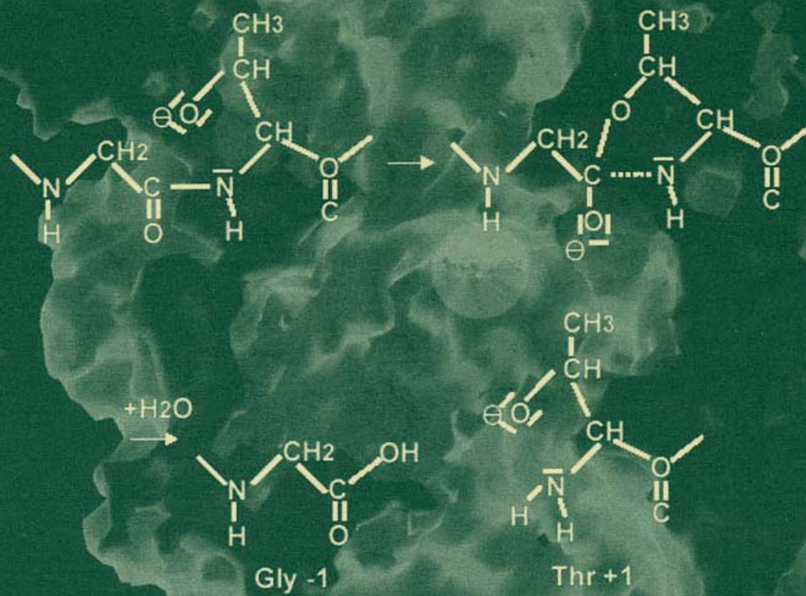
The Proteasome in Neurodegeneration



Leonidas Stefanis
Jeffrey N. Keller

The Proteasome in Neurodegeneration

Edited by
Leonidas Stefanis
Institute of Biomedical Research of the Academy of Athens
Athens, Greece

Jeffrey N. Keller
University of Kentucky
Lexington, KY, USA

With 109 Illustrations

Springer

Leonidas Stefanis
Institute of Biomedical Research
of the Academy of Athens
Athens, Greece

Jeffrey N. Keller
Sanders-Brown Center on Aging
Department of Anatomy and
Neurobiology
University of Kentucky, KY, U.S.A.

Library of Congress Control Number: 2005931437

ISBN-10: 0-387-28499-0
ISBN-13: 978-0387-28499-6

Printed on acid-free paper.

© 2006 Springer Science+Business Media, Inc.
All rights reserved. This work may not be translated or copied in whole or in part without the written permission of the publisher (Springer Science+Business Media, Inc., 233 Spring Street, New York, NY 10013, USA), except for brief excepts in connection with reviews or scholarly analysis. Use in connection with any form of information storage and retrieval, electronic adaptation, computer software, or by similar or dissimilar methodology now known or hereafter developed is forbidden.
The use in this publication of trade names, trademarks, service marks, and similar terms, even if they are not identified as such, is not to be taken as an expression of opinion as to whether or not they are subject to proprietary rights.

Printed in the United States of America. SPI/EB

9 8 7 6 5 4 3 2 1

springeronline.com

CONTRIBUTORS

Dr. Jeffrey N. Agar, Department of Chemistry, Brandeis University, Waltham, Massachusetts, U.S.A.

Mr. Subhabrata Basu, Department of Pathology, Case Western Reserve University, Cleveland, Ohio, U.S.A.

Ms. Sharmila Bose, Department of Molecular, Cellular and Developmental Biology, The University of Michigan, Ann Arbor, Michigan, U.S.A.

Dr. Pietro Calissano, Institute of Neurobiology and Molecular Medicine, National Research Center, Rome, Italy

Dr. Nadia Canu, Department of Neuroscience, University of Tor Vergata, Rome, Italy

Dr. Mark R. Cookson, Cell Biology and Gene Expression Unit, Laboratory of Neurogenetics, National Institute on Aging, NIH, Bethesda, Maryland, U.S.A.

Dr. Ted M. Dawson, Institute for Cell Engineering, Departments of Neurology, Neuroscience and Physiology, Johns Hopkins School of Medicine, Baltimore, Maryland, U.S.A.

Dr. Valina L. Dawson, Institute for Cell Engineering, Departments of Neurology, Neuroscience and Physiology, Johns Hopkins School of Medicine, Baltimore,

Dr. Miguel Diaz-Hernandez, Centro de Biología Molecular "Severo Ochoa", Consejo Superior de Investigaciones Científicas, Universidad Autónoma de Madrid, Facultad de Ciencias, Cantoblanco, Madrid, Spain

Dr. Qunxing Ding, Department of Anatomy and Neurobiology, University of Kentucky, Lexington, Kentucky, U.S.A.

Dr. Heather D. Durham, Montreal Neurological Institute, Department of Neurology/Neurosurgery, McGill University, Montreal, Quebec, Canada

Dr. Cordula Enekel, Humboldt Universität / Charité, Institut für Biochemie, Berlin, Germany

Dr. Maria Figueiredo-Pereira, Department of Biological Sciences, Hunter College of City University of New York, New York, New York, U.S.A.

Dr. Yaping Gu, Department of Pathology, Case Western Reserve University, Cleveland, Ohio, U.S.A.

Dr. Barry Halliwell, NUS Graduate School of Integrative Science and Engineering, Department of Biochemistry, Singapore, Singapore

Dr. Eunsung Junn, Center for Neurodegenerative and Neuroimmunologic Diseases, Department of Neurology, UMDNJ-Robert Wood Johnson Medical School, Piscataway, New Jersey, U.S.A.

Mr. Edor Kabashi, Montreal Neurological Institute, Department of Neurology/Neurosurgery, McGill University, Montreal, Quebec, Canada

Dr. Jeffrey N. Keller, Department of Anatomy and Neurobiology & Sanders-Brown Center on Aging, University of Kentucky, Lexington, Kentucky, U.S.A.

Dr. Jungkee Kwon, Department of Degenerative Neurological Diseases, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo, Japan

Dr. Isabelle Lang-Rollin, Department of Psychiatry, Max-Planck-Institut für Psychiatrie München, Munich, Germany

Dr. Robert Layfield, Centre for Biochemistry and Cell Biology, School of Biomedical Sciences, Queen's Medical Centre, Nottingham, U.K.

