PROTEASOME IMPAIRMENT, MITOCHONDRIA DYSFUNCTION, AND TAU PATHOLOGY IN ALZHEIMER DISEASE

by

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Advisor: Dr. Maria E. Figueiredo-Pereira

ABSTRACT

Impairment of the ubiquitin/proteasome pathway is implicated in the pathogenesis of many neurodegenerative disorders, such as Alzheimer disease (AD). This is supported by detection of ubiquitinated protein aggregates in neurofibrillary tangles (NFTs) (Ross and Poirier, 2004) and reduction of proteasome activity in autopsied brains from AD patients (Keller et al., 2000). However, the upstream events leading to impairment of the ubiquitin/proteasome pathway are not fully understood. The ubiquitin/proteasome pathway is a highly regulated and efficient pathway that is critical for degradation of most intracellular proteins (Ciechanover, 2005).

Ubiquitination, proteasome assembly and proteasome activity are energy dependent processes that require ATP binding and ATP hydrolysis (Li and Demartino, 2009). In neurons, most ATP is generated by mitochondria. Emerging evidence implicates mitochondrial dysfunction and energy deficit in many neurodegenerative disorders such as AD (Lin and Beal, 2006).

There are no current treatments that are neuroprotective in the sense that they slow or prevent further neurodegeneration in AD. To provide new insights for the development of novel and more effective therapeutic strategies that prevent/stop neurodegeneration in AD, the MAJOR AIMS of these studies were:

(1) Investigate underlying mechanisms that link mitochondrial dysfunction to impairment of the ubiquitin/proteasome pathway.

(2) Evaluate the protective effect of elevating cAMP against an endogenous product of inflammation, i.e. prostaglandin J2 that affects the ubiquitin/proteasome pathway.

In our studies we used rat E18 cerebral cortical neuronal cultures to investigate the pathogenesis of AD.

For the FIRST AIM, we mimicked mitochondrial dysfunction by treating neurons with the following mitochondrial toxins: (1) oligomycin which inhibits ATP synthase, (2) rotenone which

inhibits mitochondrial complex I, and (3) antimycin which inhibits mitochondrial complex III. We demonstrate that mitochondrial impairment affects the ubiquitin/proteasome pathway in the neurons via:

(1) Reducing polyubiquitinated protein levels by inhibiting the first step of the ubiquitination cascade that is ATP-dependent: ubiquitin adenylation by the enzyme E1, thus blocking E1-E2 transthiolation.

(2) Downregulating 26S proteasomes by promoting their disassembly. Notably, we show for the first time to our knowledge, that calpain activation is one of the factors that contributes to 26S proteasome disassembly by selectively cleaving one of its subunits, Rpn10. Calpain activation is associated with necrosis, an energy-independent cell death pathway activated upon mitochondrial dysfunction. We also demonstrate that calpain cleaves pro-caspase 3 to an inactive fragment, most likely to prevent apoptosis that is an energy-dependent cell death pathway. Furthermore, calpain cleaved the microtubule associated protein TAU that is the major component of neurofibrillary tangles in AD and other tauopathies.

(3) Promoting a rise in 20S proteasomes, which degrade most oxidized proteins as well as intrinsically disordered proteins in a ubiquitin- and energy-independent manner.

(4) Finally, we show that down-regulation of ubiquitinated proteins and 26S proteasomes with a concomitant increase in 20S proteasomes, occur upon acute (up to 16h) and long-term (up to seven days) mitochondrial dysfunction in the neurons.

Overall, these data support the notion that upon mitochondrial dysfunction, neurons initiate a series of mechanisms that converge to optimize unregulated and energy-independent turnover of randomly unfolded oxidized proteins by 20S proteasomes. This adaptive response to energy deficiency may be suitable for short-term periods. However, if maintained chronically it may lead to neurodegeneration, as regulated protein degradation by the ubiquitin/proteasome pathway is essential for neuronal survival.

For the SECOND AIM, we treated rat E18 cerebral cortical neurons with prostaglandin J2 (PGJ2), an endogenous product of inflammation that affects mitochondrial function, and inhibits the 26S proteasome by causing its disassembly. We compared the effects of PGJ2 with those of the specific and irreversible proteasome inhibitor epoxomicin. We show that treatment with both drugs induces caspase-activation, TAU cleavage at Asp421 (ΔTAU), TAU and Ub-protein aggregation, and neuronal death. Truncation of TAU at Asp421 (ΔTAU) by caspases is an early event in AD

tangle pathology. In addition, ΔTAU is detected in neurofibrillary tangles (NFTs) indicating that the apoptotic cascade is involved in NFT formation.

To prevent these deleterious events associated with proteasome inhibition, we tested two drugs that elevate intracellular cAMP: dibutyryl-cAMP (db-cAMP) and the lipophilic peptide PACAP27 that increases intracellular cAMP levels by binding to its seven transmembrane G-coupled receptor PAC1R. Previous studies from our lab showed that a single dose of db-cAMP or PACAP27, blocked caspase-activation and ΔTAU upon short-term (up to 8h) treatment with PGJ2. One single dose of these cAMP-elevating drugs however, failed to protect against longer (24h) treatments with PGJ2. In our current studies, we decided to increase the number of drug treatments to improve protection. We established that three doses of either db-cAMP or PACAP27 successfully diminished caspase-activation, ΔTAU and loss of cell viability promoted by long-term (24h) incubations, as long as these changes were mild. Based on these studies, we propose that targeting cAMP/PKA to boost proteasome activity in a sustainable manner, could offer an effective approach to avoid early accumulation of ubiquitinated proteins and later caspase-activation and TAU cleavage, possibly preventing/ delaying neurodegeneration in AD.

In summary, our data address two distinct death pathways in neurons that are associated with mitochondria and proteasome impairment: necrosis via calpain activation and apoptosis via caspase activation. We show that on one hand, mitochondria-dependent energy deficit affects ubiquitination and 26S proteasomes, and promotes cleavage of pro-caspase 3 and TAU via calpain activation. These events are associated with necrotic neuronal death. On the other hand, proteasome impairment induces the accumulation of ubiquitinated proteins and promotes TAU cleavage via caspase 3 activation. These events are associated with neuronal apoptotic death. The dual vulnerability of neurons to calpain- and caspase-mediated cell death needs to be taken into consideration when considering therapeutic approaches to prevent neuronal demise in AD and other chronic neurodegenerative diseases associated with proteasome and mitochondria dysfunction.

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Qian Huang

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LIST OF ABBREVIATIONS

ΔTau, Tau cleaved at Asp421

AD, Alzheimer disease

AIRAP, Arsenite-inducible RNA-associated protein

ALS, Amyotrophic lateral sclerosis

ATP, adenosine triphosphate

cAMP, cyclic adenosine monophosphate

CDDO, 2-Cyano-3,12-dioxooleana-1,9-dien-28-oic Acid

CHIP, Carboxyl terminus of Hsc 70-interacting protein

CNS, Central nervous system

DALIS, Dendritic cell aggresome-like induced structures

db-cAMP, dibutyryl-cAMP

DMSO, dimethyl sulfoxide

DriPs, Defective ribosomal products

DTT, dithiothreitol

DUB, deubiquitinating enzyme

E1, Ubiquitin-activating enzyme

E2, Ubiquitin-conjugating enzyme

E3, Ubiquitin ligase

E18, embryonic day number 18

ECL, enhanced chemiluminescence

Gad, Gracile axonal dystrophy

GFP, Green fluorescent protein

HD, Huntington's disease

Hsp, Heat shock protein

LC3, Light chain 3

MJD, Machado-Joseph disease

MTOC, Microtubule organizing center

MTT, 3-(4,5-dimethylthiazol

-2-yl)-2,5-diphenyl tetrazolium bromide

NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells

NFT, Neurofibrillary tangle

Nrf2, Nuclear factor-erythroid 2-related factor 2

NSAID, Non steroidal anti inflammatory drug

PA, Proteasome activator

PACAP, pituitary adenylate cyclase, activating polypeptide

PAGE, polyacrylamide gel electrophoresis

PD, Parkinson's disease

PGJ2, Prostaglandin J2

PINK1, PTEN-induced kinase 1

PolyQ Polyglutamine

PKA, cAMP-dependent protein kinase

POMP, Proteasome maturation protein

PROTACS, Proteolysis targeting chimera molecules

Rpn, 19S Regulatory particle, non ATP-dependent

s.d., standard deviation

SDS, sodium dodecyl sulfate

s.e., standard error

Suc-LLVY-AMC, succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin

UBA, Ubiquitin-associated domain

Ub-conjugates, ubiquitin conjugates

UBL, Ubiquitin-like domain

UCH-L1, Ubiquitin carboxyl-terminal hydrolase-L1

UDP, Ubiquitin-domain proteins

UIM, Ubiquitin-interacting motif

UPP, Ubiquitin proteasome pathway

CHAPTER I

INTRODUCTION

UBIQUITIN/PROTEASOME PATHWAY IMPAIRMENT IN NEURODEGENERATION: THERAPEUTIC IMPLICATIONS

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1.1. **ABSTRACT**

The ubiquitin/proteasome pathway is the major proteolytic quality control system in cells. In this review we discuss the impact of a deregulation of this pathway on neuronal function and its causal relationship to the intracellular deposition of ubiquitin protein conjugates in pathological inclusion bodies in all the major chronic neurodegenerative disorders, such Alzheimer's, Parkinson's and Huntington's diseases as well as amyotrophic lateral sclerosis. We describe the intricate nature of the ubiquitin/proteasome pathway and discuss the paradox of protein aggregation, i.e. its potential toxic/protective effect in neurodegeneration. The relations between some of the dysfunctional components of the pathway and neurodegeneration are presented. We highlight possible ubiquitin/proteasome pathway-targeting therapeutic approaches, such as activating the proteasome, enhancing ubiquitination and promoting SUMOylation that might be important to slow/treat the progression of neurodegeneration. Finally, a model time line is presented for neurodegeneration starting at the initial injurious events up to protein aggregation and cell death, with potential time points for therapeutic intervention.

1.2. INTRODUCTION

Intracellular proteolysis is a very powerful mechanism that shapes the proteome following exposure to different stress conditions. The removal of misfolded or damaged proteins is critical to recovery from adverse conditions to ensure optimal cell survival. In the cytosol and nucleus, the major proteolytic pathway used by eukaryotic cells for disposing of misfolded or damaged proteins is the ubiquitin/proteasome pathway (UPP) (Ciechanover, 2005). Following re-translocation into the cytosol, misfolded endoplasmic reticulum (ER) proteins are also degraded by the UPP. In mammalian cells it is estimated that 80-90% of protein degradation is carried out by the UPP (Lee and Goldberg, 1998). In addition, ubiquitin is likely to be used for targeting most substrates for degradation (Yewdell, 2001), since it is one of the most abundant proteins in cells present at 10^8 copies per cell (~5% of cell bulk protein) (Haas and Bright, 1985). Mitochondria and endolysosomes have their own proteolytic systems, but they account for a small percentage (10-20%) of overall protein degradation (Gronostajski et al., 1985).

Non-functional intracellular proteins must be disposed of quickly by the UPP to prevent cell damage caused by abnormal protein aggregation and/or inappropriate association with other proteins. In HeLa cells it was estimated that proteasomes are quite abundant constituting ~0.6% of the cell bulk protein and

have a half life of approximately 5 days (Hendil, 1988). Proteasomes in HeLa cells degrade defective ribosomal products (DriPs) at a rate of $1x10^6$/min, and proteins that make it to the cellular pool at a slower rate, i.e. $6x10^5$/min (Yewdell, 2001). Similar numbers were obtained for L929 cells (Princiotta et al., 2003), but proteasomal degradation rates for non-mitotic cells, such as neurons, remain to be established. It is clear that regardless of cell type, proteins defective in folding and/or intracellular location or that are not able to find a suitable partner must be turned over by the UPP quickly enough to prevent cellular demise. The UPP is thus an extremely dynamic pathway that plays a critical role in the intracellular quality control process to prevent cell death caused by proteotoxicity.

Besides removing abnormal and toxic proteins generated by a lifetime of environmental damage, the UPP controls many key cellular mechanisms critical for cell viability and function, such as cell cycle and division, signal transduction, development and transcription factor regulation (Ciechanover, 2005). In general, high levels of ubiquitinated proteins do not accumulate in healthy cells as they are rapidly degraded by the UPP. The function of the UPP can be impaired by many factors including the aging process, leading to the formation of ubiquitin protein aggregates detected in non-pathologic aging as well as in many neurodegenerative disorders, such as Alzheimer's

(AD), Parkinson's (PD) and Huntington's (HD) diseases as well as amyotrophic lateral sclerosis (ALS) to name a few [reviewed in (Alves-Rodrigues et al., 1998)]. The inability to eliminate ubiquitinated proteins can result not only from a functional failure of the UPP but also from structural changes in the protein substrates which render them inaccessible to the degradation component. The UPP plays a critical role in cellular processes such as oxidative stress, inflammation and apoptosis, all of which are implicated in abnormal protein deposition and cell death in neurodegeneration.

1.3. UBIQUITIN/PROTEASOME PATHWAY

There is a rising interest in the UPP as a pharmacological target to prevent/treat neurodegeneration. We summarize below the most important aspects of this pathway. Readers interested in more details are referred to several excellent recent reviews on the pathway in general (Jung et al., 2009b) and in the nervous system in particular (Segref and Hoppe, 2009).

