubiquitin in neuron. *Hum. Mol. Genet.*, **12**, 1945–1958.
14. Lang-Rollin, I., Rideout, H. and Stefanis, L. (2003) Ubiquitinated inclusions and neuronal cell death. *Histol. Histopathol.*, **18**, 509–517.
15. Ardley, H.C., Scott, G.B., Rose, S.A., Tan, N.G. and Robinson, P.A. (2004) UCH-L1 aggresome formation in response to proteasome impairment indicates a role in inclusion formation in Parkinson's disease. *J. Neurochem.*, **90**, 379–391.
16. Harada, T., Harada, C., Wang, Y.L., Osaka, H., Amanai, K., Tanaka, K., Takizawa, S., Setsuie, R., Sakurai, M., Sato, Y. *et al.* (2004) Role of ubiquitin carboxy terminal hydrolase-L1 in neural cell apoptosis induced by ischemic retinal injury in vivo. *Am. J. Pathol.*, **164**, 59–64.
17. Kyratzi, E., Pavlaki, M., Kontostavlaki, D., Rideout, H.J. and Stefanis, L. (2007) Differential effects of Parkin and its mutants on protein aggregation, the ubiquitin-proteasome system, and neuronal cell death in human neuroblastoma cells. *J. Neurochem.*, **102**, 1292–1303.

18. Stefanis, L., Park, D.S., Friedman, W.J. and Greene, L.A. (1999) Caspase-dependent and -independent death of camptothecin-treated embryonic cortical neurons. *J. Neurosci.*, **19**, 6235–6247.
19. Przedborski, S. and Vila, M. (2003) The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model: a tool to explore the pathogenesis of Parkinson's disease. *Ann. NY Acad. Sci.*, **991**, 189–198.
20. Lang-Rollin, I.C., Rideout, H.J., Noticewala, M. and Stefanis, L. (2003) Mechanisms of caspase-independent neuronal death: energy depletion and free radical generation. *J. Neurosci.*, **23**, 11015–11025.
21. Saigoh, K., Wang, Y.L., Suh, J.G., Yamanishi, T., Sakai, Y., Kiyosawa, H., Harada, T., Ichihara, N., Wakana, S., Kikuchi, T. *et al.* (1999) Intragenic deletion in the gene encoding ubiquitin carboxy-terminal hydrolase in gad mice. *Nat. Genet.*, **23**, 47–51.
22. Treier, M., Staszewski, L.M. and Bohmann, D. (1994) Ubiquitin-dependent c-Jun degradation *in vivo* is mediated by the delta domain. *Cell*, **78**, 787–798.
23. Jariel-Encontre, I., Pariat, M., Martin, F., Carillo, S., Salvat, C., Piechaczyk, M. *et al.* (1995) Ubiquitinylation is not an absolute requirement for degradation of c-Jun protein by the 26 S proteasome. *J. Biol. Chem.*, **270**, 11623–11627.
24. Satoh, J.I. and Kuroda, Y. (2001) Ubiquitin C-terminal hydrolase-L1 (PGP9.5) expression in human neural cell lines following induction of neuronal differentiation and exposure to cytokines, neurotrophic factors or heat stress. *Neuropathol. Appl. Neurobiol.*, **27**, 95–104.
25. Elbaz, A. and Tranchant, C. (2007) Epidemiologic studies of environmental exposures in Parkinson's disease. *J. Neurol. Sci.*, **262**, 37–44.
26. Meray, R.K. and Lansbury, P.T., Jr (2007) Reversible monoubiquitination regulates the Parkinson disease-associated ubiquitin hydrolase UCH-L1. *J. Biol. Chem.*, **282**, 10567–10575.
27. Setsuie, R., Wang, Y.L., Mochizuki, H., Osaka, H., Hayakawa, H., Ichihara, N., Li, H., Furuta, A., Sano, Y., Sun, Y.J. *et al.* (2007) Dopaminergic neuronal loss in transgenic mice expressing the Parkinson's disease-associated UCH-L1 I93M mutant. *Neurochem. Int.*, **50**, 119–129.