Dr. Gwang Lee, Brain Disease Research Center, School of Medicine, Ajou University, Suwon, Korea

Dr. José J. Lucas, Centro de Biología Molecular "Severo Ochoa", Consejo Superior de Investigaciones Científicas, Universidad Autónoma de Madrid, Facultad de Ciencias, Cantoblanco, Madrid, Spain

Ms. Xiu Luo, Department of Pathology, Case Western Reserve University, Cleveland, Ohio, U.S.A.

Ms. Richa Mishra, Department of Pathology, Case Western Reserve University, Cleveland, Ohio, U.S.A.

Dr. M. Maral Mouradian, Center for Neurodegenerative and Neuroimmunologic Diseases, Department of Neurology, UMDNJ-Robert Wood Johnson Medical School, Piscataway, New Jersey, U.S.A.

Mr. Ajitesh Ojha, Department of Pathology, Case Western Reserve University, Cleveland, Ohio, U.S.A.

Ms. Sathya Ravichandran, Graduate Program in Cellular and Molecular Medicine, Johns Hopkins School of Medicine, Baltimore, Maryland, U.S.A.

Dr. Hardy J. Rideout, Department of Neurology, Columbia University, New York, New York, U.S.A.

Dr. Thomas Schmidt-Glenewinkel, Department of Biological Sciences, Hunter College of City University of New York, New York, New York, U.S.A.

Dr. Neena Singh, Department of Pathology, Case Western Reserve University, Cleveland, Ohio, U.S.A.

Dr. Leonidas Stefanis, Section of Neurobiology, Institute for Biomedical Research, Academy of Athens, Athens, Greece

Dr. Mikiei Tanaka, Laboratory of Biochemistry, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland, U.S.A.

Mr. David M. Taylor, Montreal Neurological Institute, Department of Neurology/Neurosurgery, McGill University, Montreal, Quebec, Canada

Dr. Kostas Vekrellis, Section of Neurobiology, Institute for Biomedical Research, Academy of Athens, Athens, Greece

Dr. Keiji Wada, Department of Degenerative Neurological Diseases, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo, Japan

8

ROLE OF THE UBIQUITIN PROTEASOME SYSTEM DURING NEURONAL CELL DEATH

Nadia Canu and Pietro Calissano

1. INTRODUCTION

In recent years much effort has been devoted to understanding the nature of neuronal cell death and the proteolytic systems involved in neurodegenerative diseases. Although the precise mechanism of cell death in neurodegenerative disorders is not known, PCD (programmed cell death) has been implicated (1). Two major kinds of PCD have been proposed (2,3). Type I PCD, or classical apoptosis, is a tightly regulated process morphologically characterized by loss of cell volume, chromatin condensation, cell blebbing, neurite retraction and nuclear fragmentation. At the biochemical level a family of cytoplasmic proteases termed the caspases contributes to the execution phase of this process (4). Type II PCD, or autophagic cell death, on the other hand, is morphologically characterized by proliferation of the autophagosomal-lysosomal system and early destruction of the cytoplasm (5, 6, 3). Although biochemically and morphologically different, these two types of PCD may co-exist and account for the complex

anatomo-pathological pictures characteristic of a range of neurodegenerative diseases. These diseases are characterized by death of specific neuronal populations and by progressive accumulation of "lethal" aggregates mainly formed by a single protein such as amyloid-beta and tau protein in Alzheimer disease, prion protein in scrapie and CJD disease or α-synuclein in Parkinson's disease.

Proteinaceous deposits in such neurodegenerative diseases tend to be ubiquitinated, and constitute a visible hallmark of ubiquitin-proteasome system (UPS) impairment. There is a complex interrelationship between the deposition of aggregates, the function of the UPS and neuronal cell death. We have investigated the role of the ubiquitin-proteasome system during PCD with the aim of testing the hypothesis that its altered functions may mimic those occurring in neurodegenerative diseases. We have utilized an "in vitro" paradigm of neuronal death mimicking the process of an *in vivo* deafferentation of a neuronal population taking place either during embryogenesis or in various neurological diseases. Thus, cultured cerebellar granule cells (CGCs) undergo massive cell death when the depolarizing potassium concentration normally employed for their culturing is reduced from 25 mM to 5.0 mM. This manipulation, experimentally compared to the surgical disconnection of the nerve afferents to cerebellar granule neurons (7) activates an internal program of PCD in which biochemical and morphological elements of apoptosis and autophagy intersect and influence each other (8, 9, 10, 11). In this model, the UPS appears to be involved in channelling neurons to death through two pathways: 1) by acting as a master proteolytic system that orders caspase activation; 2) by subsequently undergoing a loss of function which, in turn, contributes to secondary damage likely due to the accumulation of pro-apoptotic molecules.

2. PROTEASOME INHIBITORS DELAY CELL DEATH

The first evidence of an active role of the UPS in PCD came from the studies of Schwartz and colleagues describing an increase in polyubiquitin and proteasome subunit gene expression during the intersegmentation of muscles and the morphogenesis of some neuronal populations in larvae of the hawk moth Manduca sexta (12). Numerous subsequent studies employing different model systems revealed that the involvement of the UPS in PCD is not always accompanied by a consistent increase in the expression of its protein components (13, 14, 15). More recently, the role of the UPS in PCD has been analyzed by the use of pharmacological inhibitors of this proteolytic system. It should be noted that the interpretation of the results may be difficult since fine tuning of this proteolytic system is apparently required to ensure cell survival, as widely underlined in the subsequent chapter of Lang-Rollin and Stefanis in this volume.

A number of studies have found that pharmacological proteasome inhibition leads to an inhibition or delay of neuronal death. For instance, proteasome inhibitors prolonged the survival of NGF-deprived sympathetic neurons (16), prevented thymocyte apoptosis induced by glucocorticoids (17), blocked MPP or rotenone-induced dopaminergic neuronal death (18), and reduced infarct volume in a rat model of focal cerebral ischemia (19). In all these cases arrest of cell death was associated with the inhibition of apoptotic markers such as the perturbation

of mitochondrial membrane, caspase activation and DNA laddering. We have confirmed a neuroprotective effect of proteasomal inhibition in CGC cultures induced to undergo apoptosis in the presence of several highly selective proteasome inhibitors such as lactacystin and epoxomicin (10, 20). The observation that the neuroprotective effect was more evident when the drugs were added within 1 hour after apoptosis induction indicated that proteasomes play a regulatory key function in the very early phase of apoptosis, as also reported in other models (16).

