Protein Surveillance Machinery in Brains with Spinocerebellar Ataxia Type 3: Redistribution and Differential Recruitment of 26S Proteasome Subunits and Chaperones to Neuronal Intranuclear Inclusions

Thorsten Schmidt, MS,1 Katrin S. Lindenberg, MS,2,3 Antje Krebs, BS,3 Ludger Schöls, MD,4 Franco Laccone, MD,5 Jochen Herms, MD,6 Martin Rechsteiner, PhD,7 Olaf Riess, MD,1 and G. Bernhard Landwehrmeyer, MD2

Intracellular aggregates commonly forming neuronal intranuclear inclusions are neuropathological hallmarks of spinocerebellar ataxia type 3 and of other disorders characterized by expanded polyglutamine-(poly-Q) tracts. To characterize cellular responses to these aggregates, we performed an immunohistochemical analysis of neuronal intranuclear inclusions in pontine neurons of patients affected by spinocerebellar ataxia type 3, using a panel of antibodies directed against chaperones and proteasome subunits. A subset of the neuronal intranuclear inclusions stained positively for the chaperones Hsp90α and HDJ-2, a member of the Hsp40 family. Most neuronal intranuclear inclusions were ubiquitin positive, suggesting degradation by ubiquitin-dependent proteasome pathways. Surprisingly, only a fraction of neuronal intranuclear inclusions were immunopositive for antibodies directed against subunits of the 20S proteolytic core, whereas most inclusions were stained by antibodies directed against subunits of the 11S and 19S regulatory particles. These results suggest that the proteosomal proteolytic machinery that actively degrades neuronal intranuclear inclusions is assembled in only a fraction of pontine neurons in end stage spinocerebellar ataxia type 3. The dissociation between regulatory subunits and the proteolytic core and the changes in subcellular subunit distribution suggest perturbations of the proteosomal machinery in spinocerebellar ataxia type 3 brains.

Ann Neurol 2002;51:302–310
DOI 10.1002/ana.10101

An expansion of CAG triplet repeats forms the genetic basis of a growing number of inherited neurodegenerative disorders and was shown to underlie at least six autosomal dominant spinocerebellar ataxias (SCAs), including SCA3 or Machado-Joseph disease.1–4 Since the CAG repeats are within the coding region, the mutant gene products are characterized by an expanded stretch of glutamines (poly-Q) in otherwise unrelated proteins. The current understanding of the mechanisms giving rise to a neurodegenerative phenotype of delayed onset is still limited and incomplete. Current evidence favors the view that the expanded poly-Q stretch confers a new property on the respective proteins: poly-Q-expanded proteins display an increased propensity to misfold and aggregate.5 One of the pathological hallmarks of these clinically and pathologically distinct neurodegenerative disorders is therefore the presence of nuclear aggregates of mutant proteins forming neuronal intranuclear inclusions (NIIs).6 The role of NIIs in the pathogenesis of poly-Q disorders is unclear.7,8 Previous work has demonstrated that the cellular response to nuclear aggregates includes (1) the recruitment of chaperones, or proteins involved in the folding of nascent translational products and in the resolubilization of aggregated polypeptides; and (2) the ubiquitination of aggregates, suggesting cellular attempts to degrade deposits of these mutant proteins via the ubiquitin-proteasomal pathway.9–11 The 26S proteasome represents the most important site for protein degradation in eukaryotic cells12 and consists of a several components: 20S (containing the catalytic sites shielded inside a barrel-shaped multienzyme complex), 11S protea-

From the 1Department of Medical Genetics, University of Tübingen; 2Department of Neurology, University of Ulm; 3Department of Neurology, University of Freiburg; 4Department of Neurology, Ruhr-University Bochum; 5Institute of Human Genetics and 6Department of Pathology, University of Göttingen, Germany; and 7Department of Biochemistry, University of Utah School of Medicine, Salt Lake City, UT.

Received May 23, 2001, and in revised form Nov 5. Accepted for publication Nov 5, 2001.