1.3.1. Ubiquitin and related enzymes

Ubiquitin is a small protein of 76 amino acids, which can form polyubiquitin chains at seven lysine residues: K6, K11, K27, K29, K33, K48 and K63. These chains are formed by the successive attachment of monomers by an isopeptide bond, most

frequently formed between the side chain of Lys48 in one ubiquitin and the carboxyl group of the C-terminal Gly76 of a neighboring ubiquitin. Attachment of K48 polyubiquitin chains to lysine residues on a protein results in at least a 10-fold increase in its degradation rate (Beal et al., 1996). Polyubiquitin chains with linkages involving lysine residues on ubiquitin other than K48 were found to play distinct roles, including DNA repair, activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), polysome stability and endocytosis [reviewed in (Hochstrasser, 2009)].

Ubiquitination of proteins is a complex process involving the following sequence of events: Initially, a high energy thioester bond is formed between ubiquitin and the ubiquitin-activating enzyme E1, an ATP-dependent reaction. Secondly, a thioester bond is formed between the activated ubiquitin and ubiquitin-conjugating enzymes (E2). Thirdly, the carboxyl terminal of ubiquitin is covalently attached to the ε-amino group of a lysine residue on protein substrates via an isopeptide bond. This reaction is carried-out by ubiquitin ligases (E3) that confer substrate specificity to the UPP [review in (Deshaies and Joazeiro, 2009)]. Finally, another family of ubiquitination factors (E4) assembles the multiubiquitin chain generating longer ubiquitin-chains (Koegl et al., 1999). In some cases, E2, instead of E3 ubiquitin ligases, transfer directly

ubiquitin to the substrate.

Removal of ubiquitin from ubiquitinated proteins and disassembly of polyubiquitin chains is mediated by de-ubiquitinating enzymes. There are more than 90 genes encoding de-ubiquitinating enzymes making them one of the largest family of enzymes of the ubiquitin pathway [reviewed in (Reyes-Turcu et al., 2009)]. De-ubiquitinating enzymes are divided into two major classes: The first class includes the ubiquitin carboxyl-terminal hydrolases that remove small amides, esters, peptides and small proteins at the carboxyl terminus of ubiquitin. The second class comprises ubiquitin-specific processing proteases which disassemble the polyubiquitin chains and edit the ubiquitination state of proteins.

The state of substrate ubiquitination depends on the balance between ubiquitinating and de-ubiquitinating enzymes acting on it. Thus, cells developed a highly dynamic strategy based on a switch-on/switch-off type of mechanism that responds promptly to cellular requirements for proteolysis by the UPP.

1.3.2. 26S proteasome and substrate recognition

Covalent binding of polyubiquitin chains to proteins targets them for degradation by the 26S proteasome, a large enzymatic complex with a native molecular mass of approximately 2000 kDa [reviewed in (Tanaka, 2009)]. The 26S proteasome

contains ATPase and proteolytic components. The ATPase component, also known as the 19S particle, contains at least 19 subunits and can be further divided into two subcomplexes: the lid and the base [reviewed in (Finley, 2009)]. The lid harbors receptors for polyubiquitin chains as well as de-ubiquitinating activity. The hexameric ATPases Associated with diverse cellular Activities (AAA^+ ATPase) base unfolds the substrates and translocates the polypeptides into the proteolytic chamber. Unfolding and threading the substrate through the catalytic barrel are rate limiting steps in proteasomal degradation.

The proteolytic component of the 26S proteasome, known as the 20S proteasome, is a cylinder-like structure only long enough to accommodate ~50 residues of a fully extended polypeptide, although a 70kDa globular protein can fit into the chamber (Rechsteiner et al., 2000). The eukaryotic 20S proteasome consists of 28 subunits arranged in four heptameric stacked rings as $\alpha_7\beta_7\beta_7\alpha_7$ and forming a barrel-shaped structure [reviewed in (Marques et al., 2009)]. The seven α-type subunits provide binding sites for regulatory particles and form the gated channel leading to the inner proteolytic chamber. Among the seven β-type constitutive subunits, only three of them bear active sites for peptide bond hydrolysis, namely β1, β2, and β5,

which are responsible for the caspase-like, trypsin-like, and chymotrypsin-like activities, respectively (Groll et al., 1997).

During degradation of one substrate molecule by the 26S proteasome 300–400 ATP molecules are hydrolyzed. ATP hydrolysis is activated upon substrate binding and promotes three events: substrate unfolding, 20S proteasome gate opening, and protein translocation (Benaroudj et al., 2003). Substrate binding to the 19S particle stabilizes gate opening and facilitates substrate channeling and their access to active sites in the 20S proteasome (Bech-Otschir et al., 2009). ATP is also required for rapid association/dissociation of the 26S proteasome from/into the 19S particle and 20S proteasome (Babbitt et al., 2005). While the 26S proteasome degrades polyubiquitinated proteins, the 20S proteasome may be sufficient to degrade oxidatively modified non-ubiquitinated proteins in an ATP-independent manner (Orlowski and Wilk, 2003;Grune and Davies, 2003).

Degradation of ubiquitinated proteins by the 26S proteasome is enhanced when more than one ubiquitin is attached to the target protein. The minimal signal for efficient degradation is a tetraubiquitin chain (Thrower et al., 2000). Removal of two ubiquitins from a tetraubiquitinated substrate by de-ubiquitinating enzymes can decrease substrate/26S proteasome affinity by approximately 100-fold, allowing the substrate to

escape degradation. Longer chains do not increase substrate/26S proteasome affinity, but optimize their binding time. The interaction of the polyubiquitin chain with the 26S proteasome involves hydrophobic patches on the surface of the tetraubiquitin chain, generated by Leu8, Ile44, and Val70 in each ubiquitin moiety, and two hydrophobic sequences with the motif LeuAlaLeuAlaLeu in subunit Rpn10 of the 19S particle (Young et al., 1998). Rpn13 is another subunit of the 19S particle identified as a receptor for polyubiquitinated proteins (Husnjak et al., 2008). Rpn13 is also a receptor for the deubiquitinating enzyme Uch37, suggesting that chain recognition and disassembly are coupled at the proteasome (Husnjak et al., 2008).

Three key factors target proteins for ubiquitination/ degradation: *(1)* misfolding due to mutation or damaging events; *(2)* constitutively active ubiquitination signals; and *(3)* post-translational modifications such as phosphorylation/ dephosphorylation events or co-factor binding. The unfolding of normal substrates precedes their degradation. This step is required to allow entry into the proteolytic chamber of the 20S proteasome through its narrow opening (Thrower et al., 2000). Unfolding activities are provided by ATPase subunits in the base of the 19S particle or by extraproteasomal chaperones. In fact, proteasomes can be recruited to ubiquitinated substrates in

conjunction with molecular chaperones (Johnston et al., 1998).

1.4. Delivery of substrates to the proteasome

One of the most extraordinary features of mammalian cells is how crowded they are, raising the question of how proteasomes encounter their substrates. In the cytoplasm, proteasomes can be found free, associated with cytoskeletal elements or bound to the endoplasmic reticulum [reviewed in (Wojcik and DeMartino, 2003)]. Proteasomes are slowly and unidirectionally transported from the cytoplasm into the nucleus but can rapidly diffuse within each compartment (cytoplasm or nucleus) without encountering selective barriers (Reits et al., 1997). Thus it was proposed that proteasomes perform quality control by continuously colliding with their substrates, degrading those that are specifically tagged or misfolded (Reits et al., 1997).

Polyubiquitinated proteins can be directly recognized by Rpn10/Rpn13 subunits of the 19S particle. However, multiple shuttling factors other than proteasome subunits were shown to bind polyubiquitinated proteins and deliver them to proteasomes for degradation [reviewed in (Welchman et al., 2005;Elsasser and Finley, 2005)]. Most of these shuttling receptors contain two important domains: *(1)* at the N-terminus a ubiquitin-like domain, such as UBL or UBX, that binds to proteasomes, and *(2)* at the C-terminus a ubiquitin-binding domain, such as ubiquitin-

interacting motifs (UIM) or ubiquitin-associated domain (UBA), that binds polyubiquitinated proteins. The sequestosome 1/p62 (p62/sqstm1), Rad23 and DSK2 are examples of such shuttling factors. These UBL (or UBX)-UBA shuttling factors can alternatively deliver substrates to Cdc48 complexes for unfolding. Cdc48 complexes comprise six identical subunits with two AAA^+ ATPase domains, where substrates can undergo unfolding before being shuttled to the proteasome for degradation. Other proteins, such as Parkin, VHL and CHIP, are ubiquitin ligases that have their own UBL domain for proteasome binding, or associate with partners that have one. This dual function (ubiquitination and/or proteasomal binding) within the same molecule or complex provides a means to respond promptly to specific cellular proteolytic requirements, by ubiquitinating and quickly delivering substrates to proteasomes. These multiple direct and indirect proteasome delivery mechanisms provide for another level of selectivity needed for regulated protein degradation by the UPP.

1.5. Paradox of protein aggregation: toxicity or protection

All major human chronic neurodegenerative diseases, such as PD, AD, HD and ALS, are characterized by ubiquitinated proteins that accumulate in abnormal intraneuronal inclusions in the respective affected areas of the CNS [reviewed in (Mayer, 2003)].

The mechanisms leading to the formation of such abnormal aggregates remain elusive. Surprisingly, the degradation rate of mature post-synthetically damaged proteins is not significantly affected by insults such as oxidative stress or increased temperature (Medicherla and Goldberg, 2008). However, degradation of newly synthesized proteins is highly affected by stress. Newly synthesized proteins go through a "fragile period" during which they are highly sensitive to degradation by the UPP. Its impairment induces accumulation of ubiquitinated proteins that develop into protein aggregates reminiscent of those detected in many neurodegenerative diseases. The role of these abnormal protein deposits in the progression of neurodegeneration is controversial. Inclusions may arise from a cellular attempt to compartmentalize accumulated proteins preventing their interference with normal cell function. Their presence may also confer cytotoxic effects that can contribute to neuronal cell damage. We will discuss some aspects of this controversy below.

1.5.1. Protein overexpression concern

Many studies addressing the effects and mechanisms involved in protein aggregation in neurodegeneration do not focus on the endogenous proteome. Instead, they rely on high and unregulated overexpression of different kinds of proteins in cells as well

as in transgenic animals, an approach that may lead to artifacts because it bypasses the internal mechanisms regulating the UPP. For example, expression of green fluorescent protein (GFP) or a GFP fused to ubiquitin caused by itself the formation of ubiquitin positive aggregates in transfected HeLa and RAW cells (Szeto et al., 2006). The transfection reagent (FuGene 6) alone was sufficient to induce aggregate formation, but to a lesser extent. Furthermore, GFP-like proteins transfected into mammalian cells show a tendency to oligomerize and aggregate into punctuate structures (Katayama et al., 2008). These structures are not cytosolic aggregates, instead they are lysosomes containing the aggregated proteins. Hence, formation of ubiquitin positive-aggregates caused by overexpressing non-resident proteins or by the transfection reagents themselves may be misleading.

In HEK 293 cells, protein aggregates generated by the transient overexpression of huntingtin or cystic fibrosis transmembrane conductance regulator (CFTR) aggregation-prone fragments were shown to directly inhibit the UPP (Bence et al., 2001). It is possible that the UPP would be inhibited by the overexpressed proteins even if they did not aggregate. Furthermore, it is not clear how cells handle the expression of high levels of proteins that in many instances do not replicate any of the cell's own proteome. For example, huntingtin is a

very large protein. Its molecular weight is ~350kDa. It is possible that expressing GFP (~30kDa), which is less than one tenth of the size of huntingtin, with an expanded polyQ will not behave in the same manner as full length huntingtin. These experimental approaches may not replicate the actual mechanisms involved in highly specific recognition, ubiquitination, deubiquitination and delivery steps that work in concert and regulate the turnover of intracellular proteins by the UPP. It is possible that they do not entirely reflect the natural intracellular responses.

1.5.2. Accumulation predicts degree of cell death independently of aggregation

Studies addressing the role of aggregate formation on cell death rely, in many instances, on transfection and overexpression of wild type or mutant forms of proteins, such as tau (Wang et al., 2009), α-synuclein (Tanaka et al., 2004), parkin (Cookson et al., 2003) or huntingtin (Arrasate et al., 2004), to list a few. In some cases the whole protein or its truncated fragments, as in the case of huntingtin, are fused to GFP. In addition the transfected cells, in many instances, are treated with proteasome inhibitors (Tanaka et al., 2004) or maintained in serum-free media which induces autophagy and/or apoptosis (Arrasate et al., 2004). Some of these studies support

the notion that aggregates are beneficial to the cell (Mitra et al., 2009) and act as "neuron protection agencies" (Orr, 2004).

The above studies involve highly overexpressed proteins that in some cases, do not mimic any intracellular protein, thus data interpretation should take these circumstances into consideration. In addition, it is likely that neurons that exhibit protein aggregates are committed to the death pathway rather than to survival. Many of the dead neurons are cleared by microglia and those that remain with aggregates are probably waiting their turn. This view is supported by the progressive nature of these neurodegenerative disorders, suggesting that groups of neurons gradually die and are cleared. Furthermore, only a few neurons with protein aggregates are detected in brains of control individuals that do not have the disease. It is thus likely that neurons with aggregates reach a point of no return, die and are cleared by microglia.

