Ensnaring Neurodegeneration

Many believe that a decrease in proteasome activity contributes to the pathogenesis of neurodegenerative disorders, such as Alzheimer's and Parkinson's disease. Thus, activation of proteasome activity has been considered a promising therapeutic strategy for treating neurodegenerative diseases. In a new study, Sharma et al. tested proteasome inhibitors in mice with neurodegeneration caused by deletion of cysteine string protein-α. Mice lacking cysteine string protein-α die early because of loss of synapses and neuronal death, which results from loss of the SNARE protein SNAP-25 (for which cysteine string protein-α is a chaperone), and a decrease in the assembly of SNARE complexes. Surprisingly, the authors found the opposite of what they expected: Instead of accelerating neurodegeneration, proteasome inhibitors alleviated neurodegeneration. This unexpected result demonstrates that at least for this form of neurodegeneration, proteasome inhibition does not represent a pathogenic mechanism, but instead can be used as a therapeutic strategy. The researchers showed that the proteasome inhibitors alleviated neurodegeneration by increasing SNAP-25 concentrations and enhancing SNARE-complex assembly. They then demonstrated that SNARE-complex assembly is impaired in human brain tissue from patients with neurodegenerative diseases. The impact of this study may go beyond neurodegeneration because systemic administration of proteasome inhibitors is currently being tested as a cancer treatment. Proteasome inhibitors also may help in the treatment of other diseases such as cystic fibrosis and nephrogenic diabetes insipidus, which are caused by proteasomal degradation of functionally important proteins.
Proteasome Inhibition Alleviates SNARE-Dependent Neurodegeneration

Manu Sharma,* Jacqueline Burré, Thomas C. Südhof*

Activation of the proteasomal degradation of misfolded proteins has been proposed as a therapeutic strategy for treating neurodegenerative diseases, but it is unclear whether proteasome dysfunction contributes to neurodegeneration. We tested the role of proteasome activity in neurodegeneration developed by mice lacking cysteine string protein–α (CSPα). Unexpectedly, we found that proteasome inhibitors alleviated neurodegeneration in CSPα-deficient mice, reversing impairment of SNARE (soluble N-ethylmaleimide–sensitive factor attachment protein receptor)–complex assembly and extending life span. We tested whether dysfunctional SNARE-complex assembly could contribute to neurodegeneration in Alzheimer’s and Parkinson’s disease by analyzing postmortem brain tissue from these patients; we found reduced SNARE-complex assembly in the brain tissue samples. Our results suggest that proteasome activation may not always be beneficial for alleviating neurodegeneration and that blocking the proteasome may represent a potential therapeutic avenue for treating some forms of neurodegenerative disease.

INTRODUCTION

Protein misfolding has been implicated in a number of neurodegenerative diseases like Alzheimer’s disease, Huntington’s disease, Parkinson’s disease, and prion diseases (1–3). Misfolded neurotoxic proteins are often ubiquitinated and hence are targeted for degradation by the proteasome (4, 5). Therefore, inhibition of proteasome activity has been proposed to be a potential cause of neurodegeneration or may exacerbate it, possibly by allowing the accumulation of toxic misfolded proteins such as amyloid-β, huntingtin, α-synuclein, and PrPsc in Alzheimer’s disease, Huntington’s disease, Parkinson’s disease, and prion disease respectively (see table S1) (6, 7). Conversely, activation of proteasome activity has been suggested as a therapeutic strategy to alleviate neurodegeneration (8). However, proteasome inhibition may also have neuroprotective effects (9, 10). Collectively, these observations raise the question of how the proteasome affects neurodegeneration associated with protein misfolding.

To address this question, we used mice lacking cysteine string protein–α (CSPα) as a model of neurodegeneration that is caused by misfolding of a neuronal protein (11, 12). CSPα is a DNA-J domain–containing co-chaperone that is associated with synaptic vesicles in nerve terminals and functions as a chaperone in a complex with Hsc70 (heat shock cognate 70) (13) and the tetratricopeptide repeat protein SGT (small glutamine-rich tetratricopeptide repeat–containing protein) (14). In humans, mutations in the CSPα gene cause autosomal-dominant adult-onset neuronal ceroid lipofuscinosis, a neurodegenerative disorder (15, 16). CSPα-deficient flies and mice develop severe neurodegeneration and have a reduced life span (17–19). CSPα-deficient mice are normal at birth but postnatally develop an impairment of synaptic release in an activity-dependent manner (17, 20) and later suffer from a fulminant form of neurodegeneration that leads to paralysis and death (17, 18).