Address correspondence to Dr Landwehrmeyer, Department of Neurology, University of Ulm, Steinhoevelstrasse 9, D-89075 Ulm, Germany. E-mail: bernhard.landwehrmeyer@medizin.uni-ulm.de
some activator, and 19S regulatory particle (a multimeric complex thought to mediate the recognition of polyubiquitin residues and the unfolding of proteins, thereby permitting access into the interior cavity of the 20S component\(^\text{[13]}\)). To characterize the cellular response to deposition of poly-Q–expanded ataxin-3, we performed an immunohistochemical analysis of nuclear inclusions in the postmortem brains of patients affected by SCA3, using a panel of 33 antibodies directed against chaperones and proteasome subunits.

**Patients and Methods**

**Tissues**

Paraffin-embedded, frozen tissue from five patients with molecular confirmation of a SCA3 mutation, an appropriate family history, and clinical findings consistent with SCA3, as well as human brain tissue from five normal donors (with clinical, macroscopic, and histological findings within normal range), were employed in this study as described previously.\(^\text{[14]}\) Paraffin-embedded sections of human umbilical vein and carcinoma of the mamma were used as positive controls for the anti-Hsp antibodies.

**Antibodies**

Details concerning the antibodies employed in the present study are summarized in the Table. Twenty-five antibodies directed against 26S proteasomal subunits used in this study were purchased from Affiniti Research Products (Exeter, United Kingdom). A total of 23 of these antibodies and antisera were subunit specific. An additional antibody directed to subunit 5a of the 19-S component was developed by one of us (M.R.). The antibodies directed against heat shock proteins were delivered by NeoMarkers (Hsp 27, HDJ-2, Hsp 60, Hsp 90α; Lab Vision Corp., Fremont, CA) and Stress-Gen (Victoria, Canada) (Hsp70/Hsc70, Hsc 70). Monoclonal and polyclonal antibodies directed against ubiquitin were purchased from Biotrend (Cologne, Germany) and DAKO (Glostrup, Denmark), respectively. The polyclonal anti-ataxin-3 antibody used has been described previously,\(^\text{[14]}\) and the monoclonal anti-ataxin-3 antibody (1H9) was kindly provided by Dr Y. Trottier.

**Immunohistochemistry**

Immunohistochemical experiments were conducted as previously described.\(^\text{[14]}\) Briefly, after deparaffination, tissue sections (7 μm) were rehydrated in a graded series of ethanols, treated for antigen retrieval,\(^\text{[15]}\) quenched (using 1% hydrogen peroxide in 40% methanol for 10 minutes), and blocked in phosphate-buffered saline (PBS) with 0.3% Triton X-100 containing 5% normal goat serum (polyclonal primary antiserum), 5% normal horse serum (monoclonal primary mouse antibody), or 5% normal rabbit serum (monoclonal primary rat antibody). Sections were incubated overnight at 4°C with the primary antibody diluted in PBS containing 3% of the appropriate normal serum (see table for dilutions) and rinsed, followed by detection of the primary antibody using the Vectastain ABC elite system (Vector Laboratories, Burlingame, CA) according to the manufacturer’s directions and 3,3′-diaminobenzidine as chromogen (Sigma-Aldrich, Deisenhofen, Germany). Some sections were lightly counterstained with hematoxylin, dehydrated in a graded series of ethanol and xylene, coverslipped using Eukitt (Kindler, Freiburg, Germany), and examined using a light microscope equipped with Nomarski optics (Leica, Bensheim, Germany). Control sections incubated exclusively with the secondary antibody developed under the same conditions did not result in immunoreactivities (IR). For figures, pictures were digitally processed using Adobe Photoshop (Adobe Systems Inc., San Jose, CA).