Several studies demonstrate that aggregate formation does not correlate with neuronal survival. The amount of diffuse, non-aggregated, huntingtin-GFP fusion products accumulating in primary rat neuronal cultures maintained in serum free media was directly related to the degree of cell death (Arrasate et al., 2004). In rat embryonic striatal cells, preventing the formation of intranuclear inclusions containing mutant huntingtin resulted in accelerated cell death (Saudou et al., 1998). Formation of

intranuclear inclusions in the latter study was halted by inhibiting ubiquitination through overexpression of a dominant negative mutant of a ubiquitin conjugating enzyme (hcDc34). This approach most likely caused deregulation of intracellular protein degradation resulting in an abnormal accumulation of intracellular proteins that could not be degraded because they were not ubiquitinated. This phenomenon could lead to proteotoxicity and thus accelerate cell death even in the absence of protein aggregates. Preventing protein aggregation by using RNA interference (RNAi) to silence the expression of p62/sqstm1, a UBA-shuttling protein, failed to protect human neuroblastoma cells from cell death induced by a product of inflammation, prostaglandin J2 (PGJ2) (Wang and Figueiredo-Pereira, 2005). P62/sqstm1knockdown prevented protein aggregation without diminishing the accumulation of endogenous ubiquitinated proteins. Furthermore, the accumulation of ubiquitinated proteins was an early response, suggesting that if cells fail to degrade the high levels of ubiquitinated proteins induced by PGJ2-treatment, apoptosis follows triggering caspase activation and tau cleavage, known to precede tau aggregation and tau pathology (Arnaud et al., 2009).

Together these studies support the notion that intracellular accumulation of proteins, rather than their aggregation, is a pathological event that accelerates cell death.

Therapeutic strategies that promote the degradation of the accumulated proteins rather than preventing their aggregation would be relevant to neurodegenerative disorders associated with protein misfolding.

1.5.3. Cytoskeletal collapse: an alternative to aggresomes

Pericentriolar structures within centrosomes are deposition sites for ubiquitinated proteins that escape UPP degradation and were named accordingly "aggresomes" (Johnston et al., 1998). The ubiquitinated proteins deposited in aggresomes resulted from overexpressing mutant CFTR or presenilin 1 or from impairing protein degradation by treating cells with proteasome inhibitors (Johnston et al., 1998;Wigley et al., 1999;Garcia-Mata et al., 1999). Centrosomes are associated with high levels of 26S proteasomes and with de-ubiquitinating activity (Fabunmi et al., 2000). While some studies suggest that the retrograde transport of ubiquitin protein aggregates to centrosomes is dependent on microtubule integrity (Johnston et al., 1998) others indicate that this process does not require intact microtubules (Fabunmi et al., 2000).

There is another possible interpretation for the deposition of aggregates at the centrosome, also known as the microtubule organizing center (MTOC). Formation of ubiquitin-protein aggregates throughout the cell could disrupt the microtubule

network causing microtubules to collapse to their site of origin, i.e. the MTOC. This collapse would cause the re-distribution onto the MTOC of aggregates, proteins and complexes, such as proteasomes, that are associated with or neighbor the cytoskeletal elements. Such events were shown to be induced by the product of inflammation PGJ2 which disrupts the structural integrity of microtubules and actin filaments (Ogburn and Figueiredo-Pereira, 2006). A similar model for the localization of protein aggregates at the MTOC could be applied to toxins such as 1-methyl-4-phenylpyridinium (MPP^+) and rotenone that strongly hinder microtubule polymerization and induce PD symptoms in animal models (Cappelletti et al., 2001;Diaz-Corrales et al., 2005;Marshall and Himes, 1978). These results suggest that preserving microtubule integrity is critical to neuronal homeostasis. Disruption of the microtubule network causes defects in axonal transport, a mechanism common to many neurodegenerative disorders [reviewed in (Roy et al., 2005)].

1.5.4. Reversible aggregates

Reversible intracellular protein aggregates form in maturing dendritic cells in response to protein damaging agents and are known as dendritic cell aggresome-like induced structures (DALIS) (Lelouard et al., 2004). Upon stimulation with the pro-inflammatory agent lipopolysaccharide followed by

treatment with the translation damaging agent puromycin, DRiPs are sorted into DALIS which are large reversible cytosolic aggregates. Unlike aggresomes, DALIS are not localized in the pericentriolar area and lack vimentin cages (Lelouard et al., 2004). In addition, DALIS contain many components of the ubiquitination machinery, including E1, E2s and E3s (Ebstein et al., 2009). When DRiPs are formed they are rapidly sequestered into DALIS where they are eventually ubiquitinated. This mechanism allows dendritic cells to regulate the degradation rate of DRiPs, an ability that is important for their immune functions (Lelouard et al., 2004).

DALIS-like structures are also present in non-dendritic cells and form in response to different types of stresses such as heat shock, the catalase inhibitor 3-amino-1,2,4-triazole (ATZ; causes oxidative stress) and sodium arsenate (uncouples oxidative phosphorylation) (Szeto et al., 2006). These reversible aggregates do not co-localize with proteasomes or γ-tubulin, are inhibited by blocking protein synthesis with cycloheximide, and form in a microtubule- and actin-independent manner confirming that they are not aggresomes.

It is possible that the formation of these two types of reversible aggregates is mediated by intracellular proteins that contain UBA domains [reviewed in (Hartmann-Petersen et al., 2003;Su and Lau, 2009)]. These proteins, such as Mud1, Rad23 and

p62/sqstm1, non-covalently bind polyubiquitin chains 300-times more tightly than mono-ubiquitin (Wilkinson et al., 2001;Madura, 2002). Due to their high affinity for polyubiquitin chains, these UBA-containing proteins could serve as receptors for binding and storing ubiquitinated proteins (Shin, 1998). Reversibly "storing" excessive amounts of polyubiquitinated proteins generated after a stress event could provide an efficient mechanism to regulate substrate access to the proteasome. This "storage" mechanism could prevent proteasomal shutdown by excessive substrate levels. The shuttling of polyubiquitinated proteins from these "storing stations" to the proteasome could be under tight regulation by mechanisms such as phosphorylation and/or ubiquitination (mono and poly). These dynamic aggregates could serve as a microenvironment for the recruitment of multimeric signaling complexes. Notably, protein aggregates were shown to be dynamic structures (Kim et al., 2002). Their composition, including transient association with chaperones, changes under a range of environmental and physiological stresses. These reversible intracellular storage aggregates could thus prevent cell damage and promote cell survival. However, aggregate size could determine their toxicity (Tran and Miller, 1999) as their expansion may interfere with cell function and thus confer fatal effects (Sharma et al., 1999).

1.5.5. Cytoplasmic versus nuclear aggregates

Not all intracellular aggregates with ubiquitinated proteins are cytoplasmic like in PD. Some accumulate in the nucleus, such as in HD. The cause of this differential subcellular aggregate distribution is not clear.

An investigation of the nuclear diffusion limit in mammalian cells, including primary neurons and neuroblastoma cells, established that large molecules (molecular masses above 70kDa) cannot freely diffuse into nuclei of intact, healthy cells (Trushina et al., 2003). It is unlikely that high molecular mass ubiquitin conjugates, most with molecular masses above 75kDa as judged by western blot analysis, passively diffuse from the cytoplasm into the nucleus and vice-versa. In the cell, the size of the aggregates may be even larger as they may be bound to proteins/complexes with UBA domains. The large aggregates could only passively enter the nucleus if the nuclear membrane was disrupted. Accordingly, nuclear migration of full-length mutant huntingtin can only occur upon deterioration of the nuclear membrane (Trushina et al., 2003). Wild type huntingtin is a cytoplasmic protein with a molecular mass of ~350kDa. Neither wild type nor mutant huntingtin have a nuclear targeting signal and thus cannot be actively transported across the nuclear membrane (Trushina et al., 2003). On the other hand, the subcellular distribution of transfected GFP (~30kDa) fused

to full length or truncated forms of wild type or mutant huntingtin is not homogeneous. These fusion proteins can accumulate in the cytoplasm, nucleus or both, depending on the size of the fusion protein, on the nuclear diffusion limit of the specific transfected cells and on their nuclear membrane integrity (Trushina et al., 2003). We conclude that aggregate size, nuclear diffusion limit and nuclear membrane integrity are some of the factors dictating the subcellular distribution of protein aggregates.

1.5.6. Host-to-graft aggregate transmission: ‘‘prion-like’’ properties or toxic microenvironment?

A new premise has emerged for a “prion-like” neuropathological propagation based on recent data involving host-to-graft studies in PD (Kordower et al., 2008a;Kordower et al., 2008b;Li et al., 2008) and HD (Cicchetti et al., 2009) patients receiving neural transplants. In the PD patients α-synuclein-positive Lewy-like inclusions were detected in long term mesencephalon transplants. In the HD cases the grafts suffered disease-like neuronal degeneration. These studies raise concerns that genetically and immunologically unrelated grafts are susceptible to the disease processes as well. To explain this host-to-graft transmission phenomenon, neuronal cells were incubated with media containing polyQ amyloids (Ren et al.,

2009), tau aggregates (Frost et al., 2009) or α-synuclein oligomers (Desplats et al., 2009). Entry of these proteins/peptides into cells most likely through endocytosis, was confirmed by immunofluorescence and/or western blot analyses. The data indicate that protein aggregates can propagate from the outside to the inside of a cell, a phenomenon potentially relevant to many neurodegenerative diseases associated with protein misfolding.

Another possible explanation for the host-to-graft transmission is the potentially toxic microenvironment into which the grafts are transplanted into. It is well established that neuroinflammation is a common denominator among diverse neurodegenerative diseases, such as AD, PD, HD and ALS (McGeer et al., 2005;Bjorkqvist et al., 2008;Boillee et al., 2006) and that chronic neuroinflammation is a critical mechanism responsible for the progressive nature of neurodegeneration. Microglia, the resident innate immune cells in the CNS, produce many factors, such as interleukin-1, tumor necrosis factor α, nitric oxide, PGJ2, superoxide, which are toxic to neurons. The chronic and unregulated activation of microglia in the affected CNS areas into which the grafts are transplanted into, could be responsible for propagating the neuronal injury to the grafts.

Regardless of which mechanisms are responsible for the host-to-graft transmission, this phenomenon raises uncertainty

about this potential therapeutical approach (Desplats et al., 2009). It is important to consider different underlying mechanisms and to explore additional therapeutic paradigms for these devastating disorders.

1.6. UPP dysfunction in neurodegenerative disorders

Most neurodegenerative disorders are associated with formation of protein aggregates, resulting ultimately in proteinaceous inclusions, such as neurofibrillary tangles in AD and Lewy bodies in PD (Lowe et al., 1988a;Lowe et al., 1988b). While the composition of these inclusion bodies varies with the disorder, a general feature is that these aggregates contain ubiquitinated proteins. These inclusions also contain components of the UPP, such as the ubiquitin carboxyl-terminal hydrolase-L1 (UCH-L1) and proteasome subunits. Proteins unrelated to the UPP are also found in these inclusions. For example, α-synuclein is a major component of PD Lewy bodies and the microtubule-associated tau protein is a major component of AD neurofibrillary tangles. Although selective sets of neurons are affected in these neurodegenerative disorders, they share the inability to degrade ubiquitinated proteins and thus UPP impairment is likely to contribute to the observed neuronal death. Support for this view comes from studies demonstrating that proteasome function is impaired in the affected brain areas

of patients with AD (Keller et al., 2000) and PD (McNaught and Jenner, 2001) and declines with age (Keller et al., 2002) and oxidative stress (Jenner, 2003). Furthermore, mutations in ubiquitin were linked to AD (van Leeuwen et al., 1998) and in the ubiquitin ligase parkin were linked to familial PD (Kitada et al., 2000) and to autosomal recessive juvenile parkinsonism (Kitada et al., 1998). A missense mutation (Ile93Met) in the *uch-l1* gene was identified in a German family with PD (Leroy et al., 1998). UCH-L1 is very abundant in the brain and the Ile93Met mutation decreases its catalytic activity (Leroy et al., 1998). Collectively, these findings indicate that the UPP may be deficient in these disorders (Moore et al., 2003). We discuss below in more detail some of the changes in UPP components directly associated with these chronic neurodegenerative disorders characterized by abnormal protein deposition.

1.6.1. Proteasome dysfunction

The most consistent risk factor for developing a neurodegenerative disorder, especially AD or PD, is increasing age (Przedborski et al., 2003). One of the most accepted theories of aging is the loss of quality control in protein turnover with the concomitant build-up of oxidatively modified proteins [reviewed in (Gray et al., 2003)]. Since proteasomes selectively degrade oxidatively damaged as well as ubiquitinated

proteins it is postulated that proteasome activity declines with aging. A loss of proteasome activity with age is supported by decreased subunit expression, alterations and/or replacement of proteasome subunits and formation of inhibitory cross-linked proteins [reviewed in (Carrard et al., 2002;Keller et al., 2002)]. Food restriction, which is one of the experimental paradigms that halts the aging process, prevents the age-dependent changes in proteasome function and structure in mice and rats, further supporting the notion that the proteasome plays a role in the aging process [reviewed in (Gaczynska et al., 2001)]. Several mechanisms explain the observed UPP changes with aging [reviewed in (Vernace et al., 2007b)]. We recently identified a unique aging-dependent mechanism that contributes to proteasome dysfunction in *Drosophila melanogaster* (Vernace et al., 2007a). We observed that the major proteasome form in old flies is the weakly active 20S proteasome, while in younger flies highly active 26S proteasomes are preponderant. Old flies also exhibited a decline in ATP levels, which is relevant to 26S proteasomes, as their assembly is ATP-dependent. The perturbation in proteasome activity in "old-age" flies most likely deprives them of the ability to effectively cope with proteotoxic damages caused by environmental and/or genetic factors.

1.6.2. UBB^{+1}

There are at least three genes (*A*, *B* and *C*) encoding human ubiquitin and two of them the *polyubiquitin B* and *C* genes, contain heat-shock promoters (Mayer et al., 1991). A mutant form of the *polyubiquitin B* gene, known as UBB^{+1}, was originally detected in brains of AD patients and not in age matched controls (van Leeuwen et al., 1998). This aberrant form of ubiquitin was also detected in patients with Down syndrome, progressive supranuclear palsy, Pick's disease, frontotemporal dementia, argyrophilic grain disease and HD but not in PD [reviewed in (van Leeuwen et al., 2006)].