The synaptic SNARE (soluble N-ethylmaleimide–sensitive factor attachment protein receptor) protein SNAP-25 (synaptosome-associated protein of 25 kD) is a key substrate of the CSPα/SGT/Hsc70 complex (11). During SNARE-mediated synaptic vesicle fusion, the CSPα/Hsc70/SNARE complex directly chaperones SNAP-25 (which is natively unfolded but becomes folded during every synaptic fusion event), promoting the assembly of SNAP-25 into SNARE complexes with its partners syntaxin-1 and synaptobrevin-2/VAMP2 (vesicle-associated membrane protein 2) (11). Even before synaptic dysfunction and neurodegeneration become apparent, the brains of CSPα-deficient mice show a decrease in the amount of SNAP-25 and exhibit a severe reduction in SNARE-complex assembly (18). In neurons from CSPα-deficient mice, dysfunctional SNAP-25 is ubiquitinated and degraded by the proteasome in a synaptic activity–dependent manner, accounting for the reduction in SNAP-25 (11). The importance of CSPα chaperone function for long-term neuronal survival is evident from the fact that reducing SNAP-25 concentration in CSPα-deficient mice worsens neurodegeneration, whereas increasing SNAP-25 relieves neurodegeneration (12).

Collectively, these studies suggest that accelerated, activity-dependent degradation of SNAP-25 by the proteasome may contribute to the development of neurodegeneration in CSPα-deficient mice. However, this hypothesis seemingly contradicts the notion that proteasomal degradation of misfolded proteins is beneficial and that neurodegeneration occurs because such degradation is impaired (see table S1 for an overview of the literature). Here, we set out to test how proteasome activity affects the neurodegenerative and molecular phenotype of mice lacking CSPα using pharmacological inhibition of proteasome function in vitro and in vivo.

RESULTS

Proteasome inhibitors alleviate neurodegeneration in CSPα-deficient mice

Biochemical studies of brains from CSPα-deficient mice revealed that proteins involved in proteasomal degradation exhibited increased expression and that proteasomal protease activity was broadly enhanced (fig. S1). Because proteasomal degradation of possibly toxic misfolded proteins is generally thought to be a protective mechanism by which
brain cells try to cope with neurodegeneration (1, 21), we hypothesized that the increased proteasome activity in CSPα-deficient brains at a time of active neurodegeneration represents a reactive response to protect against the neurodegeneration observed in these mice. To test this hypothesis, we examined whether proteasome inhibitors accelerate the neurodegenerative phenotype of CSPα-deficient mice.

We injected CSPα-deficient mice intraperitoneally with the proteasome-specific inhibitors lactacystin, epoxomicin, or vehicle every 5 days beginning at postnatal day 5 (P5) and continuously analyzed the weight and selected behavioral characteristics of the treated mice to gauge the progression of neurological symptoms. The proteasome inhibitor treatments did not appear to generally impair the CSPα-deficient mice or alter their developmental weight gain (Fig. 1A and B), but did delay the onset of behavioral symptoms in these mice. Specifically, changes in hindlimb clasping, limb strength (assessed with the grid-hanging test), and motor coordination (assessed by the righting time) occurred later in treated mice than in identically handled untreated littermate control mice (Fig. 1C to E). Force plate actometer analyses, performed every 5 days, confirmed that CSPα-deficient mice injected with proteasome inhibitors lost their mobility later than mice injected with vehicle only (fig. S2). Both proteasome inhibitors extended the survival of CSPα-deficient mice, although the effect was modest in size, possibly because of the non-optimized treatment regimen [Fig. 1F; median survival increased 10 days with lactacystin (P = 0.042) and 15.5 days with epoxomicin (P = 0.0016)].

We next probed the effect of the proteasome inhibitors on the neurodegeneration observed in CSPα-deficient mice. Previous studies showed that this neurodegeneration manifests as a loss of synapses [monitored by staining for the postsynaptic marker protein PSD95 (postsynaptic density 95)] and of neurons (examined by staining for the neuronal nuclear marker NeuN), and an increase in neuronal apoptosis (assessed by immunocytochemistry for activated caspase) and in astroglisis [measured by staining for the astroglial marker GFAP (glial fibrillary acidic protein)] (11, 12). When we applied the same tests to brains from control and treated CSPα-deficient mice at P50, we observed a marked decrease in neurodegeneration in proteasome inhibitor–treated brains using these four characteristics (Fig. 2). The two proteasome inhibitors, lactacystin and epoxomicin, produced overall similar results, although epoxomicin was slightly more effective than lactacystin at slowing neurodegeneration, possibly because it is more potent (22). Thus, systemic administration of protease inhibitors is unexpectedly therapeutically beneficial for slowing the neurodegeneration observed in CSPα-deficient mice.