Consistent with this finding, we also found that proteasome activities were slightly increased during the early phase of apoptosis. Sawada et al. (18) also found increased proteasomal activities early on after application of MPP+ or rotenone to ventral midbrain cultures.

To answer the question of whether the neuroprotective effects of proteasome inhibitors were correlated with the inhibition of classical markers of apoptosis, we determined the activity of caspase-3 in CGCs deprived of potassium. These drugs were able to prevent caspase-3 activity and pro-caspase 3 activation, suggesting that proteasomes control the activation of caspase(s) (Figure 1). Accordingly, events such as calpain/caspase-mediated cleavage of tau (21, 10), Reactive Oxygen Species (ROS) production, DNA laddering and the onset of a deficit of the antioxidant system normally occurring downstream of caspase activation were also prevented (22, 20) (Figure 1). Activation of caspases in CGCs occurs mainly through the intrinsic pathway, in which mitochondrial perturbation causes cytochrome c release in the cytosol to form the apoptosome, the promoter of the caspase cascade. We found that proteasome inhibitors were able to interfere with cytochrome c release (23) (Figure 1), suggesting that they may control proteins involved in the organization of the mitochondrial pore through which efflux of mitochondrial proteins occurs.

3. PROTEASOME-DEPENDENT DEGRADATION DURING THE EARLY PHASES OF APOPTOSIS

Given that activation and involvement of proteasomes have been described as an integral parts of the initiation phase of apoptosis, both in neuronal and non neuronal settings (16, 24, 10, 20, 25, 18, 26), three major questions arise:

3.1 How does the Proteasome Mediate the Early Steps of Neuronal Apoptosis?

The logical answer to this question is that the proteasome may be responsible for the degradation of some critical substrates that mediate neuronal survival. Thus, when the proteasome is activated early on in apoptosis, it accelerates the degradation of such pro-survival factors, leading to their relative depletion and engagement of the apoptotic pathway. Which are these potential pro-survival proteasomal substrates? In non-neuronal paradigms of apoptosis different targets of proteasome degradation have been identified. They include, amongst others, transcription factors that regulate genes coding for polypeptides involved in cell proliferation and survival (c-Fos; NFkB, AP-1, ODC), and proteins, like IAPs, that normally repress caspases (27, 28, 29, 30, 25).

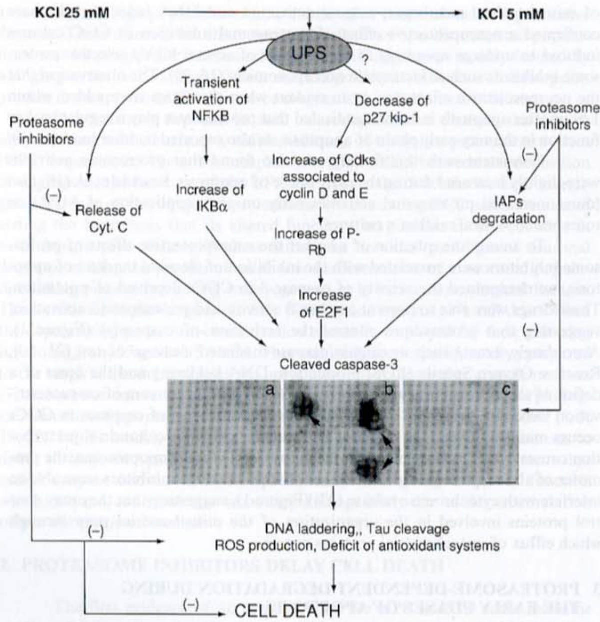


Figure 1. This drawing depicts the series of known or hypothetical events occurring in cerebellar granule cells following the apoptotic trigger. The black arrows point out the established findings underlying the role of UPS in the sequence of events leading to caspase activation, and subsequently DNA laddering, tau cleavage, ROS production, and antioxidant system failure. Gray arrows indicate the hypothesized events linked to UPS activation during the course of apoptosis and (?) indicates that the direct or indirect involvement of UPS in the decrease of p27 during CGCs apoptosis has not been investigated. The panel visible in the central part of the figure shows an immunofluorescence analysis of cleaved, activated caspase-3 (black) in control cells (a) or in potassium-deprived cells, in the absence (b) or presence (c) of the UPS inhibitor lactacystin. Notice the presence of 3 apoptotic neurons in which the staining of activated caspase 3 is clearly visible (b) and that in the presence of lactacystin such activation does not occur (c); (-): inhibition; Bar: 7 μ

As mentioned, in CGCs, the proteasome-mediated step(s) that promote(s) apoptosis occur(s) before mitochondrial changes and caspase activation. We had previously reported that ROS are involved in the release of cytochrome c in this model (31). This raised the possibility that the proteasome may down regulate the anti-oxidant system constituted by catalase, SOD, and GSH/GSSG, and thus promote apoptosis. We have indeed found that the antioxidant system increased

after proteasome inhibition before eventually declining, suggesting that the UPS may participate directly or indirectly in its turnover (20). Whether these molecules are normally degraded by the UPS or whether they acquire the ability to be UPS substrates during neuronal apoptosis remains to be established. In this regard, it must be pointed out that catalase, the major effector in the defense of aerobic cells against oxidative stress, is degraded by proteasomes in a phosphorylation-dependent fashion catalyzed by c-Abl and Arg tyrosine kinases (32). By contrast, a direct link between UPS and SOD has been demonstrated only for mutant Cu/Zn SOD whose level increases in the presence of proteasome inhibitors (33).

Other important constituents degraded by the UPS during apoptosis are IAPs (inhibitor of apoptosis proteins). IAPs are a family of proteins containing one or three BIRs (baculovirus IAP repeat) and a RING finger domain that confers ubiquitin protease ligase (E3) activity. IAPs are endowed with many functions, including the ability to bind to activated caspases and to inhibit their activity, most likely by ubiquitinating them and targeting them to the proteasomes (34). It has been reported that in thymocytes IAPs undergo autoubiquitination and degradation by the proteasome in response to apoptotic stimuli, an event critical for commitment to cell death (25).