UBB^{+1} is the product of a frameshift generated by a dinucleotide deletion in the *polyubiquitin B* mRNA resulting in a 19-amino acid extension at the C-terminus and the absence of a C-terminal Gly76, which is present in wild-type ubiquitin (Trushina et al., 2003). Without Gly76 at the *C-terminus,* UBB^{+1} fails to conjugate with other ubiquitin molecules and is deprived of the capacity to tag protein substrates. However, due to its unaffected Lys residues, UBB^{+1} by itself can form polyubiquitin chains and is recognized by substrate receptors on the proteasome. UBB^{+1}-capped polyubiquitin chains are refractory to disassembly by de-ubiquitinating enzymes and potently inhibit proteasome degradation of a polyubiquitinated substrate *in vitro* (Lam et al., 2000) and in neuronal cells (Lindsten et al., 2002).

The toxic effects of UBB^{+1} expression were demonstrated in several model systems. In yeast, expression of a protein analogous to UBB^{+1} significantly enhanced cellular susceptibility to toxic protein aggregates (Tank and True, 2009). In rodent primary neuronal cultures, transfection of UBB^{+1} impaired mitochondrial trafficking along neurites leading to activation of both the mitochondrial stress and p53-dependent cell death pathways (Tan et al., 2007). Post-natal neuronal expression of UBB^{+1} in transgenic mice reduced proteasome activity and increased ubiquitinated protein levels in the cerebral cortex, and caused a deficit in contextual memory in both water maze and fear conditioning paradigms (Fischer et al., 2009). The inhibitory activity of UBB^{+1} may thus be an important determinant of neurotoxicity and contribute to an environment that favors the accumulation of misfolded proteins. In rodent primary neuronal cultures, suppressing UBB^{+1} expression through siRNA diminished the damage caused by UBB^{+1}, suggesting a therapeutic approach for neurodegeneration associated with this aberrant form of ubiquitin (Tan et al., 2007).

1.6.3. Parkin

Mutations in the *parkin* gene (PARK2) have emerged as a major factor in familial PD (Lucking et al., 2000). The human parkin protein has a DNA binding domain, at least 15 suspected

phosphorylation sites, and has homology to ubiquitin. Mutations in the exon regions of *parkin* are associated with an autosomal recessive juvenile parkinsonism (Kitada et al., 1998) and with early-onset autosomal recessive familial PD (Lucking et al., 2000).

The human *parkin* gene contains 12 exons and encodes a 465 amino acid protein that is abundant in the brain and belongs to a group of heterogeneous proteins collectively known as "ubiquitin-domain proteins or UDPs". They bear a ubiquitin-like domain, usually at their N-terminus, but are mostly unrelated to each other in the remainder of their sequences. UDPs play an important role as adapter proteins, mediating the assembly of heteromeric complexes through their ubiquitin-like domains. Parkin binds the Rpn10 subunit of 26S proteasome through its UBL domain (Sakata et al., 2003) (Lorick et al., 1999).

Parkin also contains two RING-finger and one in between RING finger (IBR) domains at its C-terminus. RING-finger containing proteins are ubiquitin ligases and recruit E2 ubiquitin-conjugating enzymes (Ulrich and Jentsch, 2000;Lorick et al., 1999). Parkin is an E3 ubiquitin ligase that recruits the ubiquitin-conjugating enzymes UbcH7 and Ubc8 via the RING fingers (Shimura et al., 2000;Beasley et al., 2007). The ubiquitin ligase activity of parkin facilitates the assembly of both K48- and K63-linked polyubiquitin chains (Doss-Pepe et al.,

2005). The latter chains target misfolded proteins to dynein motor complexes and promote the sequestration of misfolded proteins to aggresomes with subsequent autophagic degradation (Olzmann and Chin, 2008).

In addition to mutations in the *parkin* gene, post-translational modifications are also involved in the inactivation of parkin in PD. Parkin was found to be S-nitrosylated in PD brains (Chung et al., 2004). *In vitro* studies demonstrated that S-nitrosylation of parkin by nitric oxide reduces its E3 ligase activity and impairs its protective function (Chung et al., 2004). Parkin has multiple consensus sequences for casein kinase I and II, protein kinase C (PKC), protein kinase A (PKA), and tyrosine kinase (Kitada et al., 2000). Phosphorylation of parkin by casein kinase I and cyclin-dependent kinase 5 (cdk5) decreases parkin solubility resulting in its aggregation and inactivation (Rubio de la et al., 2009). It is likely that phosphorylation/dephosphorylation events regulate parkin activity or its targeting to cellular compartments other than the cytosol or the Golgi, where it has been frequently detected (Shimura et al., 1999).

Many studies report an interaction between parkin and PTEN-induced kinase 1 (PINK1, also known as PARK6). Mutations in both genes are implicated in autosomal recessive PD. PINK1 is a serine/threonine kinase with a mitochondrial targeting sequence.

Phosphorylation of parkin by PINK1 increases the ligase activity of parkin, and enhances parkin-mediated ubiquitin signaling through the NF-κB pathway (Sha et al., 2010). While deletion of PINK1 in *Drosophila* causes mitochondrial morphological defects and loss of dopaminergic neurons, the PINK1 mutants are rescued by overexpressing parkin (Clark et al., 2006;Park et al., 2006). Parkin is essential for mitochondrial function and is selectively recruited to impaired mitochondria to promote their engulfment by autophagosomes (Narendra et al., 2008). The ubiquitin ligase protects mitochondrial DNA from oxidative damage and stimulates its repair (Rothfuss et al., 2009). Moreover, parkin regulates cytochrome c release and apoptosis, which may be relevant to the selective vulnerability of certain neuronal populations in PD (Berger et al., 2009).

The neuroprotective role of parkin is supported by different kinds of evidence. For example, parkin promotes ubiquitination and proteasomal degradation of intracellular $A\beta_{1-42}$ an effect that could prevent the formation of amyloid deposits in AD (Burns et al., 2009). The E3 ligase was also shown to negatively regulate excitatory glutamatergic synapses thus reducing the excitotoxic vulnerability of dopaminergic neurons (Helton et al., 2008). The type of RING finger architecture found in parkin, ring-IBR-ring, is also present in other proteins such as the mouse RBCK1, shown to participate in gene

expression (Morett and Bork, 1999). Notably, parkin is a p53 transcriptional repressor that interacts with the p53 promoter and reduces p53 expression and activity (da Costa et al., 2009). On the other hand, parkin depletion enhances p53 levels and activity, a phenomenon observed in autosomal recessive juvenile PD (da Costa et al., 2009).

In conclusion, parkin is a multifunctional protein that interacts with several vital cellular functions, including ubiquitination, gene expression, and molecular scaffolding for protein-protein interaction. One or more of these interactions is important for neuronal survival.

1.6.4. UCH-L1 (ubiquitin carboxyl-terminal hydrolase-L1)

The gene *uch-l1* (PARK5) encodes for PGP9.5, a de-ubiquitinating enzyme. UCH-L1 is one of the most abundant proteins in the CNS accounting for 1-2% of soluble neuronal protein (Wilkinson, 1997). This enzyme has at least four functions: removes ubiquitin from small or unfolded proteins, disassembles polyubiquitin chains for ubiquitin recycling, stabilizes monoubiquitin and was shown to exhibit ubiquitin ligase activity (Liu et al., 2002;Setsuie and Wada, 2007). Besides polyubiquitin chains, the UCH-L1 substrates remain unidentified.

An in-frame deletion including exons 7 and 8 of the *uch-l1* gene was described as the cause of gracile axonal dystrophy (*gad*) in mice, which is a condition characterized by "dying back" type axonal degeneration (Saigoh et al., 1999). This mutation results in a truncated protein lacking 42 amino acids including a possible histidine at the active site of the enzyme (Saigoh et al., 1999). This genetic model is characterized by a retrograde accumulation of amyloid β-protein and ubiquitin-conjugates in sensory and motor neurons, as seen in certain inherited human neurodegenerative diseases (Saigoh et al., 1999;MacDonald, 1999). The *gad* mouse was the first mammalian model of a hereditary neurodegenerative disorder that resulted from a mutation in a component of the UPP.

The *uch-l1* gene is also a susceptibility gene in PD. The two most studied mutations in *uch-l1* in association with PD are I93M and S18Y. I93M was originally identified in two German siblings with PD (Leroy et al., 1998). This autosomal dominant mutation leads to a reduction in the *in vitro* hydrolytic activity of UCH-L1 (Liu et al., 2002). An inverse association between the S18Y variant of the *uch-l1* gene and the risk of developing PD was found in Asian and Caucasian samples in a recent Meta-analysis (Ragland et al., 2009). However, this protection mechanism is not well understood and was suggested to be due to its reduced ligase activity (Liu et al., 2002). An

antioxidant function of the S18Y variant in neurons was also suggested to mediate its protective effect (Kyratzi et al., 2008).

In addition to mutations, UCH-L1 is a major contributor to idiopathic AD and PD (Choi et al., 2004). UCH-L1 is required for normal synaptic and cognitive function, but its activity is down-regulated in APP/PS1 mice that overexpress Aβ (Gong et al., 2006). Restoration of UCH-L1 levels rescues deficits in synaptic transmission in Aβ-treated hippocampal slices and in slices from APP/PS1 mice (Gong et al., 2006). UCH-L1 is a major target for oxidative damage. Both the familial PD-associated UCH-L1 I93M mutant and the sporadic PD-associated carbonyl-modified UCH-L1, induce an increase in α-synuclein levels. Both forms of UCH-L1 enhance an aberrant interaction with LAMP-2A (lysosome associated membrane protein type 2A), Hsc70 and Hsp90, which are components of the chaperone-mediated autophagic pathway (Kabuta et al., 2008;Kabuta and Wada, 2008). Moreover, farnesylation of a membrane-associated form of UCH-L1 was shown to promote α-synuclein neurotoxicity (Liu et al., 2009).

UCH-L1 plays an important regulatory role in the pattern, activity and plasticity of synaptic connections. Initial data with *Aplysia*, later shown to be conserved in mammals (Lopez-Salon et al., 2001), demonstrated that *uch-l1* is one of the immediate early genes essential for long-term facilitation

involved in long-term memory storage (Hegde et al., 1997).

A recent study searched for alterations in gene expression in replaceable neurons in the high vocal center of male zebra finches and in granule neurons of mouse hippocampus and olfactory bulb, two well characterized replaceable neurons in mammals (Lombardino et al., 2005). Notably, these studies established that *uch-l1* was the most consistently underexpressed gene in the replaceable neurons as compared to non-replaceable neurons. Moreover, the levels of *uch-l1* expression were increased by singing behavior in the male birds, a stimulus known to increase the survival of those specifically replaceable neurons (Lombardino et al., 2005).

Together, these studies highlight the notion that the neuronal-specific UCH-L1 plays an important role in neuronal fate: its reduced function jeopardizes survival of CNS neurons, while its up-regulation increases neuronal plasticity and survival. Based on these important findings we postulate that increasing UCH-L1 levels may prevent neurodegeneration.

1.6.5. Ataxin-3

Ataxin-3 is another de-ubiquitinating enzyme. This protein is a member of the Josephin family and contains an N-terminal catalytic Josephin domain, three C-terminal UIMs and an expandable polyQ stretch located between the second and the

third UIM (Masino et al., 2003). The normal polyQ stretch contains between 14 and 40 glutamines. Abnormal expansion to over 53 glutamines is pathological and causes Machado-Joseph disease (MJD), also known as spinocerebellar ataxia type 3 (Maciel et al., 2001). This neurodegenerative disorder is characterized by ubiquitinated intranuclear inclusions that also contain ataxin-3 (Lieberman et al., 1999).

Ataxin-3 is actively imported to and exported from the cell nucleus (Macedo-Ribeiro et al., 2009). Heat shock and oxidative stress promote nuclear localization of this de-ubiquitinating enzyme (Reina et al., 2010). Ataxin-3 interacts with the UBL domain of HHR23 proteins. The latter are human homologs of the yeast DNA repair protein Rad23 that shuttles substrates to the proteasome for degradation (Wang et al., 2000). The ataxin-3 recruitment of HHR23A to intranuclear inclusions formed by mutant ataxin-3 could contribute to the neuropathological process.

The ubiquitin-specific cysteine protease ataxin-3 binds Lys63- and Lys48-linked polyubiquitin chains, but it preferentially cleaves Lys63-linkages (Winborn et al., 2008). The cellular functions of ataxin-3 in protein quality control are modulated through ubiquitination. On one hand, its hydrolytic activity is enhanced by ubiquitination (Todi et al., 2009), and on the other hand its ubiquitination by Gp78, which

is an endoplasmic reticulum-associated E3 ligase, promotes its degradation (Ying et al., 2009).

PolyQ-expanded ataxin-3 is neurotoxic and induces neuronal apoptosis through the mitochondrial pathway (Chou et al., 2006). Suppressing caspase cleavage of ataxin-3 by caspase inhibitors in cells, or by mutating the caspase recognition sites on ataxin-3 in a *Drosophila* model of MJD, slows down neurodegeneration (Jung et al., 2009a). These data suggest that targeting ataxin-3 cleavage by caspases might slow disease progression in MJD patients.