**Proteasome inhibitors rescue impaired SNARE-complex assembly**

To explore the mechanism by which proteasome inhibitors might alleviate neurodegeneration in CSPα-deficient mice, we incubated cortical neurons cultured from CSPα-deficient mice in medium containing the proteasome inhibitors lactacystin (10 μM) and epoxomicin

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**Fig. 1.** Proteasome inhibitors alleviate the neurodegenerative phenotype of CSPα-deficient mice. Littermate CSPα-deficient mice were injected intraperitoneally every 5 days with either vehicle control (ethanol), lactacystin (0.1 mM), or epoxomicin (0.1 mM); 10 μl from P5 to P20 and 100 μl from day 25 until mice became incapacitated. (A) Images of representative treated CSPα-deficient mice at P25. (B) Body weight of control and lactacystin- or epoxomicin-treated CSPα-deficient mice as a function of age. (C to E) Age-dependent development of neurological impairments in control and in lactacystin- or epoxomicin-treated CSPα-deficient mice. Hindlimb clasping (C), grid-hanging time (D), and righting reflex times (E) were measured. For force plate analyses, see fig. S2. (F) Effect of proteasome inhibitor treatments on survival in lactacystin- or epoxomicin-treated CSPα-deficient mice. Data in (B) are means ± SEM. n.s., not significant by Student’s t test. For data in (C) and (F), *P < 0.05, **P < 0.01, ***P < 0.001 by Mantel-Cox test. Data in (D) and (E) are means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 by two-way ANOVA (n = 6 mice for each treatment; control = two male, four female; lactacystin = three male, three female; epoxomicin = four male, two female).
Astrogliosis (GFAP/DAPI) at P50

D

C

B

(10 μM) for 36 hours. Subsequent measurements of protein levels revealed a large increase in SNAP-25 (which is selectively decreased by enhanced degradation in CSPα-deficient mice) in neurons from treated mice (18, 11) but no increases in other SNARE proteins (Fig. 3A). In addition, the proteasome inhibitor treatment elevated the levels of several proteins that are characteristically induced by stress responses, such as Hsp90 (heat shock protein 90), Hsc70, BIP (heat shock–binding protein), and HSF-1 (heat shock transcription factor-1); this elevation was accompanied by an increase in tau, phospho-tau, and Triton X-100–insoluble tau, and a decrease in the autophagy protein LC-3 (Fig. 3A and fig. S4A). Moreover, as expected, both proteasome inhibitors caused an accumulation of polyubiquitinated proteins (fig. S4A).

We next measured the effect of the proteasome inhibitor treatments of cultured CSPα-deficient neurons on SNARE-complex assembly (Fig. 3, B and C). SNARE complexes were monitored either as SDS-resistant high–molecular weight complexes on gels (23) or by communoprecipitation of SNARE proteins (18). Markedly, proteasome inhibitors increased SNARE-complex assembly by at least 200% as measured by both methods (Fig. 3, B and C). Although the observed absolute enhancement of SNARE-complex assembly varied between methods and antibodies, all measurements indicated a large effect.

Proteasome inhibitors boost SNARE-complex assembly in CSPα-deficient mice

Is the in vitro rescue of SNARE-complex assembly in CSPα-deficient neurons by proteasome inhibitors indicative of the in vivo action of these agents? To address this question, we analyzed the levels of critical proteins in the brains of proteasome inhibitor–treated and control untreated CSPα-deficient mice at P20, after only four intraperitoneal injections of the proteasome inhibitors. Transgenic overexpression of α-synuclein can rescue neurodegeneration and SNARE-complex assembly deficits in CSPα-deficient mice without affecting SNAP-25 amounts (18). However, proteasome inhibitors did not affect α-synuclein levels either in cultured neurons or in vivo (fig. S4). Similar to what we had observed in cultured neurons (Fig. 3A), we observed that the proteasome inhibitors induced a large increase in SNAP-25 concentration in vivo (Fig. 4A). Again, proteins related to stress responses, as well as tau, phospho-tau, and Triton X-100–insoluble tau, were increased, whereas the levels of the LC-3 proteolytic fragment that indicates activation of autophagy were decreased (Fig. 4A and fig. S4B).

We then measured the effect of the proteasome inhibitors on SNARE-complex assembly in vivo. Just as in cultured neurons, proteasome inhibitors caused a large increase in SNAP-25 (Fig. 4B) with no increases in other SNARE proteins (Fig. 4C). In addition, the proteasome inhibitor treatment elevated the levels of several proteins that are characteristically induced by stress responses, such as Hsp90 (heat shock protein 90), Hsc70, BIP (heat shock–binding protein), and HSF-1 (heat shock transcription factor-1); this elevation was accompanied by an increase in tau, phospho-tau, and Triton X-100–insoluble tau, and a decrease in the autophagy protein LC-3 (Fig. 4A and fig. S4A). Moreover, as expected, both proteasome inhibitors caused an accumulation of polyubiquitinated proteins (fig. S4A).