<table>
<thead>
<tr>
<th>Antibody/Subunit</th>
<th>Type</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody against ataxin-3</td>
<td>mAb</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Antibody against ubiquitin</td>
<td>pAb</td>
<td>1:5,000</td>
</tr>
<tr>
<td>Antibodies against heat shock proteins/chaperones</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hsp 27</td>
<td>mAb</td>
<td>1:500</td>
</tr>
<tr>
<td>Hsp 40 (HDJ-2)</td>
<td>mAb</td>
<td>1:500</td>
</tr>
<tr>
<td>Hsp 60</td>
<td>mAb</td>
<td>1:500</td>
</tr>
<tr>
<td>Hsp 70/Hsc 70</td>
<td>mAb</td>
<td>1:200</td>
</tr>
<tr>
<td>Hsc 70</td>
<td>mAb</td>
<td>1:50</td>
</tr>
<tr>
<td>Hsp 90α (Hsp 86)</td>
<td>pAb</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Antibodies against 26S proteasome</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20S core</td>
<td>pAb</td>
<td>1:1,000</td>
</tr>
<tr>
<td>20S α-type subunits</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subunits α1, 2, 3, 5, 6, and 7</td>
<td>mAb</td>
<td>1:500</td>
</tr>
<tr>
<td>Subunit α2 (HC3)</td>
<td>mAb</td>
<td>1:100</td>
</tr>
<tr>
<td>Subunit α3 (HC9)</td>
<td>mAb</td>
<td>1:200</td>
</tr>
<tr>
<td>Subunit α4 (XAPC-7)</td>
<td>mAb</td>
<td>1:100</td>
</tr>
<tr>
<td>Subunit α5 (zeta)</td>
<td>mAb</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Subunit α6 (HC2)</td>
<td>mAb</td>
<td>1:2,000</td>
</tr>
<tr>
<td>Subunit α7 (HC8)</td>
<td>mAb</td>
<td>1:1,000</td>
</tr>
<tr>
<td>20S β-type subunits</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subunit β1 (Y)</td>
<td>mAb</td>
<td>1:2,500</td>
</tr>
<tr>
<td>Subunit β1i (Lmp2)</td>
<td>pAb</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Subunit β2 (Z)</td>
<td>mAb</td>
<td>1:2,000</td>
</tr>
<tr>
<td>Subunit β2i (MECL-1)</td>
<td>pAb</td>
<td>1:2,500</td>
</tr>
<tr>
<td>Subunit β3 (HC10)</td>
<td>mAb</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Subunit β5i (Lmp7)</td>
<td>pAb</td>
<td>1:2,000</td>
</tr>
<tr>
<td>Subunit β7 (HN3)</td>
<td>mAb</td>
<td>1:1,000</td>
</tr>
<tr>
<td>11S (PA 28) regulator subunits</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subunit α (PA28 α)</td>
<td>pAb</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Subunit β (PA28 β)</td>
<td>pAb</td>
<td>1:1,000</td>
</tr>
<tr>
<td>19S (PA 700) regulator subunits</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subunit 4 (mts2)</td>
<td>pAb</td>
<td>1:500</td>
</tr>
<tr>
<td>Subunit 5a</td>
<td>pAb</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Subunit 6a (Tbp1)</td>
<td>pAb</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Subunit 6b (Tbp7)</td>
<td>pAb</td>
<td>1:2,000</td>
</tr>
<tr>
<td>Subunit 7 (Mss1)</td>
<td>pAb</td>
<td>1:2,000</td>
</tr>
<tr>
<td>Subunit 8 (p45)</td>
<td>pAb</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Subunit 10a (p44)</td>
<td>pAb</td>
<td>1:2,000</td>
</tr>
<tr>
<td>Subunit 10b (p42)</td>
<td>pAb</td>
<td>1:500</td>
</tr>
</tbody>
</table>

Nomenclature of the proteasome subunits according to Baumeister et al.\(^\text{[12]}\)

mAb = monoclonal mouse antibody; mAb = monoclonal rat antibody; pAb = polyclonal rabbit antiserum.
viewed using a Leica microscope at ×40 magnification equipped with a video camera (Dage, Michigan City, MI) and an image processing system (MCID, Imaging Research, St. Catharines, Ontario, Canada). In some stainings nuclear inclusions were of rather small apparent size; in these instances counting was repeated at ×100 magnification. In an appropriate number of random fields of view, the total number of neurons and the number of neurons containing unambiguous nuclear inclusion bodies were counted by three independent observers until at least 100 neuronal profiles were encountered. The fields of view were randomly chosen but systematically distributed over the entire section. The percentage of neurons containing nuclear inclusions for each experiment was estimated by calculating the mean of all respective counts. To determine the subcellular redistribution of 20S core antigens, sections containing pontine neurons were inspected using ×20 magnification until at least 100 neuronal profiles were encountered.