28. Kwon, J., Wang, Y.L., Setsuie, R., Sekiguchi, S., Sato, Y., Sakurai, M., Noda, M., Aoki, S., Yoshikawa, Y., Wada, K. *et al.* (2004) Two closely related ubiquitin C-terminal hydrolase isozymes function as reciprocal modulators of germ cell apoptosis in cryptorchid testis. *Am. J. Pathol.*, **165**, 1367–1374.
29. Choi, J., Levey, A.I., Weintraub, S.T., Rees, H.D., Gearing, M., Chin, L.S., Li, L. *et al.* (2004) Oxidative modifications and down-regulation of ubiquitin carboxyl-terminal hydrolase L1 associated with idiopathic Parkinson's and Alzheimer's diseases. *J. Biol. Chem.*, **279**, 13256–13264.
30. Gong, B., Cao, Z., Zheng, P., Vitolo, O.V., Liu, S., Staniszewski, A., Moolman, D., Zhang, H., Shelanski, M., Arancio, O. *et al.* (2006) Ubiquitin hydrolase Uch-L1 rescues beta-amyloid-induced decreases in synaptic function and contextual memory. *Cell*, **126**, 775–788.
31. Gong, B. and Leznik, E. (2007) The role of ubiquitin C-terminal hydrolase L1 in neurodegenerative disorders. *Drug News Perspect.*, **20**, 365–370.
32. Naze, P., Vuillaume, I., Destee, A., Pasquier, F. and Sablonniere, B. (2002) Mutation analysis and association studies of the ubiquitin carboxy-terminal hydrolase L1 gene in Huntington's disease. *Neurosci. Lett.*, **328**, 1–4.
33. Xue, S. and Jia, J. (2006) Genetic association between Ubiquitin Carboxy-terminal Hydrolase-L1 gene S18Y polymorphism and sporadic Alzheimer's disease in a Chinese Han population. *Brain Res.*, **1087**, 28–32.
34. Stefanis, L., Larsen, K.E., Rideout, H.J., Sulzer, D. and Greene, L.A. (2001) Expression of A53T mutant but not wild-type alpha-synuclein in PC12 cells induces alterations of the ubiquitin-dependent degradation system, loss of dopamine release, and autophagic cell death. *J. Neurosci.*, **21**, 9549–9560.
35. Jabado, O., Wang, Q., Rideout, H.J., Yeasmin, M., Guo, K.X., Vekrellis, K., Papantonis, S., Angelastro, J.M., Troy, C.M., Stefanis, L. *et al.* (2004) RAIDD aggregation facilitates apoptotic death of PC12 cells and sympathetic neurons. *Cell Death Differ.*, **11**, 618–630.
36. Rideout, H.J., Zang, E., Yeasmin, M., Gordon, R., Jabado, O., Park, D.S., Stefanis, L. *et al.* (2001) Inhibitors of trypsin-like serine proteases prevent DNA damage-induced neuronal death by acting upstream of the mitochondrial checkpoint and of p53 induction. *Neuroscience*, **107**, 339–352.
37. Rideout, H.J. and Stefanis, L. (2002) Proteasomal inhibition-induced inclusion formation and death in cortical neurons require transcription and ubiquitination. *Mol. Cell Neurosci.*, **21**, 223–238.
38. Rukenstein, A., Rydel, R.E. and Greene, L.A. (1991) Multiple agents rescue PC12 cells from serum-free cell death by translation- and transcription-independent mechanisms. *J. Neurosci.*, **11**, 2552–2563.
39. Lecoeur, H., Chauvier, D., Langonne, A., Rebouillat, D., Brugg, B., Mariani, J., Edelman, L., Jacotot, E. *et al.* (2004) Dynamic analysis of apoptosis in primary cortical neurons by fixed- and real-time cytofluorometry. *Apoptosis*, **9**, 157–169.
40. LeBel, C.P., Ischiropoulos, H. and Bondy, S.C. (1992) Evaluation of the probe 2′,7′-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. *Chem. Res. Toxicol.*, **5**, 227–231.
41. Hendil, K.B., Khan, S. and Tanaka, K. (1998) Simultaneous binding of PA28 and PA700 activators to 20 S proteasomes. *Biochem. J.*, **332** (Pt 3), 749–754.
42. Hendil, K.B., Hartmann-Petersen, R. and Tanaka, K. (2002) 26 S proteasomes function as stable entities. *J. Mol. Biol.*, **315**, 627–636.