1.6.6. Sequestosome 1 (p62): overseeing substrate delivery to proteasomes or autophagosomes

Sequestosome 1, also known as p62 (p62/sqstm1), is a protein that is implicated in the UPP due to its ability to bind to proteasomes as well as to polyubiquitinated proteins. P62/sqstm1 has at its C-terminus a UBA domain that binds non-covalently to polyubiquitin chains. For example, this UBA domain interacts with K63-linked polyubiquitinated tau protein and delivers it to proteasomes for degradation (Babu et al., 2005). Loss of p62/sqstm1 led to hyperphosphorylation of tau and accumulation of polyubiquitinated tau (Ramesh et al., 2008).

P62/sqstm1 contains additional structural motifs that promote its interaction with other proteins: a ZZ type zinc

finger that binds the receptor interactive protein (RIP) involved in TNFα-induced apoptosis (Sanz et al., 1999;Wooten et al., 2001), a binding site for the RING-finger protein tumor necrosis factor receptor-associated factor 6 (TRAF6) that is an E3 ubiquitin ligase (Sanz et al., 2000;Wooten et al., 2001) and two PEST (proline, glutamic acid, serine, threonine) sequences [reviewed in (Geetha and Wooten, 2002)]. An additional 22 amino acid structural motif known as LC3-interacting region (LIR), binds to LC3 that is an autophagosomal marker (Pankiv et al., 2007). This LC3-interacting region is thought to target p62/sqstm1 to autophagosomes for lysosomal degradation.

At its N-terminus, p62/sqstm1 has a PB1 domain, which is a protein-protein interaction domain that binds the atypical PKC ζ (Puls et al., 1997). The PB1 domain assumes an ubiquitin-like folding and can directly bind to proteasomes and other PB1-containing proteins including itself (Wooten et al., 2006). The PB1 domain also interacts with a mutant form of superoxide dismutase 1 (SOD1) and delivers it to autophagosomes for degradation (Gal et al., 2009).

Its ability to bind proteasomes as well as autophagasomes supports the notion that p62/sqstm1 selectively sequesters polyubiquitinated proteins and decides their proteolytic fate. P62/sqstm1 could target the polyubiquitinated proteins to autophagy and, ultimately, to degradation by the lysosome when

proteasomes are impaired or overwhelmed (Seibenhener et al., 2007;Pankiv et al., 2007). P62/sqstm1 may thus be a candidate for the missing link between the UPP and the autophagy-lysosome pathway.

P62/sqstm1 was detected in ubiquitin-containing intraneuronal and intraglial inclusions in a variety of neurodegenerative disorders (Kuusisto et al., 2001;Zatloukal et al., 2002;Furukawa et al., 2004;Nakano et al., 2004). For example, increased p62 immunoreactivity was detected early during neurofibrillary pathogenesis and was consistently present in neurofibrillary tangles but absent in neuropil threads and senile plaques in AD patients (Kuusisto et al., 2002). Furthermore, p62/sqstm1 expression in neuronal cells is induced by serum withdrawal conditions that trigger apoptosis and by proteasome inhibitors, (Kuusisto et al., 2001;Nakaso et al., 2004) as well as by products of inflammation, such as PGJ2 (Wang and Figueiredo-Pereira, 2005) and by expression of expanded pathologic polyglutamine repeats (Nagaoka et al., 2004). These findings suggest that p62/sqstm1 is involved in the cellular defense mechanism triggered by the accumulation of misfolded and/or ubiquitinated proteins to enhance their degradation and/or aggregation.

The role played by p62/sqstm1 in the aggregation of ubiquitinated proteins was addressed by *in vitro* studies in

which expression of this protein was inhibited by specific antisense oligonucleotides or by siRNA (Nan et al., 2004;Nakaso et al., 2004;Wang and Figueiredo-Pereira, 2005). These studies established that abolishing p62/sqstm1 up-regulation prevents aggregation of ubiquitinated proteins thus indicating that p62/sqstm1 promotes protein aggregation. Notably, increased oxidative damage to the p62/sqstm1 promoter was found to correlate with a decline in protein level observed in many neurodegenerative disorders such as AD, PD and HD brains (Du et al., 2009). It was proposed that pharmacological means that increase p62/sqstm1 expression could be beneficial in delaying the onset of neurodegeneration. The reasoning is that p62/sqstm1 up-regulation would increase trafficking of polyubiquitinated proteins for proteasomal degradation and/or their sequestration into aggregates for autophagic removal. However, whether protein aggregation is beneficial or detrimental to cells is a highly controversial issue as discussed above.

1.7. Therapeutic approaches targeting the UPP

For the prevention/treatment of neurodegenerative disorders it is of therapeutic interest to find strategies to activate the UPP in order to avoid the accumulation/aggregation of ubiquitinated proteins. The challenge rests on developing strategies that will enhance the degradation of misfolded and

aggregation-prone proteins without compromising the normal function of the UPP. We discuss below some potential therapeutic strategies that aim to activate proteasome activity, ubiquitination or SUMOylation to prevent neurodegeneration associated with protein misfolding, accumulation and aggregation.

1.7.1. Activate proteasomes

One of the most appealing targets for therapeutic intervention is the proteasome. In the case of cancer, the aim is to inhibit the proteasome. However, for neurodegenerative diseases the emphasis is to activate not inhibit the proteasome. Notably, ~36% of cancer patients treated with the proteasome inhibitor bortezomib develop peripheral neuropathy (Richardson et al., 2005), implicating proteasome dysfunction in neurological impairment. We will discuss endogenous, genetic and pharmacological approaches to increasing proteasome activity.

(1) Endogenous activators of the proteasome: There are three endogenous activators of the 20S proteasome: PA700, PA200 and PA28. The PA700 is also known as the 19S regulatory particle, a multimeric complex comprising 9 subunits in the lid and 10 subunits in the base. The 19S regulatory particle activates 20S proteasomes in a ubiquitin- and ATP-dependent manner. We discussed its properties above under “26S proteasome and substrate recognition”.

PA200 is a large nuclear protein that binds to 20S proteasomes as a 200kDa monomer (Ustrell et al., 2002). It activates proteasomal hydrolysis of peptides rather than proteins, by opening the axial gate of the α-rings (Ortega et al., 2005). PA200 forms hybrid proteasomes with the 19S regulatory particle and 20S proteasomes. Upon exposure of cells to ionizing radiation PA200-20S-19S hybrid proteasomes are recruited to chromatin with enhanced proteolytic activity to participate in DNA repair (Blickwedehl et al., 2008).

Like PA200, PA28 activates peptide hydrolysis by the 20S proteasome by associating with its α rings (Rechsteiner et al., 2000). This activation is independent of ATP and ubiquitin, therefore it is not involved in degrading ubiquitinated proteins. PA28, also known as 11S regulatory particle (REG), can be either a heteroheptamer composed of PA28α and PA28β subunits primarily localized in the cytoplasm, or a homoheptamer composed of PA28γ predominantly in the nucleus (Mao et al., 2008). PA28α/β is upregulated by interferon-γ and is required for the assembly of immunoproteasomes (Schwarz et al., 2000). Upregulation of immunoproteasome was detected in injured (Ferrington et al., 2008), AD (Mishto et al., 2006) and HD brains (Diaz-Hernandez et al., 2003), suggesting that it plays a role in neuronal protection and damage repair. Notably, PA28γ overexpression recovered proteasome activity in skin fibroblasts from HD

patients, and improved the viability of striatal neurons expressing mutant huntingtin (Seo et al., 2007).

The most successful therapeutical approach would be to target the PA700 (19S particle) in order to increase the degradation of polyubiquitinated proteins. Molecules known as ubistatins block proteasome-polyubiquitin chain interaction (Verma et al., 2004b). A search for molecules that have the opposite effect and enhance this interaction could stimulate substrate degradation and prevent protein aggregation (Hol et al., 2006).

(2) Genetic activation of the proteasome: It is known from studies in yeast that the cellular abundance of proteasomes is controlled by the zinc finger transcription factor Rpn4 which allows the concerted induction of all proteasome subunits via interaction with PACE (proteasome associated control elements) sequences [reviewed in (Hanna and Finley, 2007)]. In humans a similar coordinated regulation of proteasome subunits must exist. Accordingly, stable overexpression of the β5 subunit in primary human fibroblasts resulted in elevated levels of other β subunits, and increased the levels of all three proteasome activities (Chondrogianni et al., 2005). This genetic manipulation resulted in increased survival against oxidants and a delay in senescence. In a follow up study proteasomal up-regulation was achieved via overexpression of the proteasome

maturation protein (POMP), the accessory factor for proteasome assembly in humans (Chondrogianni and Gonos, 2007). POMP overexpression in fibroblasts led to increased levels of assembled and functional proteasomes, and enhanced the capacity to effectively cope with various oxidative stressors.

An alternate therapeutic approach for maintenance of proteasome function would be to overexpress arsenite-inducible RNA-associated protein (AIRAP) as it dramatically stabilizes proteasome activity in the absence of ATP (Stanhill et al., 2006). In addition, AIRAP containing proteasomes show a higher rate of hydrolysis using model substrates. Together, these data strengthen the therapeutic prospect of genetic manipulation of the proteasomal system.

(3) Natural and synthetic activators: The detergent SDS and some fatty acids such as oleic, linoleic and linolenic acids stimulate proteasome activity *in vitro* by favoring the open conformation of the proteasome (Dahlmann et al., 1985;Watanabe and Yamada, 1996). Some synthetic compounds such as peptidyl alcohols, esters, p-nitroaniliades and nitriles reversibly stimulate proteasome activity probably by binding to the same site as PA28 (Wilk and Chen, 1997). In addition, some proteasome-activating hydrophobic peptides bind as modifiers at noncatalytic sites, thus mimicking the effect of the PA28 complex by opening the gate of the α-rings (Kisselev et al.,

2002). Oleuropein, the most abundant phenolic compound in *Olea europaea* leaf extract, olive oil, and olives has a stimulatory impact on proteasome activity in *vitro*, probably acting through conformational changes of the gate of 20S α-ring (Katsiki et al., 2007). Some natural antioxidants such as dithiolethione and sulforaphane, were also shown to enhance mammalian proteasome expression through the Keap1/Nrf2 (Kelch-like ECH-associated protein 1/nuclear factor-erythroid 2-related factor 2) signaling pathway, resulting in increased protection against various oxidants (Kwak et al., 2003;Kwak et al., 2007). Some synthetic triterpenoid derivatives, such as 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO), its methyl ester (CDDO-Me) and imidazolide (CDDO-Im) derivatives, are Nrf2 activators (Thimmulappa et al., 2007) and could also be considered as proteasome activators. Betulinic acid, a lupene-type pentacyclic triterpene derived from many plants, such as birch trees, is a potent activator of the chymotrypsin-like activity of the proteasome (Huang et al., 2007). Notably, C-3 modifications on betulinic acid change it from a proteasome activator into a proteasome inhibitor (Huang et al., 2007).

As a group these natural and synthetic proteasome activators might be important to slow down the progression of neurodegenerative diseases by enhancing proteasome activity and

preventing the abnormal accumulation/aggregation of ubiquitinated proteins.

4) Concern: Proteasome inhibitors are currently used as anti-cancer drugs mostly because they induce apoptosis preferentially in transformed cells [reviewed in (Orlowski and Kuhn, 2008)]. Based on the anti-carcinogenic effect of proteasome inhibition, one could argue that increasing proteasome function to prevent/treat neurodegenerative diseases could have the opposite effect and thus induce carcinogenesis (Hol et al., 2006). However, studies with human senescent fibroblasts strongly oppose this view. Overexpressing the β5 subunit of the 20S proteasome (Chondrogianni et al., 2005) or its assembly chaperone POMP (Chondrogianni and Gonos, 2007) in the fibroblast enhanced proteasome activity without increasing cell proliferation. Interestingly, fibroblasts from healthy centenarians exhibited proteasome characteristics (expression levels and activity) similar to younger rather than elderly individuals (Chondrogianni et al., 2000). These studies strengthen the prospect that increasing/maintaining proteasome function as we age is a promising therapeutical strategy to prevent neurodegeneration.

1.7.2. Enhance ubiquitination

1) "Chemical knockdown" of specific proteins by PROTACS: The development of PROteolysis TArgeting Chimera moleculeS (PROTACS) is a strategy with potential for selectively inducing ubiquitination and proteasomal degradation of specific substrates. Basically, PROTACS are heterobifunctional molecules comprising a ligand for the target protein, a linker moiety, and a ligand for an E3 ubiquitin ligase (Sakamoto et al., 2001). PROTACS function as a bridge between the target protein and an E3 ubiquitin ligase. Upon binding to a PROTAC, the E3 ubiquitin ligase promotes the synthesis/attachment of a polyubiquitin chain to the target protein, followed by its recognition and degradation by the 26S proteasome (Schneekloth et al., 2008). Potential advantages of PROTACS are *(a)* their selectivity based on a unique site on the target protein and recruitment of the E3 ubiquitin ligase with subsequent enhanced ubiquitination and proteasomal degradation of the target protein; *(b)* tissue-specific knockdown of a protein since some E3 ubiquitin ligases are expressed in a tissue-specific manner. PROTACS represent a novel approach for small molecule-induced targeted proteolysis through the UPP in intact cells. One could envision designing specific PROTACS for degradation of disease-promoting proteins, such as α-synuclein, truncated tau and huntingtin, in order to

prevent their accumulation/aggregation and promote neuronal survival.

2) Parkin gene therapy: This genetic manipulation might be an effective approach for patients with loss-of-function mutations in parkin associated with the demise of dopaminergic neurons in autosomal recessive juvenile PD. Prior to clinical trials preliminary studies were conducted in primates. The serotype-1 recombinant adeno-associated virus (rAAV1) vector was used to deliver α-synuclein or α-synuclein plus parkin unilaterally into striatum of monkeys (Yasuda et al., 2007). Overexpression of parkin diminished the accumulation of α-synuclein.