Results

Frequency of Neuronal Intranuclear Inclusions in Pontine Neurons in SCA3

To assess the frequency of NIIs, we stained pontine sections from SCA3 patients with a monoclonal antibody directed to the carboxyterminus of ataxin-3, which is known to label NIIs with high intensity.\textsuperscript{14,16} In brains unaffected by SCA3, incubation with 1H9 (Fig 1A) resulted in a neuronal staining, preferentially in the cytoplasmatic compartment. In pontine neurons of SCA3 patients, however, an intense IR of NIIs was observed. NIIs as visualized by 1H9 (see Fig 1A) appeared round, with a mean cross-sectional area of 5.2 μm\textsuperscript{2} (standard deviation [SD], 2.1; range, 1.6–8.7). In numerous neuronal nuclei, two NIIs forming a figure 8 could be seen. In all regions of the

Fig 1. Immunohistochemical staining of pontine neurons in spinocerebellar ataxia type 3 (SCA3) brains demonstrated recruitment of chaperones and 19S proteasomal subunit antigens to neuronal intranuclear inclusions (NIIs). (A) Anti-ataxin-3 immunoreactivity (IR) using the monoclonal antibody 1H9. Note the double inclusion distinct from the nucleolus. (B) Anti-ubiquitin-IR in a pontine neuron counterstained with hematoxylin. IR is confined to NIIs. (C) Anti-Hsp90-IR. (D) Anti-subunit-5a-IR. Subunit 5a is thought to be a 19S subunit in the lid portion of the 19S particle mediating binding to polyubiquitin chains. (E) Anti-subunit-7-(Msi1)-IR. Subunit 7 is part of the base of the 19S particle and has adenosine triphosphatase activity (F) Anti-subunit-10a-IR. Subunit 10a is a component of the 19S particle without adenosine triphosphatase activity. Bar = 10μm. Nomarski optics.
ventral pons, 1H9-IR-NIIs were found in pontine neurons with no obvious regional clustering. NIIs were observed in most pontine neurons; in 100 neuronal profiles sampled at random, three independent observers counted 72 (± 11) neurons bearing NIIs (Fig 2).

Staining with polyubiquitin antibodies (Fig 1B) in adjacent sections disclosed a somewhat lower frequency of ubiquitin-IR NIIs (51 ± 10 per 100 neuronal profiles; see Fig 2). Ubiquitin-IR-NIIs tended to be smaller than 1H9-IR-NIIs (mean cross-sectional area, $2.1 \mu m^2$ SD, 1.4; range, 0.8–5.1). Thus, it appeared that immunohistochemical staining with the 1H9 antibody disclosed the highest number of NIIs, setting the gold standard for the estimation of NII frequency. Therefore, in the following paragraphs, NIIs as labeled by other antibodies will be expressed as percentages of 1H9-IR-NIIs.

**Antibodies against Heat Shock Protein Stain**

**Neuronal Intranuclear Inclusions in SCA3**

Immunohistochemical staining of pontine sections with antibodies to heat shock proteins demonstrated an intense staining of a subset of NIIs by a polyclonal antibody to Hsp90α (Fig 1C). In 100 pontine neurons sampled in 5 random sets of 20 neurons, we observed an average of 15 neurons containing Hsp90α-IR-NIIs; this number corresponded to a staining of 20% of NIIs. However, a monoclonal antibody to HDJ-2, which is a member of the Hsp40 family, displayed IR only in a small subset of NIIs. No staining of NIIs was observed with monoclonal antibodies for Hsp27, Hsp60, Hsp70, or Hsc70, despite clear immunoreactive signals in control tissues processed in a similar manner. Compared with control brains, there was no obvious redistribution of Hsp-IR aside from an intense labeling of some NIIs: as in controls, Hsp90α, Hsc/Hsp70, and Hsp40 IR were preferentially observed in cytoplasm. There was no evidence of a marked increase in intensity of cytoplasmatic Hsp-IR in SCA3 brains compared with controls; antibodies resulting in faint or no labeling in control brain tissue (eg, Hsp70) did not give rise to more intense labeling in SCA3 brains.