Fig. 2. Proteasome inhibitors delay neurodegeneration in CSPα-deficient mice. (A) Synapse density in the cortex of CSPα-deficient mice treated with a control regimen or lactacystin or epoxomicin as described for Fig. 1 was analyzed by immunocytochemistry for the postsynaptic marker protein PSD95 at P50. Merged representative images of brain sections double-labeled for PSD95 (red) and nuclei [4′,6-diamidino-2-phenylindole (DAPI); blue] are shown on the left, and summary graphs of synapse density are shown on the right. Data are means ± SEM. *P < 0.05, **P < 0.01 by Student’s t test comparing the lactacystin and epoxomicin treatment conditions to control (n = 3 mice per treatment; control = one male, two female; lactacystin = two male, one female; epoxomicin = one male, two female; with control = 24 sections; lactacystin = 25 sections; epoxomicin = 29 sections). For individual images and complete histograms, see fig. S3. (B) Same as (A), except that the density of neurons was visualized by staining for the neuron-specific transcription factor NeuN. (C) Same as (A), except that astrogliosis was measured by immunostaining for GFAP.

Proteasome inhibitors increase SNARE-complex assembly in cortical neurons cultured from CSPα-deficient mice. (A) Protein measurements. Cortical neurons cultured from newborn CSPα-deficient mice were incubated at 10 days in vitro with vehicle (dimethyl sulfoxide; control) or the proteasome inhibitors lactacystin (10 μM) and epoxomicin (10 μM), and were harvested 36 hours later. Protein concentrations were measured by quantitative immunoblotting with 125I-labeled secondary antibodies and normalized for guanine-nucleotide dissociation inhibitor (GDI) as a loading control. Protein concentrations are shown as percent of control concentrations (n = 6 cultures). Synt-1, syntaxin-1; Syb2, synaptobrevin-2; LC3 (II), microtubule-associated proteins 1A/1B light chain 3A–fragment II (see also fig. S4). (B and C) Measurements of SNARE-complex assembly in cultured neurons treated as in (A). SNARE complexes were quantitated as high–molecular mass bands immunoreactive for SNARE proteins (B) (normalized to GDI) or by coimmunoprecipitation of SNARE proteins (C) [recovered proteins relative to the input were normalized to the immunoprecipitated (IP) protein concentrations]. All data are shown as percent of control (n = 3 independent cultures). Data are means ± SEM. **P < 0.01, ***P < 0.001 by Student’s t test comparing the lactacystin and epoxomicin treatment conditions to control.

Proteasome inhibitors increase SNARE-complex assembly in CSPα-deficient mice in vivo. (A) Measurements of protein concentrations in the brains of littermate CSPα-deficient mice that were treated with control, lactacystin, or epoxomicin as described for Fig. 1. Protein concentrations were measured in the cortex of mice at P20 with quantitative immunoblotting with GDI as an internal loading control (for immunoblots and further protein quantitation, see fig. S4). (B and C) Measurements of SNARE-complex assembly in the brain cortex from control and lactacystin- and epoxomicin-treated mice [see (A)]. SNARE complexes were quantitated as high–molecular mass bands immunoreactive for SNARE proteins (B) (normalized to GDI) or by coimmunoprecipitation of SNARE proteins (C) [recovered proteins relative to the input were normalized to the immunoprecipitated protein concentrations]. All data are shown as percent of control. Protein concentrations were determined by phosphorimager analysis with 125I-labeled secondary antibody. Data are means ± SEM. **P < 0.01, ***P < 0.005 by Student’s t test comparing the lactacystin and epoxomicin treatment conditions to control (n = 6 mice per treatment; control = three male, three female; lactacystin = two male, four female; epoxomicin = four male, two female).
inhibitors markedly increased SNARE-complex assembly as measured either with the SDS-resistance assay (Fig. 4B) or with the coimmunoprecipitation assay (Fig. 4C). These data are consistent with the notion that the proteasome inhibitors alleviate neurodegeneration in vivo by increasing SNARE-complex assembly.