3) Modulation of carboxyl terminus of Hsc 70-interacting protein (CHIP): Like parkin, the E3 ubiquitin ligase CHIP is a good candidate for enhancing ubiquitination. CHIP is also a co-chaperone with Hsp70 and Hsp90 and one of its roles is to ubiquitinate client proteins. CHIP is a dimeric protein containing a U-box domain at the C-terminus as its ubiquitin ligase domain, and a tetratricopeptide repeat (TPR) domain at the N-terminus as its chaperone binding domain (Zhang et al., 2005).

Overexpression of CHIP is neuroprotective, while its deletion is neurotoxic in cell and animal models of a variety of neurodegenerative diseases. In a cell model of PD,

overexpression of CHIP reduced α-synuclein levels by promoting its proteasomal and lysosomal degradation via the TPR and U-box domains respectively, and suppressed α-synuclein aggregation (Shin et al., 2005). Furthermore, CHIP up-regulation protects against mutant *leucine-rich repeat kinase-2 (LRRK2)*-induced toxicity, which is one of the most common causes of autosomal dominant PD (Ko et al., 2009). *In vivo* studies of AD brains demonstrate that higher CHIP levels correlate with less tau aggregation and fewer neurofibrillary tangles in early stages of AD (Sahara et al., 2005). Restoring CHIP levels in a transgenic mouse model of AD rescued Aβ-induced effects on tau pathology (Oddo et al., 2008). On the other hand, CHIP deletion in transgenic mice induced the accumulation of non-aggregated, ubiquitin-negative, hyperphosphorylated tau species (Dickey et al., 2006). In cell models of HD and MJD, CHIP overexpression promoted ubiquitination and degradation of the polyglutamine-expanded proteins huntingtin and ataxin-3, and suppressed their aggregation as well as cell death (Jana et al., 2005).

Besides its roles as a chaperone and E3 ubiquitin ligase, CHIP was proposed to play a role in the aggresome pathway (Sha et al., 2009). As such, CHIP interacts with the inducible form of nitric oxide synthase, promotes its ubiquitination and degradation by the proteasome as well as its sequestration into aggresomes, and enhances its interaction with histone

deacetylase 6, a linker between ubiquitinated proteins and the dynein motor.

Collectively these studies suggest that CHIP is an important regulator for ubiquitination of multiple substrates. Enhancing CHIP levels may thus be a promising therapeutic approach to facilitate ubiquitination and degradation of misfolded proteins, prevent their aggregation, and slow the neurodegenerative process.

1.7.3. Promote SUMO conjugation

SUMO (small ubiquitin-like modifier) conjugation is a reversible pathway that provides a rapid and effective manner for regulating subcellular localization, activity and stability of many substrates [reviewed in (Matunis and Pickart, 2005;Dorval and Fraser, 2007)]. Several proteins implicated in neurodegenerative disorders such as tau in AD, α-synuclein in PD, and huntingtin in HD are SUMOylated suggesting that this post-translational modification is involved in the neurodegenerative process [reviewed in (Dorval and Fraser, 2007)]. Two recent studies demonstrate that SUMOylation attenuates aggregation and cell toxicity of proteins that contain expanded polyQs, such as ataxin 7 (Janer et al., 2010) and the androgen receptor (Mukherjee et al., 2009). Apparently SUMOylation functions at least in part as a steric impediment to the formation of higher

order polyglutamine β-sheet structures thus preventing misfolding of these proteins. It is conceivable that enhancing SUMOylation could be of clinical value for preventing/treating neurodegenerative disorders associated with high order protein aggregation.

1.8. Conclusions

In order to survive under various environmental stress conditions or those induced by mutations, cells have a repertoire of mechanisms that they can activate or inhibit according to their needs. The UPP is the ultimate mechanism that cells use to ensure the selective destruction of misfolded or damaged proteins (Goldberg, 2003). We reviewed a large body of evidence linking UPP impairment with neuronal loss in most chronic neurodegenerative diseases such as AD, PD, HD and ALS. When functional, the UPP provides neurons with the fundamental ability to protect themselves from a certain amount of damage before there is a severe disruption of function and viability (Figure 1). The life or death of individual neuronal populations depends on the overall intracellular burden of accumulated misfolded or aggregated proteins [reviewed in (Gorman, 2008)]. Neuronal survival demands a dynamic, effective and safe capacity to deal with this abnormal protein burden.

It is critical to understand the causes and establish tools for early pre-symptomatic diagnosis to slowdown or halt the underlying neurodegenerative process. Current therapies for neurodegenerative diseases treat only the symptoms and not the causes of these disorders. Among potential strategies, preventing apoptosis with caspase inhibitors failed to prevent neurodegeneration (Waldmeier et al., 2006). Likewise, it is not clear if targeting the removal of protein aggregates will promote neuronal survival. It is possible that like for apoptosis, when cells develop protein aggregates the damage is too far advanced for the therapy to be effective. When protein aggregates develop cells may be already at a point of no return and committed to the death pathway.

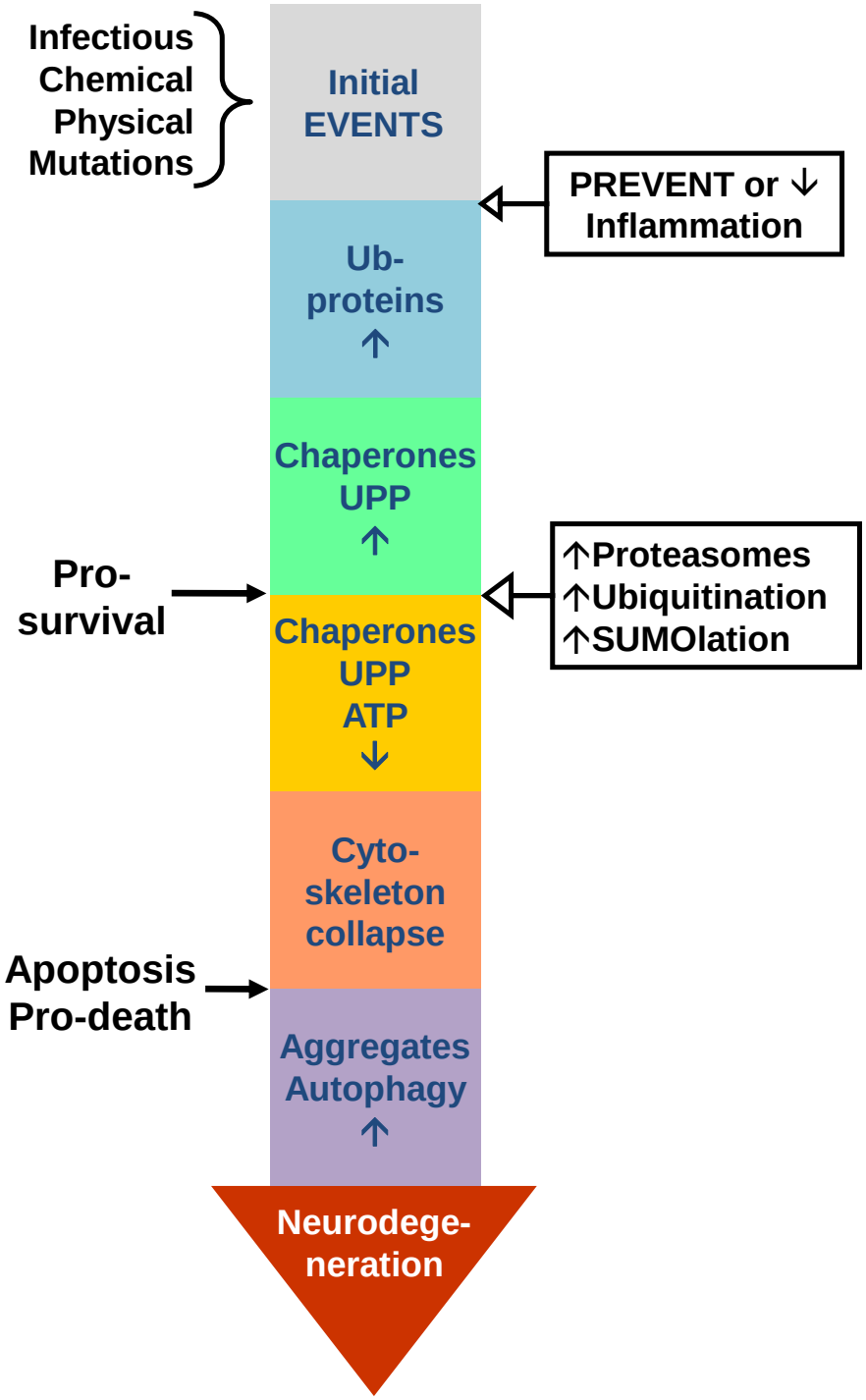
Strategies that focus on enhancing proteasome activity in general, and ubiquitination and/or SUMOylation of particular proteins may prove to be a worthwhile endeavor. The genetic and pharmacological therapeutical approaches targeting the UPP and relevant to neurodegeneration that we discussed in this review focus on modulating different aspects of the UPP. A critical requirement for any of these degradation-enhancing therapies is the ability to cross the blood brain barrier. The latter is particularly relevant to diseases of the CNS.

Neuroinflammation is likely to be one of the mechanisms that contributes to the cascade of events leading to the

accumulation of ubiquitinated proteins and UPP impairment in the neurodegenerative process [reviewed in (Schmidt-Glenewinkel and Figueiredo-Pereira, 2006)]. Neuroinflammation is viewed as a process that occurs in the CNS and that involves primarily non-cell-autonomous pathological mechanisms mediated by activated glia leading to progressive neurodegeneration (Streit et al., 2008). During neuroinflammation activated microglia migrate to sites of neuronal injury, phagocytose neighboring cells, and produce large amounts of oxygen free radicals and other toxic products that elicit protein misfolding and impair the UPP. This process of microglia activation involving cell-to-cell interaction may explain the host-to-graft transmission phenomenon observed in patients receiving neural transplants. The host-to-graft transmission phenomenon is reviewed in (Brundin et al., 2008). Interestingly, one of the mechanisms used by microglia to withstand such oxidative challenges is to up-regulate the proteasome through PARP activation (Ullrich et al., 2001). Microglia can elicit pathological cell-cell interactions causing vulnerable neurons to become dysfunctional and at risk for degeneration. Therapeutic strategies aimed at down-regulating these inflammatory processes might help to slow down the progression of neurodegeneration. However, a better understanding of the complex function of microglia [reviewed in (Lucin and Wyss-Coray, 2009)] is required in order to provide a

basis for therapeutically targeting neuroinflammation associated with neurodegeneration.

Fig. 1 – Time line for neurodegeneration: The following sequence of events is proposed for neurodegeneration associated with protein misfolding. The CNS is subjected to all kinds of injury, such as infectious, chemical and physical stimuli. In many instances, these insults induce inflammation resulting in astrocytes and microglia activation. Activated glia release both protective and toxic factors. Protective factors, such as BDGF, GDNF, IGF-1 and VEGF, act in concert to repair the damage. However, if the toxic factors, such as nitric oxide, IL6, TNFα, reactive oxygen species and PGJ2, prevail they generate a toxic microenvironment that damages neurons by causing protein misfolding. In addition, mutations and the initial insults themselves can act in/on neurons and also promote protein misfolding. Ubiquitination of these damaged proteins and removal by the UPP is essential for cell survival. However, if due to mitochondrial dysfunction ATP levels decline and the UPP and chaperones activities also deteriorate, the ubiquitinated proteins accumulate, the cytoskeleton collapses and apoptosis is activated. All of these events contribute to protein aggregation. Autophagy activation is an attempt to remove the aggregates, but at this point the cells may have reached a point of no return leading to neurodegeneration. Abolishing/diminishing the toxic effects associated with inflammation and/or activating the UPP early in the process seem to be attractive approaches to prevente the neurodegenerative cascade before the damage is too far advanced for the therapy to be effective and the cells are committed to die resulting in neurodegeneration.


Infectious
Chemical
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PREVENT or ↓
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Ub-
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Pro-
survival
Chaperones
UPP
ATP
↓
Cyto-
skeleton
collapse
Apoptosis
Pro-death
Aggregates
Autophagy
↑
Neurodege-
neration

Figure 1

CHAPTER II

LINK BETWEEN PROTEASOME AND CALPAIN

UPON MITOCHONDRIAL DYSFUNCTION IN NEURONS

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2.1. ABSTRACT

Proteasome and mitochondria dysfunction are linked to many neurodegenerative conditions. Proteasomes are one of the major intracellular protein degradation machineries. Mitochondria generate ATP, sequester excess cytoplasmic calcium, and produce and detoxify reactive oxygen species. To investigate the impact of mitochondria dysfunction on the proteasome we treated rat E18 cerebral cortical neurons with oligomycin, antimycin, or rotenone, which inhibit different elements of the electron transport chain. Firstly, we observed a reduction in the levels of ubiquitinated proteins caused by a blockade of the initial steps of the ubiquitination cascade. These steps are ubiquitin activation via an adenylate intermediate catalyzed by the E1 enzyme, and E1-E2 transthiolation. Secondly, we established that 26S proteasomes were disassembled with an ensuing reduction in activity. Notably we demonstrate, to our knowledge for the first time, that one of the factors contributing to 26S proteasome demise is calpain-dependent selective cleavage of Rpn10, one of the proteasome subunits. Calpain activation is linked to necrosis, which is induced upon energy depletion. Calpain also cleaved caspase 3 to an inactive fragment, thus preventing apoptosis, an energy dependent cell death pathway. In addition, calpain cleaved the microtubule associated protein TAU, a major component of neurofibrillary tangles in Alzheimer disease (AD)

and other tauopathies. Thirdly, we detected a rise in 20S proteasomes, which degrade most oxidized proteins as well as intrinsically disordered proteins in a ubiquitin- and energy-independent manner. Finally, we show that down-regulation of ubiquitinated proteins and 26S proteasomes with a simultaneous increase in 20S proteasomes, occurs upon acute (up to 16h) or long-term (up to seven days) mitochondrial impairment in the neurons. These data suggest that upon mitochondrial dysfunction, neurons initiate a series of mechanisms that converge to optimize unregulated and energy-independent turnover of randomly unfolded oxidized proteins by 20S proteasomes. This adaptive response to energy deficiency may be suitable for short-term periods. However, if maintained chronically it may lead to neurodegeneration, as regulated protein degradation by the ubiquitin/proteasome pathway is essential for neuronal survival.