**A Small Subset of Neuronal Intranuclear Inclusions in SCA3 Displays Proteasome 20S-Subunit-IR**

Using a polyclonal 20S core antibody raised against a proteasomal preparation isolated from red blood cells, we observed IR in a small subset of NIIs (approximately 3%). A monoclonal antibody recognizing a peptide sequence shared by six of the seven subunits forming the α-ring of the 20S proteosome resulted in a similarly low frequency of immunoreactive NIIs (approximately 5%). Immunohistochemical staining using monoclonal antibodies selective for α-subunits 2–7 under conditions resulting in distinct signals in control brains disclosed IR-NIIs for antibodies selective for α2, α5, α6, and α7 at relatively low abundance, ranging from 4 to 15% of NIIs. Numerous neuronal profiles in pontine sections of SCA3 brains displayed no NII staining when antibodies selective for α-subunits were used. No NIIs were encountered in sections stained with antibodies against α3 and α4. Antibodies to subunits of the β-rings of the 20S particle resulted in a similarly low number of labeled NIIs. In comparison, the highest percentage of NIIs was seen in sections stained with antibodies selective for β2 and β3 (less than 20% of NIIs; Fig 3). Antibodies directed to three alternative β-subunits (β1i, β2i, and β5i) inducible by γ-interferon did not result in staining of NIIs. Taken together, antibodies directed to 20S subunits labeled an average of 5 ± 3 NIIs per 100 neuronal profiles (see Fig 2).

**Most Neuronal Intranuclear Inclusions in SCA3 Exhibit 19S- and 11S-Proteasome-IR**

In marked contrast to the results obtained with the panel of antibodies directed to 20S proteasomal subunits, subunit-specific antibodies directed to the 19S particle consistently resulted in labeling of numerous NIIs (Fig 1D, E, and F). The eight subunit-specific antibodies used stained an average of 37 ± 10 NIIs per 100 neuronal profiles (see Fig 2). For example, the antibody recognizing the structural subunit 5α, which is thought to mediate binding of the polyubiquitin chain (see Fig 1D), labeled a mean number of 35 NIIs per 100 neuronal profiles. Antibodies directed to the subunit 5α stained even higher numbers of NIIs (57 ± 7) (see Fig 1E). This subunit has been shown to be associated with adenosine triphosphatase activity and is presumably involved in unfolding proteins prior to degradation by the 20S core particle. In yeast, the homologue of subunit 5α...
is part of the base portion of the 19S particle. Similar numbers were observed with an antibody to the structural subunit S10a (see Fig 1F) of the lid portion of the 19S particle (56 ± 15 NIIs; see Fig 3).

Antibodies to the two subunits constituting the 11S particle, PA28α and β, resulted in preferentially cytoplasmatic staining in pontine neurons of normal brain (Fig 4A and C) and labeled a substantial number of NIIs (Fig 4B and D; see Fig 2), approaching the number observed with antibodies to ubiquitin (see Fig 2). A summary of the labeling of NII by proteasomal subunits is provided in Fig 3.

Pontine Neurons in SCA3 Demonstrate Redistribution of Proteasome 26S-Subunit-IR

In both control and SCA3 brains, the overall cellular staining pattern obtained with antibodies to proteasomal antigens varied substantially from one neuronal profile to the next. In some neurons a cytoplasmatic staining pattern predominated, while neighboring neurons displayed prominent nuclear staining, presumably reflecting different functional states of the respective cells. Overall nuclear IR obtained with 26S antibodies in pontine neurons was less prominent in SCA3 patients (13%; SD ± 16) than in controls (67%; SD ± 20), suggesting a relative depletion of proteasomal subunits in the nuclear compartment in at least a subset of pontine neurons. In neurons containing NII, nuclear IR was concentrated to NII at the expense of overall nuclear staining in 54% of neuronal profiles (Fig 5A–D). In contrast, cytoplasmatic IR in pontine neurons of SCA3 patients was overall more prominent than in control brains, consistent with redistribution or upregulation of components of the 26S proteasome (see Fig 5A–D). Counting more than 2,000 pontine neurons stained with 20S antibodies demonstrated a higher percentage of neurons with predominantly cytoplasmatic staining in SCA3 (34%; SD ± 25) compared with controls (3%; SD ± 7). In addition, using antibodies selective for inducible β-subunits of the 20S core component, we observed a cytoplasmatic IR in many pontine neurons of SCA3 brains, well in excess of the signals obtained in control brains (Fig 5E and F): 64% of pontine neurons in SCA3 brains but only 6% of pontine neurons in control brains displayed a predominantly cytoplasmatic IR using an antibody to β2i.

Discussion

In this study we provide what we believe is thus far the most comprehensive immunohistochemical analysis of chaperones and proteasomal subunits in pontine neurons of SCA3 brains, using a total of 33 antibodies.