**SNARE-complex assembly deficits are observed in human neurodegenerative disorders**

Although not necessarily causative, a deficit in SNARE-complex assembly might generally develop in neurodegenerative disorders and contribute to the pathology. This possibility is raised by the fact that a presynaptic terminal needs to sustain continuous membrane traffic throughout the life of an organism, with continuous SNARE-complex assembly and disassembly. Thus, any loss of cellular energy stores to fuel the chaperones involved (especially CSPα and N-ethylmaleimide-sensitive factor) likely causes rapid impairment of SNARE protein folding, resulting in defective SNARE-complex assembly, leading to neurodegeneration. As a first step toward exploring this possibility, we analyzed postmortem brain tissue samples from the cerebral cortex of Alzheimer’s and Parkinson’s disease patients and age-matched controls with non-neurological causes of death (n = 5; for the anatomical sample area, ages, and gender distributions, see fig. S5). We measured the levels of selected synaptic proteins in brain lysates (Fig. 5A and fig. S6) and found that the levels of most proteins analyzed were surprisingly similar in the three groups of patients. A modest reduction in the total concentrations of synaptobrevin-2, α-synuclein, synaptophysin, vGaT [vesicular γ-aminobutyric acid (GABA) transporter], and tau was detected in the brains of both Alzheimer’s and Parkinson’s disease patients (Fig. 5A and fig. S6), similar to the previously reported decreases in synaptic proteins in brain tissue from dementia patients (24, 25), and possibly caused by a loss of neurons and synapses. Alternatively, reduced levels of free functional α-synuclein may be present in the brains from Alzheimer’s and Parkinson’s disease patients as a result of the aggregation of α-synuclein. Furthermore, increased levels of phospho-tau and Triton X-100–insoluble tau were observed in Alzheimer’s disease patients, consistent with Alzheimer’s disease pathology. A massive increase in the amount of GFAP and significant increases in the microglial marker CD11b and polyubiquitinated proteins were observed in Alzheimer’s and Parkinson’s disease brains, as expected for a neurodegenerative disorder (Fig. 5A and fig. S6). There were small yet significant increases in HSF-1 and multiple chaperone proteins in the Alzheimer’s and Parkinson’s disease brains (Fig. 5A), possibly a reaction to neurodegenerative stress.

We next examined SNARE-complex assembly in the brain homogenates from Alzheimer’s and Parkinson’s disease patients using both the coimmunoprecipitation assay and the SDS-resistance assay (Fig. 5B and fig. S6). Notably, we detected an about twofold decrease in SNARE-complex assembly in most antibody combinations using the coimmunoprecipitation assay, a finding that was confirmed by the SDS-resistance assay. Because these assays for SNARE-complex assembly are completely independent of absolute protein levels and thus not as susceptible to postmortem artifacts as measurements of protein levels, these data suggest that SNARE-complex assembly may be generally reduced in neurodegenerative diseases.

**DISCUSSION**

Reduced SNAP-25 concentration and defective SNARE-complex assembly are responsible, at least partly, for neurodegeneration in CSPα-deficient mice (11, 12). Here, we show that treatment of CSPα-deficient mice with proteasome inhibitors ameliorates the neurodegeneration and extends the life span of these mice, most likely by increasing SNAP-25 levels and SNARE-complex assembly. Although our data do not exclude the general notion that proteasomal degradation of misfolded proteins is an important defense mechanism preventing neurodegeneration, our data suggest that inhibiting proteasomal activity may be a more viable therapeutic strategy than activating proteasomal activity. Moreover, our results with human postmortem brain tissue samples suggest that a decrease in SNARE-complex assembly may generally occur in neurodegenerative diseases, not necessarily as the primary disease process but as a secondary pathogenic mechanism that enhances disease progression. If confirmed, these results would indicate that a proteasome inhibitor treatment strategy may be a possible consideration for treating human neurodegenerative diseases.

Our observations may have implications beyond neurodegenerative disorders. Targeted degradation of functionally important proteins may be a more viable therapeutic strategy than activating proteasomal activity and extends the life span of these mice, most likely by increasing SNAP-25 levels and SNARE-complex assembly. Although not necessarily causative, a deficit in SNAP-complex assembly are responsible, at least partly, for neurodegeneration in CSPα-deficient mice. Here, we show that treatment of CSPα-deficient mice with proteasome inhibitors ameliorates the neurodegeneration and extends the life span of these mice, most likely by increasing SNAP-25 levels and SNARE-complex assembly. Although our data do not exclude the general notion that proteasomal degradation of misfolded proteins is an important defense mechanism preventing neurodegeneration, our data suggest that inhibiting proteasomal activity may be a more viable therapeutic strategy than activating proteasomal activity. Moreover, our results with human postmortem brain tissue samples suggest that a decrease in SNARE-complex assembly may generally occur in neurodegenerative diseases, not necessarily as the primary disease process but as a secondary pathogenic mechanism that enhances disease progression. If confirmed, these results would indicate that a proteasome inhibitor treatment strategy may be a possible consideration for treating human neurodegenerative diseases.
teins by the proteasome is a hallmark of a wide range of metabolic disorders, such as cystic fibrosis (26), nephrogenic diabetes insipidus (27), Charcot-Marie-Tooth disease (28), and multiple lysosomal storage diseases (29, 30). These disorders may be ameliorated by inhibiting the degradation of mutant yet functional proteins by the proteasome. In combination with induction of chaperones, this might be a viable therapeutic approach.