To determine how proteasome inhibitors prevent CGC death, we examined whether proteasomes degraded these anti-apoptotic proteins and found that proteasome inhibitors, but not caspase inhibitors, stabilized and increased the levels of IAPs (Nadia Canu, unpublished observation). This finding suggests that IAPs are targeted to proteasome for degradation during the CGC apoptosis, thus favouring caspase activity as reported in other settings (25), and likely increasing the amount of pro-apoptotic IAP substrates (see also article by Lang-Rollin and Stefanis in this volume). It should be noted however that such an increase of IAPs would not be expected to lead to inhibition of cytochrome c release, as IAPs generally function downstream of the mitochondrial checkpoint.

Studies carried out in the same experimental model suggest that proteasomes could also be operative at other levels. A peculiar case is that of NF-kB. The processing of this transcription factor from an inactive precursor to an active form involves the degradation of part of the precursor and the release from the inhibitory effect of IkBα and β. Both these functions are mediated by the proteasome (35, 36). Thus, this transcription factor has been found to be activated by the UPS in the early phase of apoptosis (37), presumably via limited proteolysis of the NFkB precursor protein (35). The role of NF-kB in cell death in the nervous system is controversial. It is clear that it can be activated both following pro-apoptotic (38, 39) and pro-survival signals (40). In most settings, inhibiting such activation appears to be associated with pro-death effects (40, 41, 42), but the converse has also been observed (43, 39). It appears that cell type, intensity, type and duration of the death stimulus and duration and temporal relationship of NF-kB inhibition to the death stimulus are important variables (43, 39). If, as in some models (43, 39), NF-kB acts as a death mediator when activated early on in apoptosis, it is possible that the inhibition of its induction by proteasomal inhibitors may account in part for their early protective activity in potassium-deprived CGCs, although this has not been specifically tested.

Proteasomes might also have a crucial role in the pathway involving p27kip that, in certain non-neuronal settings is a proteasome substrate (44). Interestingly, Padmanabhan et al. (45) have reported that in KCl deprivation-evoked death of CGCs a significant decrease in the level of this cyclin inhibitor occurs. Moreover, an enhanced activity of cyclin D1 and E-associated kinases and, more importantly, a transient increase in phosphorylation of Rb (a known substrate of these cyclins, which gets inactivated by phosphorylation), were reported. Consequently, it has been postulated that the Rb function of binding and repressing the transcriptional activity of E2F would be lost, a hypothesis reinforced by the finding that E2F-1 induces and modulates CGC death (46). The changes reported above were prevented by the cyclin-dependent kinase inhibitor flavopiridol but not by caspase inhibitors. It would therefore be interesting to ascertain whether proteasome inhibitors in this system mediate their protective effects through p27kip increase.

Altogether these findings suggest that the events depicted in Figure 1 play a role in the mediation of apoptosis following potassium deprivation in CGCs.

3.2. How is the Proteasome Activated During PCD?

An attempt to answer this question was carried out by Kroesen et al. (26). They reported that B cell receptor activation initiates an apoptotic program characterized by sphingolipid-dependent activation of proteasomes and degradation of IAPs that were prevented by an ISP-1/myriocin, a potent inhibitor of ceramide formation. Proteasome activation occurred likely via sphingolipid-dependent phosphorylation of its C8 and C9 subunit (47). Whether a similar mechanism governs proteasome activation during neuronal apoptosis remains to be established.

3.3. What Specific Signal(s) Channel any Potential Proteasome Substrate Toward Degradation During Apoptosis?

As far as this question is concerned, a particular case is the degradation of IAPs. These inhibitors of death must be inactivated in cells that are doomed to die. Studies in Drosophila demonstrated that this is accomplished by proteins such as Reaper, Hid and Grim. It has been reported that Reaper, a small 65aa protein that is specifically expressed in cell undergoing apoptosis induces apoptosis by specifically stimulating the auto-ubiquitination and degradation of IAPs (48). In mammalian cells a similar role might be played by mitochondrial proteins such as Diablo/Smac and the serine protease HrtA2/Omi (49).

4. PROTEASOME CHANGES DURING THE LATE PHASES OF APOPTOSIS

After such early involvement we found that the proteasome undergoes a series of changes resulting in its inactivation. Before apoptosis is triggered in CGC cells, proteasomes were present in the nuclei as well as in the cytoplasm (50, 51). Upon chromatin condensation, nuclear proteasomes were found mainly in the cytosol. The movement of the UPS is likely due to its involvement in cell

shape changes, since proteasomes have been found in apoptotic bodies and cytoplasmic vesicles (52). Other studies indicate an active role of the UPS through the regulation of ezirin turnover. Ezirin is a cytoskeletal protein involved in anchoring actin to the cell membrane. It is thus involved in the control of cell blebbing, rounding-up, and overall cellular size. Whether the movement of proteasomes to the cytosol in CGCs is correlated to a UPS-mediated rearrangement of the cytoskeleton and to the organization of the apoptotic bodies has not been specifically addressed.

At the biochemical level one of the most impressive proteasome changes is the progressive decline of its function at late stages of apoptosis. Indeed, we found that proteasomes became part of a generalized cellular failure that affects the major activities of the apoptotic neurons after caspase activation. Cell extracts from apoptotic CGCs showed a decrease in proteasome chymotrypsin-like, trypsin and post-acidic-like activities, correlating with the degree of apoptosis observed, similar to findings reported for dexamethasone-induced apoptosis of thymocytes (53). These changes were observed at the time of the execution phase and were prevented by the general caspase inhibitor z-VAD-fmk, suggesting that caspase-dependent proteolysis inactivates proteasome functions (10). It has been previously reported that the impairment of the chymotrypsin-like activity of the proteasome caused an accumulation of ubiquitinated proteasome substrates (54, 55). Therefore, we asked whether the progressive failure of proteasome activity, during PCD, was accompanied by changes in protein ubiquitination. We found that proteasome failure during CGC apoptosis occurred with concomitant increase in the amount of high-molecular mass ubiquitin conjugates, as detected by immunofluorescence analysis (Figure 2) and Western immunoblot (10). Two apoptotic neurons filled with ubiquitin-conjugated proteins are clearly shown in Figure 2.