Distinct Chaperones Colocalize with a Subset of Neuronal Intranuclear Inclusions in SCA3 Brains

We observed labeling of NIIs with antibodies to heat shock protein (Hsp) 90α and HDJ-2, a member of the Hsp 40 family of chaperones. Both chaperones stained
only a subset of NIIs. The presence of Hsp90 in NIIs of SCA3 brains has not been reported previously; no labeling of NIIs in pontine SCA3 neurons was observed in an earlier study that used a different immunoreagent for Hsp90. Hsp90 can prevent protein aggregation and participates in the folding of a specific subset of proteins, e.g., signal-transducing molecules, presumably by binding unstable folding intermediates and maintaining them in a foldable state. In addition, Hsp90 has been implicated in the degradation of certain mutant proteins and copurifies with the proteasome. Hsp90 is thought to act in association with several cochaperones, including Hsp40 and 70, giving rise to discrete subcomplexes (see Caplan for a review). In SCA3 pontine neurons, a small subset of NIIs was labeled by an antibody to HDJ-2, in agreement with independent observations. In our material we failed to detect NIIs labeled with the Hsp/Hsc70 antibodies used. Chai and colleagues demonstrated rare pontine neurons in SCA3 containing Hsp70 positive NIIs; in addition, these cells displayed elevated cytoplasmatic IR for Hsp70, suggesting a heat shock response. These observations and similar findings in SCA1 indicate the relative paucity of NIIs decorated by the Hsp40 and Hsp70 family of chaperones in human poly-Q brain (in contrast to a transgenic animal model for SCA7, in which NIIs immunoreactive to Hsp40 and Hsp70 represented most if not all NIIs). A number of recent studies indicate that overexpression of Hsp40 and Hsp70 reduces poly-Q toxicity while altering but not abolishing NIIs. The paucity of NIIs decorated by Hsp40 and Hsp70 and the lack of evidence of chaperone up-regulation suggest that this potential mechanism of reducing poly-Q toxicity is employed by only a minority of neurons in end stage SCA3 brains.

**Accompaniment of Neuronal Intranuclear Inclusions in SCA3 by Proteasomal Subunits**

The ubiquitination of most NIIs in SCA3 brains and the association of components of the 26S proteasome complex with NIIs indicate that the ubiquitin-proteasome pathway is involved in degrading mutant ataxin-3. The present study extends previous reports, however, by disclosing that (1) the immunohistochemical composition of NIIs in pontine neurons of SCA3 is not uniform, and (2) only a small fraction of NIIs in pontine neurons of end stage SCA3 assemble the proteasomal machinery thought to be required for protein degradation.

To study the association of the catalytic 20S component consisting of four stacked, heptameric rings with NIIs, we used antibodies to all seven subunits of two outer rings of β-subunits, six of which were subunit specific, and seven mono- and polyclonal antibodies to probe the two inner rings of β-subunits. An average of 8% of NIIs were immunoreactive to the 15 antibodies directed to 20S (see Fig 2 and 3). Since these antibodies were directed to different epitopes of the 20S core component, epitope masking
is unlikely to account for this disproportionally low labeling compared with 11S- and 19S-associated epitopes. A similar low labeling using 20S antibodies was observed in brains with Huntington’s disease (Krebs et al, unpublished results) and a cellular model of Kennedy’s disease,9 suggesting that the proteasomal machinery that actively degrades NIIs is assembled in only a fraction of neurons. A failure to recruit the essential catalytic 20S particle to most NIIs may contribute to the impaired clearance and gradual accumulation of mutant ataxin-3 in SCA3 brains. In addition, the shredder-like function of the 20S component may be impeded when encountering poly-Q–expanded polypeptides, since they contain no predicted cleavage sites.31

Prior to proteolytic cleavage, polypeptides have to be unfolded and then threaded through the narrow central openings of the outer β-rings of the 20S core particle.13,32 The 19S component of the 26S proteasome is thought to mediate this process in an adenosine triphosphate–consuming fashion.13 Polyclonal antibodies selective for all six adenosine triphosphatases resulted consistently in the staining of numerous NIIs, suggesting that aggregates of mutant ataxin-3 resist unfolding or at least require the prolonged or persistent recruitment of 19S subunits. Aside from its function in unfolding polypeptides, the 19S complex is critical in recognition of ubiquitinated substrates and contains sites for tight binding of polyubiquitin chains. An antibody to this putative “polyubiquitin receptor,” S5a, as well as an antibody to a structural subunit located in the lid portion of the 19S particle, S10a, resulted in staining of many NIIs, suggesting the recruitment of the entire 19S particle (and not only fragments thereof, eg, the base portion) to NIIs.