2.2. INTRODUCTION

Proteasome function is essential for neuronal homeostasis and survival. Neurons are also critically dependent on mitochondria for energy production necessary for maintaining homeostasis, neurotransmission and plasticity. There is a mutual dependence between mitochondrial and proteasomal function (Livnat-Levanon and Glickman, 2011). Mitochondria provide most ATP required for 26S proteasomal degradation of ubiquitinated

proteins, some of which are mitochondrial proteins. Emerging evidence implicates proteasomal and mitochondrial dysfunction in ageing and neurodegenerative disorders such as Alzheimer disease (AD) (Ding et al., 2006;Stranahan and Mattson, 2012). In neurons this is particularly important because, on the one hand neurons have a limited glycolytic capacity (Reddy, 2007) thus are particularly sensitive to the ageing-associated decline in mitochondrial bioenergetic capacity (Beal, 2005). On the other hand intraneuronal accumulation of ubiquitinated proteins, indicative of proteasome malfunction, is a hallmark of most chronic neurodegenerative diseases including AD (Bedford et al., 2009). Notably, the impact of mitochondrial impairment on proteasome function remains poorly defined.

One of the principal mechanisms by which mitochondrial dysfunction contributes to ageing and neurodegeneration is via a net increase in the production of reactive oxygen species (Eckert et al., 2011). Oxidative stress induced by reactive oxygen species (ROS) alters the structure of cellular proteins (Stadtman and Berlett, 1998) which, if not repaired, must be removed by proteolysis to prevent their accumulation and aggregation. One of the major roles of the proteasome is to degrade oxidatively modified proteins, but whether ubiquitination is required remains elusive (Shang and Taylor, 2011). Some studies support the notion that oxidatively modified

proteins in cells are removed by the 20S proteasome independently of ubiquitination (Shringarpure et al., 2003). Others demonstrate that upon oxidative stress there is an increase in the levels of ubiquitinated proteins, and in ubiquitin-activating and ubiquitin-conjugating enzyme activities, suggesting that the ubiquitination machinery is recruited to degrade oxidatively modified proteins (Shang et al., 2001). This is an important issue since there is ample evidence that neural tissue is especially vulnerable to oxidative stress, which plays an important role in many neurodegenerative disorders (Lin and Beal, 2006). Impaired clearance of oxidatively modified proteins can cause their aggregation and directly promote progression of the neurodegenerative process (Grune et al., 2004).

Another deleterious mechanism associated with mitochondrial dysfunction is the limitation in ATP production that can cause an energy crisis in neurons (Nicholls, 2008). Most ATP in neurons is produced in mitochondria from oxidative phosphorylation derived from glucose metabolism under aerobic conditions. Brain glucose hypometabolism is detected early in Alzheimer patients and has been implicated in the initiation and progression of Alzheimer pathology (Mosconi et al., 2008). Degradation of proteins by the 26S proteasome is highly dependent on ATP binding and hydrolysis (Liu et al., 2006). Both deleterious consequences of mitochondrial impairment, i.e.

restricted ATP generating capacity and ROS production, are likely to contribute to impaired proteasome-dependent proteolysis in neurons. Interestingly, neurons exhibit a higher sensitivity to proteasome inhibition than astrocytes, mostly because they undergo increased levels of oxidized proteins (Dasuri et al., 2010).

It is postulated that in neurons even a modest restriction of ATP production by mitochondria far outweighs the negligible effects of reactive oxygen species, although the underlying mechanisms are not yet clearly understood (Nicholls, 2008). In the current study we demonstrate an adaptive response of the ubiquitin/proteasome pathway to a decline in ATP synthesis by mitochondria in neurons: there is down-regulation of ubiquitination and 26S proteasomes, both of which are energy-dependent, with a simultaneous up-regulation of 20S proteasomes. This adaptive response occurred upon acute or long-term mitochondrial impairment. Notably, calpain activation is a contributing factor to 26S proteasome demise via cleavage of selective proteasome subunits including Rpn10. We propose that under acute mitochondrial stress, unregulated protein degradation via 20S proteasomes is beneficial to promote turnover of randomly unfolded oxidized proteins. Regulated protein degradation by the ubiquitin/proteasome pathway, however, is essential for long-term neuronal survival.

2.3. MATERIALS AND METHODS

2.3.1. Materials - Inhibitors: oligomycin A, antimycin A, rotenone, and chloroquine (Sigma-Aldrich, St. Louis, MO); epoxomicin (Peptides International Inc., Louisville, KY); calpain inhibitor III and calpeptin (Calbiochem/EMD Bioscience, Gibbstown, NJ). Substrate: Suc-LLVY-AMC (BACHEM Bioscience Inc., King of Prussia, PA). Primary antibodies: rabbit polyclonal anti-ubiquitinated proteins (1:1,500, cat# Z0458, Dako North America, Carpinteria, CA); rabbit polyclonal anti-β5 (1:5,000, cat# PW8895), mouse monoclonal anti-Rpt6 (1:2,000, cat# PW9265), anti-Rpn10 (1:2000, cat# PW9250), anti-α5 (1:2,000, cat# PW8125), anti-Rpn2 (1:2000, cat# PW9270), anti-Rpt5 (1:2000, cat# PW8770), (all from ENZO Life Sciences, Inc., Farmingdale, NY); mouse monoclonal anti-β-actin (1:10,000, cat# A-2228, Sigma, St. Louis, MO); rabbit polyclonal anti-caspase 3 (1:1000, cat# 9662), anti-UBE1a (cat# 4890, 1:1000), and anti-E2-25K/Hip2 (cat# 3847, 1:1000) (all from Cell Signaling Technology, Danvers, MA); mouse monoclonal anti-spectrin α chain (1:5000, clone AA6, cat# MAB1622, Millipore, Billerica, MA); mouse monoclonal anti-βIII-tubulin (1:10,000, cat# MMS-435P, Covance, Oakland, CA); mouse monoclonal TAU C5 (1:50,000; detects all TAU isoforms; ep: a.a. 210-241), courtesy of Dr. L. Binder (Northwestern University, Chicago, IL, USA). Secondary antibodies with HRP conjugate (1:10,000, Bio-Rad Laboratories, Hercules, CA).

2.3.2. Cell cultures - Dissociated cultures from Sprague Dawley rat embryonic (E18, both sexes) cerebral cortical neurons were prepared as follows: the isolated cortices free of meninges were digested with papain (0.5mg/ml from Worthington Biochemical Corp., Lakewood, NJ) in Hibernate E without calcium (BrainBits LLC., Springfield, IL) at 37°C for 30min in a humidified atmosphere containing 5% CO_2. After removal of the enzymatic solution, the tissues were gently dissociated in Neurobasal media (Invitrogen, Carlsbad, CA). Dissociated tissues were centrifuged at 300X*g* for 2min. The pellet was resuspended in Neurobasal media without antibiotics and plated on 10cm dishes pre-coated with 50µg/mL poly-D-lysine (Sigma, Saint Louis, MO). Cells were plated at a density of $6X10^6$ cells per 10cm dish, or $2.5X10^5$ cells per well on 24-well plates (cell viability only). Cultures were maintained in Neurobasal media supplemented with 2% B27 and 0.5mM L-Glutamax (all from Invitrogen) at 37°C in a humidified atmosphere containing 5% CO_2. Half of the medium was changed every 4 days.

2.3.3. Culture treatments – Cortical neurons were treated acutely (2h, 4h, 8h, or 16h) with DMSO or with the different drugs in DMSO added directly to DMEM without serum supplemented with 0.5mM L-Glutamax and 1mM sodium pyruvate (all from Invitrogen). The final DMSO concentration in the medium was 0.5%.

For long-term (one day to 7 days) studies, neurons were maintained for the entire time in DMEM with 2% B27 supplemented with 0.5mM L-Glutamax and 1mM sodium pyruvate.

2.3.4. ATP assay - Steady state ATP content was measured with a kit using the sensitive luciferin/luciferase system (Molecular Probes, Carlsbad, CA). This assay is based on luciferase requiring ATP for light production using luciferin as a substrate. Cells were harvested with 4% trichloroacetic acid followed by centrifugation (19,000X*g*, 15min at 4°C). ATP steady state levels were determined in cleared supernatants upon neutralizing the samples with 10mM Tris-HCl, pH 8.0. Samples were then added to the reaction buffer containing luciferin and assayed using a Luminoskan Ascent microplate luminometer (Thermo Electron Corporation, Waltham, MA). Protein concentration was determined with the bicinchoninic acid assay kit (Pierce, Rockf., IL) upon resuspending the pellet with buffer (10mM Tris-HCl, pH 8.0 and 1% SDS), and sonication. ATP levels were normalized for protein concentration.

2.3.5. Cell viability assay - Cells were treated under various conditions for 2h, 4h, 8h or 16h. Cell viability was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described in (Mosmann, 1983).

2.3.6. Western blotting – After treatment, cells were rinsed twice with PBS and harvested by gently scraping into hot (100°C) SDS buffer (0.01M Tris-EDTA, pH 7.5 and 1% SDS) to make sure all intracellular proteins were included. Samples were subjected to a 5min boil at 100°C followed by brief sonication. After determination of the protein concentration with the bicinchoninic acid assay kit (Pierce, Rockf., IL) the following was added to each sample (final concentrations): β-mercaptoethanol (4%), bromophenol blue (0.005%), and glycerol (4%). Following SDS-PAGE on 6%, 8%, 10% or 12% polyacrylamide gels, proteins were transferred to an Immobilon-P membrane (Millipore, Bedford, MA). The membranes were probed with the respective antibodies and antigens were visualized by a standard chemiluminescent horseradish peroxidase method with the ECL reagent. Semi-quantification of protein detection was done by image analysis with the ImageJ program (Rasband, W.S., ImageJ, U.S. NIH, Maryland, http://rsb.info.nih.gov/ij/, 1997-2006). Relative intensity (no units) is the ratio between the value for each protein and the value for the respective loading control.

2.3.7. Evaluation of endogenous E1- and $E2_{25k}$-ubiquitin thiol esters – Upon treatment with vehicle (control, DMSO) or oligomycin, cortical neurons were washed twice with PBS, harvested with a thiol stabilizing buffer [5mM Tris-HCl, pH 7.8,

8.7M urea, 1% Nonidet P-40, 20mM N-ethylmaleimide, 3mM EDTA, 2% protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO)], and kept on ice for 15min for lysing. As described in (Jha et al., 2002), samples were sonicated for 10s, centrifuged at 19,000X*g* for 15min at 4°C, mixed (30µg) 1:2 (volume) with thiol ester gel buffer (33mM Tris-HCl, pH 6.8, 2.7M urea, 2.5% SDS and 13% glycerol), and boiled for 5min. After determination of the protein concentration with the Bradford assay (Bio-Rad Laboratories, Hercules, CA), the normalized samples were separated into reducing [with β-mercaptoethanol (4%)] and non-reducing (no β-mercaptoethanol) aliquots for SDS-PAGE, followed by western blotting with anti-E1 and anti-E2 antibodies, as described above.

2.3.8. In gel proteasome activity and levels - Upon treatment with vehicle (control, DMSO) or the respective drugs, cells were washed twice with PBS and harvested for the in gel assay as described in (Myeku et al., 2011). The native gels loaded with 30µg protein/lane, were run at 150V for 120min. The in gel proteasome activity was detected by incubating the native gel on a rocker for 10min at 37°C with 15ml of 300µM Suc-LLVY-AMC followed by exposure to UV light (360nm). Gels were photographed with a NIKON Cool Pix 8700 camera with a 3-4219 fluorescent green filter (Peca Products, Inc). Proteins on the native gels

were transferred (110mA) for 2h onto PVDF membranes. Immunoblotting was carried-out for detection of the 20S and 26S proteasomes with the anti-Rpt6 and anti-β5 antibodies, which react with subunits of the 19S or the 20S particles, respectively, thus detecting 26S and 20S proteasomes. Antigens were visualized by a chemiluminescent horseradish peroxidase method with the ECL reagent.

2.3.9. Glycerol density gradient centrifugation – Cells were harvested in 25mM Tris-HCl, pH 7.5, 2mM ATP and 1mM DTT. Following homogenization and sonication the lysates were centrifuged (19,000xg for 15min) at 4°C. The cleared supernatants (one mg of protein/sample) were subjected to centrifugation (83,000xg for 24h) at 4°C in a Beckman SW41 rotor in a 10-40% glycerol gradient (fractions 1 to 24) made in the same lysis buffer. Following centrifugation 24 fractions (500μl each) were collected. Aliquots (50μl) of each fraction were assayed for chymotrypsin-like activity with the substrate Suc-LLVY-AMC. After 3h of incubation at 37°C samples were read with a spectrofluorometer. In addition, proteins were precipitated with acetone from 450μl of each fraction and subjected to western blot analysis (10% gels). The membranes were probed with the respective antibodies and antigens were visualized by a standard

chemiluminescent horseradish peroxidase method with the ECL reagent.