Our observation that infrequent proteasome inhibitor administration—one dose every 5 days—significantly delayed the development of neurodegeneration in CSPα-deficient mice and altered protein levels in the brains of these mice is surprising, given the relatively discontinuous administration of the inhibitors. How do the proteasome inhibitors work? In our experiments, proteasome inhibitors produced at least two different effects in CSPα-deficient neurons: a large increase in SNAP-25 with a resulting enhancement of SNARE-complex assembly, and elevation of the levels of stress-response proteins such as Hsp90, BiP, and HSF-1. The fact that proteasome inhibitors increased functional SNAP-25 concentration, and not just ubiquitinated SNAP-25, was surprising because the proteasome inhibitors were only expected to result in an accumulation of ubiquitinated SNAP-25. This observation shows that the proteasome inhibitors directly affect degradation of SNAP-25. It is possible that accumulation of ubiquitinated SNAP-25 may produce deubiquitination or a feedback inhibition of further SNAP-25 ubiquitination, thereby slowing the enhanced degradation of SNAP-25 that leads to the decrease in SNAP-25 levels and SNARE-complex assembly. We have previously shown that boosting SNARE-complex assembly in CSPα-deficient mice is sufficient to prevent neurodegeneration (12, 18), and thus, the increase in SNAP-25 and SNARE-complex assembly suffices to account for the therapeutic effect of the proteasome inhibitors. Moreover, we observed an induction of stress responses by the proteasome inhibitors, consistent with previous studies (31, 32). The stress responses may contribute to the beneficial effect of proteasome inhibitors by enhancing the refolding of nonspecific chaperones, thereby preventing its enhanced degradation. Independent of the mechanism by which the proteasome inhibitors increase SNAP-25 levels and SNARE-complex assembly, it is likely that these actions are responsible for their beneficial effects in CSPα-deficient mice.

The neuroprotective effect of proteasome inhibitors in CSPα-deficient mice limited by possible toxic effects of proteasome inhibitors, which would eventually kill the treated mice? Although the impact of long-term systemic proteasome inhibition may be detrimental to a normal animal, proteasome inhibitors have been used in cancer therapy for almost 10 years (33–35). A number of new proteasome inhibitors have gone through clinical trials where no significant side effects did occur (36). As in the case of cancer therapeutics, if a functionally important protein is being eliminated by the proteasome severely affecting survival, the benefits of proteasome inhibitors may outweigh the side effects.

MATERIALS AND METHODS

Mouse husbandry and treatments
CSPα-deficient mice (17) were bred as heterozygotes on a C57BL/6 background; all experiments were approved by the Institutional Animal Care and Use Committee at Stanford University. CSPα-deficient mouse pups were injected intraperitoneally with Hamilton syringes every 5 days with proteasome inhibitors beginning at P5, with 10-μl injections from P5 to P20, and 100-μl injections from P25 until the animal could not reach food and water because of neurological symptoms and was sacrificed. Injection solutions contained vehicle control (50% ethanol in H2O), lactacystin (Calbiochem; 100 μM) (which covalently modifies MB1 subunit of proteasome) (37), or epoxomicin (Calbiochem; 100 μM) [which modifies LMP7 (low–molecular mass protein 7)], X, MECL1 (multicatalytic endopeptidase complex subunit 1), and Z catalytic subunits of proteasome (38). Approximate dosages were as follows: day 5, lactacystin (0.09 mg/kg) and epoxomicin (0.14 mg/kg); day 10, lactacystin (0.07 mg/kg) and epoxomicin (0.11 mg/kg); day 15, lactacystin (0.06 mg/kg) and epoxomicin (0.09 mg/kg); day 20, lactacystin (0.05 mg/kg) and epoxomicin (0.08 mg/kg); day 25, lactacystin (0.47 mg/kg) and epoxomicin (0.69 mg/kg); day 30 until death, lactacystin (0.31 mg/kg) and epoxomicin (0.46 mg/kg). Injections were performed immediately after behavioral studies, before mice were returned to their home cage. All experiments were performed in a “blinded” fashion on cohorts of littermate mice.

Behavioral studies
Mice were analyzed by weight measurements and neurological tests as follows: Hindlimb clasping (39) was used to assess onset of motor impairment. Mice were suspended from the tail for 1 min to induce limb clasping. Onset is defined as the first day when hindlimb clasping was observed. For the grid-hanging test (40), animals were placed on top of a wire mesh grid. The grid is shaken lightly three times to cause the mouse to grip the wires, and then turned upside down and held ~20 cm above the home cage litter to prevent the mouse both from easily climbing down and from hurting itself when falling. A stopwatch was used to record the time the mouse held onto the grid. The latency to fall was calculated from three trials per mouse performed with 1-min intertrial intervals. The righting reflex (18) was used to assess the level of paresis/paralysis. A mouse was lifted by its tail and placed on one side, and the time it took for the mouse to right itself was recorded with a stopwatch. Force plate actometry, a sensitive, quantitative method (41), was used to document changes in locomotor activity induced by proteasome inhibitors. Pups, beginning at P10, were individually tested every 5 days until death by placing them on the force plate (28 cm × 28 cm) for 30 min. From the force plate traces/coordinate records, we calculated the total distance traversed per 30-min session, and the number of low-mobility bouts per session (defined as periods of no locomotion outside of a 30-mm-diameter circle for 10.24 s) was determined.