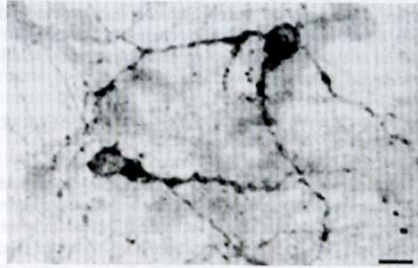


Figure 2. Ubiquitin immunostaining in cerebellar granule neurons undergoing apoptosis analyzed by confocal microscopy. Apoptosis in CGCs was induced by potassium and serum withdrawal. Immunostaining of ubiquitin was performed 6 hr after induction, using a polyclonal anti-ubiquitin antibody. Notice that two apoptotic neurons are filled with ubiquitinated proteins (black) both in the soma and along the neurites, while healthy neurons are barely stained with anti-ubiquitin antibodies. Bar: 7 μ

Interestingly, this event was abolished by caspase inhibitors, confirming that impairment of proteasome function occurs downstream of caspase activation, a conclusion also supported by data from other "in vitro" cellular systems exposed to diverse apoptogenic stimuli (56, 57).

What could be the functional consequences of proteasomal inhibition at the late phases of apoptosis? This subject is dealt in more detail by Lang-Rollin and Stefanis in the subsequent chapter, where the significance of the up regulation of particular potentially deleterious proteins is analyzed. In the case of CGC apoptosis, after the initial increase of NF-kB activity, there is a decrease, mediated largely by the prolonged stability of its endogenous inhibitor IkBα, as its proteasomal degradation is hindered (58, 37). The importance of an elevated level of IkBα in CGCs has been suggested by the findings that over-expression of a stabilized form of this protein renders CGCs more vulnerable to apoptotic stimuli (37) and that during proteasomal inhibition-induced cell death the amount of this inhibitor increases (59, 60). These data suggest that at these late phases NF-kB may act as a survival factor and that inhibiting its activation via proteasomal impairment may accelerate death.

It must also be considered that the accumulation of unique or multiple unspecified and ubiquitinated proteins inside the cells, usually in organized structures referred to as aggresomes, can act as possible apoptotic triggers. Indeed, it has been reported that protein aggregation causes impairment of the UPS and therefore depletes cells of this pivotal and vital proteolytic system, thus causing cellular deregulation and death (61). In this regard, although some findings support a protective role for some of these inclusions, as in the case of cytoprotection exerted by aggresomes formed by alpha-synuclein and synphilin-1 (62, see also chapter 4 in this volume) other studies suggest that some neuronal populations are highly vulnerable to the accumulation of ubiquitinated proteins under the form of aggregates, as found in many neurodegenerative diseases (61, 63, 64). Therefore, we can envisage a vicious circle that starts when accumulated proteins block the interior of proteasomes, thereby reducing their activity and eventually adding noxious inputs to neurons already committed to death. For a more detailed discussion of the topic of protein aggregation and how it influences survival, refer to section 2, chapters 3-5 of this volume.

5. COMPONENTS OF THE PROTEASOME SYSTEM DEGRADED BY CASPASES

Since induction of apoptosis in CGCs resulted in diminished activity of the UPS that was prevented by caspase inhibitors, we asked whether the proteasome itself was a victim of caspase-dependent attack. Interestingly, we found that the amount of the α-2-α7 subunits (10) and the β1-β7 subunits (Canu, unpublished observation) of the core 20S proteasome did not change during CGC apoptosis. By contrast, the amount of the ATPase subunit S6 of the 19S regulator of the 26S proteasome, which is necessary for ATP-dependent proteolysis by the proteasome, was reduced in a caspase-dependent manner during apoptosis of CGCs (Figure. 3). This finding is in line with results recently reported for Jurkat cells treated with etoposide or actinomycin and for PCD in flies (57, 65). In these

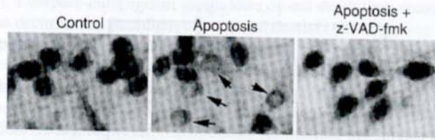


Figure 3. Immunofluorescence analysis of the ATPase subunit S6 of the 19S regulator of UPS. Notice the presence of 4 apoptotic neurons (arrows) in which the immunostaining of this subunit is strongly reduced while in the presence of the caspases inhibitor z-VAD-fmk the staining is comparable to control.

cases proteasomal subunits, in particular S6, S1 and S5a of 19 S, are cleaved by caspases both in vitro and in vivo under different apoptotic stimuli (57, 65). It has been speculated that caspase-mediated cleavage of these subunits may affect the structural integrity and activity of the proteasome, interfering either with the stabilization of the interaction of the lid and base of the 19S subunit (66, 67) or with the recognition and interaction with multiubiquitin chains in intact proteasomes (68, 69, 70, 71).

Another mechanism contributing to impairment of proteasome activity in CGCs is the finding that histone H2A is deubiquitinated (Canu et al. unpublished observation), suggesting that depletion of free ubiquitin takes place in our experimental model as already reported in other systems (72). The availability of free ubiquitin for the ubiquitination reaction is guaranteed by the synergistic activities of different deubiquitinating (DUB) enzymes. We measured these activities in cell extracts of CGCs undergoing apoptosis using as substrate a mixture of (Ub)4, (Ub)3, and (Ub)2 oligomers that are converted by the action of DUB enzymes into monomeric ubiquitin UB1 (73). These activities were markedly and progressively impaired in CGCs undergoing apoptosis, and more importantly, their loss was prevented by a caspase inhibitor (Figure. 4).

DUB enzymes are encoded by two gene families: the UCH family (ubiquitin C-terminal hydrolases, with molecular weight of ~30 kDa, hydrolyzing small C-terminal derivatives) and the UBP family (ubiquitin-specific processing proteases, with molecular weight of ~110 kDa, hydrolyzing large derivatives of ubiquitin). UCHL-1 is one of the most abundant enzymes in the brain, comprising up to 2% of total brain proteins (74). A partial loss of UCHL-1 activity, due to a missense mutation, has been implicated in proteasome failure and aggregation of ubiquitinated proteins in familial cases of Parkinson's disease (74). For more details on UCH-L1 and its role in UPS dysfunction and neurodegeneration, see the chapter by Kwon and Wada in this volume. We found that the amount of this enzyme does not change during CGC apoptosis (Nadia Canu, unpublished observation), suggesting that other caspase-mediated modifications of UCHL-1 may account for an impairment of its activity, or, more likely, that other DUB enzymes are responsible for this deficit. This latter conclusion is supported by the demonstration that isopeptidase T, an enzyme belonging to the UBP family, is cleaved by caspase-3, with loss of function, both in vitro and in vivo (65).