Another regulator of proteasome function is the 11S regulatory particle, a heterodimeric complex composed of three pairs of alternating subunits called PA28α and

Fig 5. Immunohistochemical staining of pontine neurons in normal brain (A, C, E) and spinocerebellar ataxia type 3 (SCA3) brain (B, D, F) using subunit-specific antibodies directed to subunits of the 20S core component of the proteasome demonstrated reduced nuclear staining and more prominent cytoplasmatic staining in SCA3 tissues. (A) Anti-α5-IR in normal brain. (B) Anti-α5-IR in SCA3 brain. (C) Anti-β2-IR in normal brain. (D) Anti-β2-IR in SCA3 brain. (E) Anti-β2i-IR in normal brain. (F) Anti-β2i-IR in SCA3 brain. Bar = 20μm.
β, which can form a cap at either end of the 20S core (see Fig 3). We used antibodies selective for each subunit and observed a surprisingly high number of NIIs labeled with either antibody, suggesting the accompaniment of most NIIs by the 11S particle. The association of 19S, 20S, and 11S with NIIs may indicate the recruitment of heterocomplexes of proteasomes, in which a 19S complex caps one opening of the 20S core and an 11S particle the other opening. Alternatively, the PA28 complex in association with NIIs may have a function independent from 20S: PA28 has a critical role in Hsp90-dependent protein refolding.

Proteasomal Redistribution in Pontine Neurons of SCA3

There is evidence for a redistribution of proteasomal antigens in SCA3 brains. In neurons containing NIIs, nuclear IR obtained with subunit-specific proteasomal antibodies was often concentrated in NIIs at the expense of overall nuclear staining, suggesting a depletion of certain proteasomal subunits. The apparent depletion was particularly obvious for antibodies to 19S subunits and some 20S subunits, resulting in intense nuclear staining under physiological conditions. Clearly, the assumption that the apparent sequestration of some proteasomal subunits into NIIs results in a functional depletion of some proteasomal subunits in the nuclear compartment requires biochemical confirmation.

Aside from these alterations in the nuclear staining pattern, overall cytoplasmatic staining in pontine neurons of SCA3 patients appeared more intense than in control brains (see Fig 4). In particular, cytoplasmatic staining with antibodies to inducible β-subunits of 20S was more prominent than in control brains, compatible with redistribution or upregulation of some components of the 26S proteasome. A quantitative confirmation using Western blot analyses and mRNA studies is required; we could not carry out these studies due to a lack of suitable material. The functional consequences of this apparent redistribution are currently unknown. However, the appearance of inducible catalytic 20S β-subunits in the cytoplasmatic compartment suggests that protein catabolism in the cytoplasmatic compartment may be enhanced, possibly contributing to atrophy of affected neurons similar to muscle wasting in cachexia.

Perturbations of Proteasomal Degradation Pathways

Taken together, these observations suggest a perturbation of proteasomal protein degradation in SCA3 brains. Recent studies of cellular models of poly-Q disorders indeed support impaired proteasomal function in the presence of poly-Q-expanded proteins. Cellular consequences of impaired proteasomal function may include alterations in gene expression (as described in poly-Q disorders) caused by interference with the degradation of transcriptional regulators or with mitochondrial functions, potentially leading to neuronal death. Since protein deposits are a pathological hallmark of many neurodegenerative diseases, including Alzheimer’s disease, Pick’s disease, Parkinson’s disease, and prion disorders, these disorders may be viewed as proteinopathies in which disease-specific proteins misfold and eventually form aggregates tagged by ubiquitin and associated with proteasomal antigens. Perturbations of the proteasomal machinery as demonstrated in this study for SCA3 may therefore be part of the pathophysiology of other neurodegenerative diseases as well.

References


This study was supported by grants from the Deutsche Forschungsgemeinschaft (Ri6827/1 awarded to OR, and SFB 505, TPC2, awarded to GBL); the German Hereditary Ataxia Foundation (awarded to OR); and the Graduierten-Kolleg für Molekulare Medizin (awarded to KSL).

We thank the families of the patients, whose generosity made this research possible. We also thank Dr Y. Trottier for providing antibodies directed to ataxin-3.