Cultured cortical neurons were obtained from newborn mice essentially as described (42, 43). Cortices were dissected in ice-cold Hank’s balanced salt solution, dissociated by trypsinization (0.0.5% trypsin-EDTA for 10 min at 37°C), triturated with a siliconized pipette, and plated (100 μl) onto a 12-mm coverslip (for immunofluorescence) or on 12-well plastic dishes, coated for at least 30 min with Matrigel (BD Biosciences). Plating medium [minimum essential medium (MEM) (Gibco) supplemented with glucose (5 g/liter), NaHCO3 (0.2 g/liter) (Sigma), transferrin (0.1 g/liter) (Calbiochem), insulin (0.25 g/liter) (Sigma), t-glutamine (0.3 g/liter) (Gibco), and 10% fetal bovine serum] was replaced with growth medium [MEM (Gibco) containing glucose (5 g/liter), NaHCO3 (0.2 g/liter) (Sigma), transferrin (0.1 g/liter) (Calbiochem), t-glutamine (0.3 g/liter) (Gibco), 5% fetal...
bovine serum, 2% B-27 supplement (Gibco), and 2 μM cytosine arabinoside (Sigma)] 24 to 48 hours after plating.

**Human postmortem brain samples**

Frozen cortical sections from the frontal lobe (second or third slab) were obtained from the Human Brain and Spinal Fluid Resource Center (Los Angeles, CA) with HSB identifiers, patient histories, and pathological findings. Control brains were from patients who had died of non-neurological causes and displayed no signs of brain pathology. Notably, one patient categorized under Alzheimer’s disease (sample 8) was also diagnosed with Parkinson’s disease, and a patient categorized under Parkinson’s disease (sample 13) had Lewy body dementia with marked depigmentation and neuron loss in the substantia nigra. Brain samples were shipped on dry ice and stored at −80°C until they were homogenized in ice-cold phosphate-buffered saline (PBS) supplemented with EDTA-free protease inhibitor cocktail (Roche). Homogenates were kept at −80°C until proteins were solubilized for immunoblotting or immunoprecipitation experiments. Human materials used followed Stanford University guidelines (http://humansubjects.stanford.edu/research/medical/medical.html).

**Antibodies**

The following monoclonal antibodies were used: 14-3-3 (610543, BD Transduction), β-actin (A1978, Sigma), calnexin (610523, BD Transduction), CD11b (ab8878, Abcam), GDI (cl. 81.2, SYSY), glycine receptor (146111, SYSY), GFAP (MAB360, Millipore), Hsc70 (cl. 3C5, SYSY; 1B5, Affinity BioReagents; SPA-815, Stressgen), Lamp-2 (cl. 72.2, SYSY), synaptobrevin-2 (cl. 69.1, SYSY), synaptophysin (cl. 72.2, SYSY), synaptotagmin-1 (604.1, SYSY), syntaxin-1 (HPC1, SYSY), BiP (M1/70.15.11.5.2, DSHB), complexin 1/2 (122002, SYSY), vGAT (131013, SYSY), and vGluT1 (vesicular glutamate transporter 1) (135302, SYSY). The following polyclonal antibodies were used: CD11b (M1/70.15.11.5.2, DSHB), complexin 1/2 (122002, SYSY), BiP (ab21685, Abcam), GABA receptor (06-868, Upstate), HS-1 (4356S, Cell Signaling), Hsp90 (ADI-SPA-846, Enzo), Hsc70 (A903), LC-3 (L7543, Sigma), MAP2 (microtubule-associated protein 2) (AB5622, Millipore), NeuN (MAB377, Millipore), PSD95 (MA1046, Thermo), Sgt (CHAT133), SNAP-25 (P913), synaptobrevin-2 (P939), synapsin (E028), syntaxin-1 (438B), α-synuclein (T2270), tau (MAB361, Millipore), vGluT1 (131013, SYSY), and vGlut1 transporters (135302, SYSY).