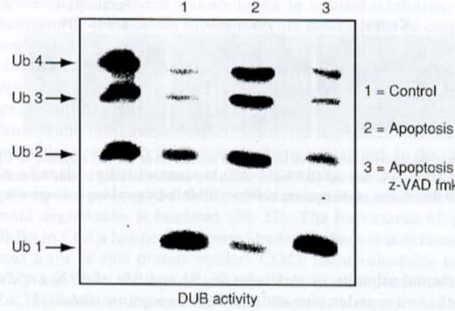


Figure 4. Deubiquitinating activity in granule neurons undergoing apoptosis. Cultures at 6 DIV were induced to undergo apoptosis in the absence or in the presence of the general caspase-inhibitor z-VAD-fmk for12 hr. At the time indicated after the induction of apoptosis, 5 fg of supernatants were incubated with 1 fg of multi-ubiquitin chains, substrates for DUB enzymes at 22°C for 10 min, and immunoblotted with anti-ubiquitin antibody (10).

How may a decrease in DUB activity impair proteasome function? It has been demonstrated that DUB enzymes remove ubiquitin from various cellular adducts, thus playing an important role both in the editing of the ubiquitination state of proteins as well as in the recycling of ubiquitin (73). The recycling of free ubiquitin from poly-ubiquitin remnants is required for the continued activity of the UPS. In fact, insufficiently disassembled polyUb chains bind avidly to, and inhibit, the 26 S proteasome complex, presumably via competition with polyubiquitinated substrates (75, 76). It is also possible that the proteasome efficacy is additionally hampered by post-translational modifications of the proteasome and /or by the generation, during apoptosis, of cross-linked or aggregated proteins. These polypeptides, which are poor substrates for proteolysis and may physically impede proteasome entry by blocking the catalytic site, may be generated as a consequence of the disruption of intracellular sulfhydryl homeostasis (77). The finding that the level of heat shock proteins, which operate in the trafficking of misfolded proteins on their way to the proteasome, are increased during CGC apoptosis (78) supports the hypothesis that the generation of misfolded and eventually aggregated proteins or structures may occur during CGC apoptosis.

6. APOPTOSIS, AUTOPHAGY AND PROTEASOME INHIBITION

In the presence of proteasome inhibitors, CGCs deprived of KCl did not manifest classical hallmarks of apoptosis and remained alive for 12-15 h (10). At later time points, the neuroprotective effect was less evident and at longer time points it was no longer detectable. This situation likely reflects the block of a pivotal proteolytic system as well as the inability of proteasome inhibitors to counteract

autophagy, a caspase-independent mechanism of cell death that, recently, has been reported to occur in this paradigm of neuronal death (11).

Apoptosis and autophagic degeneration are two morphologically and biochemically distinct modes of programmed cell death described in embryogenesis and tissue renewal in adults. A substantial body of evidence has revealed the simultaneous presence of apoptosis and autophagic elements in neurodegenerative disorders (79, 1). However, it is not clear yet whether autophagy is an attempt to protect the cell from apoptosis, or to hasten cellular demise. Data from diverse in vitro models of neuronal death support this latter hypothesis.

Autophagy not only co-exists with apoptosis but may precede and influence it in a process that is induced by apoptotic stimuli (80, 81). In CGCs undergoing apoptosis, activation of autophagy appears to occur very early after the apoptotic stimuli, before any classical hallmark of apoptosis is manifested, although it is more evident in neurons displaying nuclear condensation. We have found that autophagy controls the release of cytochrome C, caspase activation and, more importantly, mediates a caspase-independent process of cell death. Block of autophagy rescues CGCs from apoptosis (11). Similar results have been reported in newly isolated sympathetic neurons deprived of NGF or treated with cytosine arabinoside (80) as well as in PC12 cells deprived of serum or in delayed neuronal death occurring in the CA1 pyramidal layer of the gerbil hippocampus after brief forebrain ischemia (82). These findings suggest that autophagy is likely a mode to initiate apoptosis in different settings.

What is the role of the proteasome system in autophagic cell death? To answer this question we have visualized autophagic vesicles with a specific marker in CGCs undergoing apoptosis in the presence of proteasome inhibitors and found that these inhibitors not only do not cause disappearance of autophagic vesicles (Figure. 5), but that the autophagosome content is quite different in the two experimental settings.

Thus autophagosomes of CGCs treated with proteasome inhibitors contain ubiquitinated neurofilaments suggesting that additional and different mechanisms operate to induce formation of autophagosomes in CGCs undergoing apoptosis in the presence of proteasome inhibitors. Moreover, preliminary

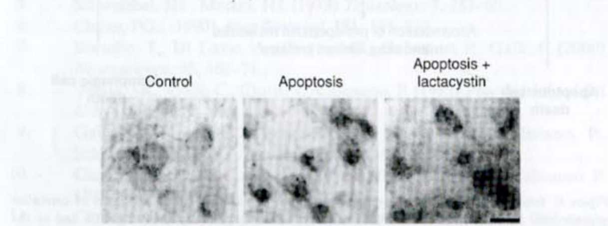


Figure 5. Staining of autophagosomes performed with monodansyl cadaverine, a specific marker of these vescicles, in CGCs undergoing apoptosis in the absence or in the presence of lactacystin. Notice that this proteasome inhibitor does not abolish the autophagosome proliferation that occurs during apoptosis. Bar: 7 µ

15. Young, F M., Illingworth, PJ., and Fraser, HM (1998) J. *Reprod. Fertil.* **114**, 163–168.
16. Sadoul, R., Fernandez, PA., Quiquerez, AL., Martinou, I., Maki, M., Schroter, M., Becherer, JD., Irmler, M., Tschopp, J., Martinou, JC. (1996) *EMBO. J* **15**, 3845–3852.
17. Grimm, LM., Goldberg, AL., Poirier, GG., Schwartz, LM., Osborne, BA. (1996) *EMBO J.* **15**, 3835–3844.
18. Sawada, H., Kohno, R., Kihara, T., Iz, Y., Sakka, N., Ibi, M., Nakanishi, M., Nakamizo, T., Yamakawa, K., Shibasaki, H., Yamamoto, N., Akaike, A., Inden, M., Kitamura, Y., Taniguchi, T., Shimohama S (2004) *J Biol Chem.* **279**, 10710–9.
19. Phillips, JB., Williams, AJ., Adams, J., Elliott, PJ., Tortella, FC. (2001) *Stroke.* **31**, 1686–93.
20. Atlante, A., Bobba, A., Calissano, P., Passarella, S., Marra, E. (2003) *J Neurochem.* **84**, 960–71.
21. Canu, N., Dus, L., Barbato, C., Ciotti, MT., Brancolini, C., Rinaldi, AM., Novak, M., Cattaneo, A., Bradbury, A., Calissano, P (1998) *J Neurosci.* **18**, 7061–74.
22. Schulz, JB., Welle, M., Klockgether, T. (1996) *J Neurosci.* **16**, 4696–706.
23. Bobba, A., Canu, N., Atlante, A., Petragallo, V., Calissano, P., Marra, E. (2002) *FEBS Lett.* **515**, 8–12.
24. Hirsch, T., Dallaporta, B., Zamzami, N., Susin, SA., Ravagnan, L., Marzo, I., Brenner, C., Kroemer, G. (1998). *J Immunol.* **161**, 35–40.
25. Yang, Y., Fang, S., Jensen, JP., Weissman, AM., Ashwell, JD (2000) *Science* **288**, 874–7.
26. Kroesen, BJ, Jacobs, S., Pettus, BJ., Sietsma, H., Kok, JW., Hannun, YA., de Leij, LF (2003) *J Biol Chem.* **278**, 14723–31.
27. He H., Qi X. M., Grossmann J., Distelhorst C. W (1998) J. *Biol. Chem.* **273**, 25015–9.
28. Ivanov V. N., Nikolic-Zugic J. (1998) *Int. Immunol.* **10**, 1807–17.
29. Saitoh T, Nakayama M, Nakano H, Yagita H, Yamamoto N, Yamaoka S (2003) *J Biol Chem.* **278**, 36005–12.
30. Grassilli E., Benatti F., Dansi P., Giammarioli A. M., Malorni W., Franceschi C., Desiderio M. A (1998) *Biochem. Biophys. Res. Comm.* **250**, 293–7
31. Bobba, A., Atlante, A., Giannattasio, S., Sgaramella, G., Calissano, P., Marra, E. (1999) *FEBS Lett.* **457**, 126–30.
32. Cao, C., Leng, Y., Liu, X., Yi, Y., Li, P., Kufe, D. (2003) *Biochemistry* **42**, 10348–53.
33. Hoffman, EK., Wilcox, HM., Scott, RW., Siman, R. (1996) *J Neurol Sci.* **139**:15–20.
34. Suzuki, Y., Nakabayashi, Y., Takahashi, R. (2001) *Proc Natl Acad Sci U S A.* **98**, 8662–7
35. Palombella, VJ., Rando, OJ., Goldberg, AL., Maniatis, T. (1994). *Cell.* **78**, 773–85.
36. Traenckner, E.B., Wilk, S., and Baeuerle, P.A. (1994) *EMBO J.* **13**; 5433–41.

37. Kovacs, AD., Chakraborty-Sett, S., Ramirez, SH., Sniderhan, LF., Williamson, AL., Maggirwar, SB. (2004) *Eur J Neurosci.* **20**, 345–52.
38. Stephenson, D., Yin, T., Smalstig, E.B., Hsu, M.A., Panetta, J., Little, S., and Clemens, J. (2000) *J Cereb Blood Flow Metab.* **20**, 592–603
39. Aleyasin, H., Cregan, SP., Iyirhiaro, G., O'Hare, M.J., Callaghan, S.M., Slack, R.S., Park, D.S. (2004). *J Neurosci* **24**, 2963–73
40. Maggirwar, S,B,, Sarmiere, P.D., Dewhurst, S., and Freeman, R.S. (1998) *J Neurosci* **18**, 10356–65.
41. Fridmacher, V., Kaltschmidt, B., Goudeau, B., Ndiaye, D., Rossi, F.M, Pfeiffer, J., Kaltschmidt, C., Israel, A., and Memet, S. (2003) *J Neurosci* **23**, 9403–8
42. Culmsee, C., Siewe, J., Junker, V., Retiounskaia, M., Schwarz, S., Camandola, S., El-Metainy, S., Behnke, H., Mattson, M.P., and Krieglstein, J. (2003) *J Neurosci* **23**, 8586–95
43. Lin, K.I., Baraban J.M., and Ratan, R.R. (1998) *Cell Death Differ.* **5**, 577–83
44. Pagano, M., Tam, SW., Theodoras, AM., Beer-Romero, P., Del Sal, G., Chau, V., Yew, PR., Draetta, GF., Rolfe, M. (1995) *Science.* **269**:682–5.
45. Padmanabhan, J., Park, DS., Greene, LA., and Shelanski, ML (1999) *The Journal of Neurosci.* **19**, 8747–8756.
46. O'Hare, MJ., Hou, ST., Morris, EJ., Cregan, SP., Xu, Q., Slack, RS., Park, DS (2000) *J Biol Chem.* **275**, 25358–64.
47. Arizti, P., Arribas, J., Castano, JG. (1993) *Enzyme Protein.* **47**, 285–95.
48. Vucic, D., Kaiser, WJ., Harvey, AJ., Miller, LK. (1997) *Proc Natl Acad Sci U S A.* **94**, 10183–8.
49. Vaux, DL., Silke, J. (2003) *Biochem Biophys Res Commun.* **304**:499–504.
50. Machiels, BM., Henfling, ME., Schutte, B., van Engeland, M., Broers JL, and Ramaekers FC. (1996) *Eur. J. Cell Biol.* **70**, 250–259
51. Pitzer, F., Dantes, A., Fuchs, T., Baumeister, W., Amsterdam, A. (1996) *FEBS lett.* **394**, 47–50.
52. Amsterdam, A., Dantes, A., Selvaraj, N., and Aharoni, D. (1997) *Steroids* **62**, 207–211
53. Beyette, J., Mason, GG., Murray, R.Z., Cohen, GM., and Rivett, AJ. (1998) *Biochem. J.* **332**, 315–320.
54. Heinemeyer, W., Kleinschmidt, JA., Saidowsky, J., Escher, C., Wolf, DH. (1991) *EMBO J.* **10**, 555–62.
55. Figueiredo-Pereira, ME., Berg, KA., Wilk, S. (1994) *J Neurochem* **63**, 1578–1581.
56. Soldatenkov, VA., Dritschilo, A. (1997) *Cancer Res.* **18**, 3881–5
57. Sun, XM., Butterworth M., MacFarlane, M., Dubiel W., Ciechanover, A., Cohen, GM. (2004) *Mol Cell.* **14**, 81–93.
58. Alkalay, I., Yaron, A., Hatzubai, A., Orian, A., Ciechanover, A., Ben-Neriah, Y. (1995) *Proc Natl Acad Sci U S A.* **92**, 10599–603.
59. Piccioli, P., Porcile, C., Stanzione, S., Bisaglia, M., Bajetto, A., Bonavia, R., Florio, T., Schettini, G. (2001) *J Neurosci Res.* **66**, 1064–73.
60. Porcile, C., Piccioli, P., Stanzione, S., Bajetto, A., Bonavia, R., Barbero, S., Florio, T., Schettini, G. (2002) *Ann N Y Acad Sci.* **973**, 402–13.