**Immunofluorescence labeling experiments**

Anesthetized mice were perfused with ice-cold 4% paraformaldehyde in PBS, followed by removal of the brains and overnight fixation in 4% paraformaldehyde in PBS (room temperature). Fixed brains were cryopreserved in 30% sucrose in PBS for 2 days and frozen in TissueTek OCT embedding medium (Sakura Finetechical). Sagittal brain sections (20 μm) were cut at −20°C (Leica CM3050S cryostat), placed onto slides, and heat-adhered at 37°C for 30 min. Slides were incubated in blocking solution (3% bovine serum albumin, 0.1% Triton X-100 in PBS) for 1 hour followed by overnight incubation with primary antibodies (4°C), washed three times in PBS (5 min each), and incubated in blocking buffer containing Alexa Fluor 488–, Alexa Fluor 546–, or Alexa Fluor 633–coupled secondary antibodies (Molecular Probes) for 3 hours at room temperature. After six washes in PBS, slides were mounted with Vectashield hard-set mounting medium with DAPI (Vector) followed by fluorescence microscopy.

**Proteasome activity measurements**

Fresh homogenates from the CSF–deficient and wild-type littermate mouse brains (P40) were solubilized in isotonic (150 mM NaCl) tris-HCl buffer (pH 7.4) containing 1% NP-40. The postnuclear supernatant was used immediately in the quantification of the chymotrypsin-like, trypsin-like, and peptidylglutamyl hydrolase activities of the proteasomal enzymatic sites with fluorometry-based microplate assays (Chemicon). Lactacystin (10 μM) was added as a specific proteasomal inhibitor, indicating the baseline nonproteasomal activity.

**Immunoblotting and immunoprecipitations**

Either brain homogenates or cultured neurons were solubilized in 50 mM tris-CI buffer (pH 7.4) containing 150 mM NaCl and 0.1% Triton X-100. After centrifugation at 16,000g for 10 min at 4°C, the clarified lysate was used for immunoblotting (after addition of 2× SDS sample buffer containing 10% β-mercaptoethanol) or subjected to immunoprecipitations as described (18, 42). For measurements of insoluble tau levels, Triton X-100–insoluble pellet was extracted with 2× SDS sample buffer containing 10% β-mercaptoethanol and boiled for 20 min. Immunoprecipitations was performed with the indicated primary antibodies and 30 μl of a 50% slurry of protein G–Sepharose beads (Amersham) for monoclonal immunoglobulin G, or protein A–Sepharose beads (GE Healthcare) for polyclonal rabbit sera, for 2 hours at 4°C. Control immunoprecipitations were performed with brain lysates with no antibody (for monoclonal antibodies) or with preimmune serum (for polyclonal rabbit sera). After five washes with 1 ml of the extraction buffer, bound proteins were eluted with 2× SDS sample buffer containing 10% β-mercaptoethanol and boiled for 15 min at 100°C. Coprecipitated proteins were separated by SDS–PAGE, with 5 to 10% of the input in the indicated lane.

**Protein quantitation**

All quantitative immunoblotting experiments were performed with iodinated secondary antibodies as described (44). Samples were separated by SDS–PAGE and transferred onto nitrocellulose membranes. Blots were blocked in tris-buffered saline containing 0.1% Tween 20 (Sigma) and 5% fat-free milk for 2 hours at room temperature. The blocked membrane was incubated in blocking buffer containing primary antibody for 1 hour, followed by three to five washes. The washed membrane was incubated in blocking buffer containing either horseradish peroxidase (HRP)–conjugated secondary antibody (MP Biomedicals, 1:8000) for 2 hours at room temperature or 125I-labeled secondary antibody (PerkinElmer, 1:1000) overnight at room temperature. HRP immunoblots were developed with enhanced chemiluminescence (GE Healthcare). 125I blots were exposed to a phosphorimager screen (Amersham) for 1 to 7 days and scanned with a Storm scanner (GE Healthcare), followed by quantification with ImageQuant software (GE Healthcare).

**Statistical analyses**

Prism software (GraphPad) was used to plot the survival and hindlimb clasping curves, followed by log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests to assess statistical significance. All other data shown are means ± SEM and were analyzed by either two-way analysis of variance (ANOVA) or Student’s unpaired two-tailed t test to...
compare the data groups with Prism software. n refers to the number of different cultures or mice used in each group in separate experiments.

SUPPLEMENTARY MATERIALS

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List of abbreviations

Fig. S1. CSPz-deficient mouse brains exhibit increased expression of stress-response genes and increased general proteasome activity.

Fig. S2. Proteasome inhibitors delay development of neurological symptoms in CSPz-deficient mice.

Fig. S3. Proteasome inhibitors delay neurodegeneration in CSPz-deficient mice in vivo.

Fig. S4. Effect of proteasome inhibitor treatments of CSPz-deficient mice on protein concentrations in cultured neurons and in brain tissue.

Fig. S5. Origin of the Alzheimer’s and Parkinson’s disease patient materials.

Fig. S6. Quantitation of protein concentrations and SNARE-complex assembly in homogenates of postmortem cortical tissue from Alzheimer’s and Parkinson’s disease patients.

Table S1. Studies correlating proteasome inhibition with neurodegeneration.

REFERENCES AND NOTES

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