61. Bence, NF., Sampat, RM., Kopito, RR (2001) **292**, 1552–5.
62. Tanaka, M., Kim, YM., Lee, G., Junn, E., Iwatsubo, T., Mouradian, MM. (2004) *J Biol Chem.* **279**, 4625–31
63. Wood, JD., Beaujeux, TP., Shaw, PJ. (2003) *Neuropathol Appl Neurobiol.* **29**, 529–45.
64. Hoshi, M., Sato, M., Matsumoto, S., Noguchi, A., Yasutake, K., Yoshida, N., Sato, K (2003) *Proc Natl Acad Sci U S A.* **100**, 6370–5.
65. Adrain, C., Creagh, EM., Cullen, SP., Martin, SJ. (2004) *J Biol Chem.* **279**, 36923–30
66. Ferrell, K., Wilkinson, CR., Dubiel, W., and Gordon, C. (2000) *Trends Biochem Sci.* **25**, 83–8
67. Glickman, MH., Ciechanover, A. (2002) *Physiol Rev.* **82**, 373–428.
68. Deveraux, Q., Ustrell, V., Pickart, C., Rechsteiner, M. (1994) *J Biol Chem.* **269**, 7059–61.
69. Lam, YA., Lawson, TG., Velayutham, M., Zweier, JL., Pickart, CM. (2002) *Nature* **416**, 763–7.
70. Elsasser S., Gali RR., Schwickart M., Larsen CN., Leggett DS., Muller B., Feng MT., Tubing F., Dittmar GA., Finley D. (2002) *Nat Cell Biol.* **9**,725–30.
71. Saeki, Y., Sone, T., Toh-e A., Yokosawa, H. (2002) *Biochem Biophys Res Commun.* **296**, 813–9.
72. Mimnaugh, EG., Kayastha, G., McGovern, NB., Hwang, SG., Marcu, MG., Trepel, J., Cai, SY., Marchesi, VT., Neckers, L. (2001) *Cell Death Differ* **8**, 1182–1196.
73. Wilkinson, K. (1997) *FASEB J.* **11**, 1245–1246
74. Leroy, E., Boyer, R., Auburger, G., Leube, B., Ulm, G., Mezey, E., Harta, G., Brownstein, MJ., Jonnalagada, S., Chernova, T., Dehejia, A., Lavedan, C., Gasser, T., Steinbach, PJ., Wilkinson, KD., Polymeropoulos, MH. *Nature* **395**, 451–2.
75. Hadari, T., Warms, JV., Rose, IA., Hershko, A. (1992) *Biol Chem.* **276**, 719–27.
76. Amerik, Ayu., Swaminathan, S., Krantz, BA., Wilkinson, KD., Hochstrasser, M. (1997) *EMBO J.* **16**, 4826–38.
77. Figueiredo-Pereira, ME., Yakushin, S., Cohen, G (1998) *J Biol Chem* **273**, 12703–12709
78. Alavez, S., Pedroza, D., Moran J. (2000) *Neurochem Res.* **25**, 341–7.
79. Anglade, P., Vyas, S., Hirsch, EC., Agid, Y. (1997) *Histol Histopathol.* **12**, 603–10.
80. Xue, L., Fletcher, GC., Tolkovsky, AM. (1999) *Mol Cell Neurosci.* **14**, 180–98.
81. Guimaraes CA, Benchimol M, Amarante-Mendes GP, Linden R. (2003) *J Biol Chem.* **278**, 41938–46.
82. Uchiyama, Y. (2001) *Arch Histol Cytol.* **64**, 233–46.

9

PATHWAYS OF NEURONAL CELL DEATH INDUCED BY PROTEASOMAL INHIBITION

Isabelle Lang-Rollin and Leonidas Stefanis

1. INTRODUCTION

Neuronal cell death occurs normally in the developing nervous system. A term that has been used to describe this death is Programmed Cell Death (PCD). The predominant morphological form of neuronal PCD is that of apoptosis, although other morphologies do occur. Neuronal cell death also occurs during various disease states in the nervous system, either following acute, subacute insults, such as trauma or stroke, or more chronic insults, such as those that take place during neurodegenerative diseases. There is substantial evidence that in animal models that mimic such conditions, as well as in diseased human tissue, elements of PCD, either apoptotic or non-apoptotic, are activated. The classical morphological features of apoptosis correspond to a defined biochemical pathway, whose signature event is the activation of the cysteine proteases caspases. Other morphologies of neuronal cell death, such as autophagy and necrosis, may also be elicited following the application of injurious stimuli to the nervous