2.3.10 Statistical analysis – Statistical significance was estimated using one-way ANOVA (Tukey-Kramer multiple comparison test) with the Instat 2.0, Graphpad Software (San Diego, CA).

2.4. RESULTS

2.4.1. Oligomycin, antimycin, and rotenone as mitochondrial inhibitors – To investigate the link between mitochondrial impairment and loss of neuronal viability, rat E18 cerebral cortical neuronal cultures were treated with three different mitochondrial inhibitors: (1) rotenone, which binds to ND1 and inhibits NADH–ubiquinone reductase activity of complex I (Degli, 1998), (2) antimycin A, binds to the quinone reduction site of complex III (ubiquinol-cytochrome c oxidoreductase) inhibiting the reduction of cytochrome c (Huang et al., 2005), and (3) oligomycin, which binds to a polypeptide in the F0 baseplate and blocks ATP synthesis by the F0/F1 mitochondrial ATP synthase (Liu and Schubert, 2009). As shown in Fig. 1A, the three drugs decrease ATP levels in a concentration dependent manner, with oligomycin being the most efficient, at 5nM lowering ATP levels to ~75% control. We postulate that the 25% ATP that remains upon oligomycin-treatment is generated in a mitochondria-independent

manner by glycolysis. Not surprisingly the reduction in ATP levels induced a loss of neuronal viability (Fig. 1B) assessed with the MTT assay, which in most cells seems to be reduced largely within the cytoplasm (Berridge and Tan, 1993;Liu et al., 1997).

2.4.2. The decline in polyubiquitinated proteins induced by the three mitochondrial inhibitors is linked to E1 failure – Chronic neurodegenerative disorders linked to mitochondrial dysfunction, like AD, are also characterized by accumulation/aggregation of ubiquitinated (Ub) proteins (Oddo, 2008). We thus examined the effect of the three mitochondrial inhibitors on Ub-protein levels in the cortical neurons. Upon treatment with the three drugs, Ub-proteins decreased in a concentration (Fig. 2 A and B, *panels 1*) and time (established for oligomycin, Fig 2C, *panel 1*) dependent manner. In neurons treated with oligomycin, the decline in polyubiquitinated proteins coincided with a rise in free ubiquitin levels, observed in a concentration (Fig. 2B, *panel 2*) and time (Fig. 2C, *panel 2*) dependent manner. For comparison, the effect of the proteasome inhibitor epoxomicin on Ub-proteins in neurons is shown in Fig. 2E. It is clear that with epoxomicin the levels of Ub-proteins increased, contrary to what was observed with the mitochondria inhibiting drugs. Since the three mitochondrial

toxins had similar effects, in the subsequent study we concentrated on oligomycin.

To address a mechanism mediating the drop in Ub-proteins induced by oligomycin, we focused on the E1A ubiquitin activating enzyme, and on the E2 conjugating enzyme E2-25K. E1 activity requires ATP for formation of a thiol ester adduct with ubiquitin, a process that is sensitive to reducing agents such as β-mercapthoethanol (Jahngen-Hodge et al., 1997). In principle, if E1 activity is decreased all protein ubiquitination including E1-E2 transthiolation, should be diminished. To assess E1- and E2-ubiquitin thiol ester levels, we subjected the samples to SDS-PAGE under reducing (with β-mercapthoethanol) and non-reducing (absence of β-mercapthoethanol) conditions. As shown in Fig. 2D (*panel 1, three left lanes*), under non-reducing conditions E1-ubiquitin thiol ester (~126kDa) migrated ~9kDa above native E1 (117kDa), consistent with the additional mass of ubiquitin. Oligomycin-treatment abolished the E1-ubiquitin thiol ester, reflecting the loss of the ubiquitin monomer. A similar pattern was observed for the E2 conjugating enzyme E2-25K. Its E2-ubiquitin thiol ester (~33kDa), which is dependent on E1 transthiolation activity, was diminished in neurons treated with oligomycin (Fig. 2D, *panel 2, three left lanes*). Under reducing conditions (*panel 1 and 2, three right lanes*) only native E1 and E2 were detected.

Together these data demonstrate that upon oligomycin treatment, ATP depletion in neurons prevents E1 from forming thiol ester intermediates with ubiquitin, resulting in an overall down-regulation of Ub-proteins.

2.4.3. Perturbing mitochondria in neurons causes a decline in 26S proteasomes and a concomitant increase in 20S proteasomes – The activity and assembly of 26S proteasomes are highly dependent on ATP binding and hydrolysis (Eytan et al., 1989;Liu et al., 2006). We thus assessed with the native in gel assay, the effects of the three mitochondrial inhibitors on proteasome activity and levels in the cortical neurons. The native in gel assay provides a means to detect the three native forms of the proteasome: 26S proteasomes with either two regulatory caps [26S (2)] or just one cap [26S(1)], and the 20S core particle alone (20S). Proteasome activity was determined with the substrate Suc-LLVY-AMC, which assesses the chymotrypsin-like activity (Fig. 2A, B and C, *panels 1*). Under control conditions (*lanes marked with "0"*), the activity of the 20S proteasome is substantially lower than that of the 26S, because the 20S is a latent form of the proteasome (Goldberg, 2007). Proteasome levels were established by immunoblotting with the anti-Rpt6 antibody that reacts with an ATPase subunit of the 19S particle (Fig. 2A, B and C, *panels 2*), and with the anti-β5 antibody (Fig. 2A, B and

C, *panels 3*). The β5 subunit is a component of the 20S core, thus its antibody detects both 26S and the 20S proteasomes. It is clear that the three mitochondria drugs inhibited the 26S proteasome in a concentration (Fig. 3A and B, *panels 1*) and time (oligomycin, Fig. 3C, *panel 1*) dependent manner. Furthermore, the three drugs caused the disassembly of the 26S proteasome, as its levels decreased while those of the 20S proteasome increased (Fig. 3A, B and C *panels 2 and 3*).

We corroborated by glycerol gradient fractionation that mitochondrial dysfunction in neurons causes a shift from 26S to 20S proteasomes (Fig. 4). Total extracts from cells treated with vehicle (DMSO, control) or oligomycin (5nM, 8h) were fractionated by glycerol density gradient centrifugation. Fractions were analyzed for Suc-LLVY-AMC hydrolysis which reflects the chymotrypsin-like activity (*graph*). Compared to control, the chymotrypsin-like activity of oligomycin-treated cells was significantly reduced in the fractions corresponding to the elution of the 26S proteasomes: fractions 12-16 (*peak for 26S*), compare oligomycin-treatment (*white squares*) with control (*black squares*). In contrast, the activity of 20S proteasomes (fractions 7-9, *peak for 20S*) was increased in the oligomycin-treated cells.

To confirm the proteasome elution pattern, aliquots from each fraction were subjected to western blot analyses with the

anti-Rpt6 antibody that reacts with an ATPase subunit of the 19S particle (*rows 1 and 3*, control and oligomycin-treated, respectively), and the anti-α5 antibody that reacts with a subunit of the 20S core particle, (*rows 2 and 4*, control and oligomycin-treated, respectively). From these experiments we can conclude that an 8h treatment with 5nM oligomycin disassembles/inhibits 26S proteasomes with a parallel increase in 20S proteasomes.

2.4.4. Mitochondria dysfunction causes a selective decline in the Rpn10 subunit of the 19S particle that concurs with calpain but not caspase activation – We considered that the decrease in 26S proteasome levels induced by oligomycin could be caused by a reduction in subunit levels. We thus investigated the effect of oligomycin on subunits of the 19S (Rpn2, Rpn10, Rpt5, and Rpt6) and 20S (α5 and β5) proteasome particles. Notably, from all of the proteasome subunits tested, only Rpn10 levels were reduced by oligomycin in a concentration (Fig. 5A, *top row*) and time (Fig. 5 B, *top row*) dependent manner. Since oligomycin triggers a loss of neuronal viability, we contemplated the possibility that apoptosis was induced and the ensuing caspase activation cleaved Rpn10, thus decreasing its levels. However, we did not observe cleavage of pro-caspase 3 (33kDa) to its active form (17kDa) in neurons treated with

oligomycin (Fig. 5C, *panel 1*). Instead, pro-caspase 3 was truncated to a much larger fragment (Cl-caspase 3, 29kDa) not reflective of caspase 3 activation. We next asked if calpain was activated upon mitochondrial dysfunction by assessing cleavage of one of its substrates, α-spectrin. Oligomycin-treatment clearly induced the cleavage of α-spectrin (280kDa) to a ~150/145kDa doublet, which is indicative of calpain activation (Fig. 5C, *panel 2*). Oligomycin also brought about cleavage of another calpain substrate, the microtubule associated protein TAU, which is the major component of neurofibrillary tangles in AD (Fig. 5D, *panels 1 and 2*). Together, these results suggest that the selective decline in Rpn10 induced by mitochondrial dysfunction could be caused by calpain-mediated degradation.

2.4.5. Upon mitochondrial impairment, Rpn10 cleavage is mediated by calpain and not by proteasomes or lysosomes – To sort out which proteolytic activity cleaves Rpn10 upon treatment with oligomycin, we pretreated neurons with inhibitors of the proteasome (epoxomicin), lysosome (chloroquine), and calpain [calpain inhibitor III (Z-Val-Phe-CHO) and calpeptin (Z-Leu-Nle-CHO)]. From all the protease inhibitors tested, only calpain inhibitors (Fig. 6A, *dashed box*) partially prevented the decline in Rpn10 (*panel 1*). Furthermore, calpain inhibitors blocked cleavage of pro-caspase 3 (*panel 2*), α-spectrin (*panel 3*), and

TAU (*panel 4*). Most importantly, pre-treatment with calpeptin diminished 26S proteasome disassembly brought about by oligomycin (Fig. 6B, *dashed boxes*). It is clear that calpain inhibition opposed the reducing effect of oligomycin on 26S proteasome activity (*panel 1*) and levels (*panels 2 and 3*). Interestingly, treatment of neurons with calpeptin alone (*not shown*) or in combination with oligomycin increased 20S proteasome activity (*panel 1*).

2.4.6. Long-term (7 days) incubations with lower doses (1nM) of oligomycin mimic the effects of acute (up to 16h) treatment with higher (5nM) doses – In the previous studies we investigated the effects of short-term (up to 16h) incubations with oligomycin (5nM). We now treated neurons with a low (one nM) dose of oligomycin for 7 days, to mimic the effect of chronic mitochondrial impairment. It is clear that the same phenomena were observed upon the long 7 day treatment (Fig. 7): 26S proteasome activity (A, *panel 1*) and levels (B, *panel 1*), Ub-proteins (C), and ATP (D) declined. Concurrently, a raise in 20S proteasome activity (A, *panel 2*) and levels (B, *panel 2*) were detected. Note that to be able to detect the 20S proteasome activity, 0.04% SDS was added to the reaction mixture (A, panel 2).

2.5. DISCUSSION

In this study we characterize some of the mechanisms by which mitochondrial toxins (oligomycin, antimycin, and rotenone) affect the ubiquitin/proteasome pathway in cortical neurons. In the first place, we show that mitochondrial impairment slows down the ubiquitination cascade by blocking its first step, i.e. ubiquitin activation via an adenylate intermediate catalyzed by the enzyme E1. Furthermore, E1-E2 transthiolation is halted. These results have implications for the sequence of events leading to chronic neurodegenerative diseases, such as AD, that are characterized by mitochondrial and UPP dysfunction. It is clear that these two phenomena are related in the aforementioned disease states. For therapeutic purposes, it would be important to determine which occurs upstream, UPP or mitochondrial impairment. On one hand, our data show that treatment with the three mitochondrial toxins blocks the ubiquitination cascade thus preventing accumulation of ubiquitinated proteins. On the other hand, it is well established that proteasome inhibition leads to accumulation of ubiquitinated proteins and affects mitochondrial function (Sullivan et al., 2004;Papa and Rockwell, 2008). Together these studies support the notion that UPP impairment may precede mitochondrial dysfunction at least in the neurodegenerative process that leads to accumulation of ubiquitinated proteins.

In the second place, we demonstrate that mitochondrial impairment downregulates 26S proteasomes by promoting their disassembly. Notably, the demise of 26S proteasomes upon treatment of neurons with oligomycin coincides with selective depletion of Rpn10, the only one among all of the proteasome subunits that we tested. Rpn10 is a subunit of the 19S regulatory particle that is a receptor for polyubiquitinated proteins (Glickman et al., 1998;Verma et al., 2004a), and was mapped to the apex of 26S proteasomes (Sakata et al., 2012). Proteasome subunits including Rpn2, Rpn10, and Rpt5 were reported to be cleaved in a caspase-mediated manner to facilitate the apoptotic program (Sun et al., 2004). We show, for the first time to our knowledge, that the decline in Rpn10 upon treatment with oligomycin is calpain-dependent, as it is prevented by pre-treatment with calpain inhibitors and not by proteasome or lysosomal inhibitors. Calpain-activation is linked to ATP-depletion and necrosis, a cell death pathway characterized by a bioenergetic catastrophe (Zong and Thompson, 2006). It is not surprising then that upon ATP-depletion caused by oligomycin-treatment, neurons activate “programmed” necrosis that involves calpain activation. In fact, calpain activation was shown to be induced by electron transport chain inhibitors, such as the ones used in our studies: rotenone, antimycin and oligomycin (Shell and Lawrence, 2012). Besides Rpn10, we

observed calpain-mediated cleavage of two other proteins that are relevant to AD: caspase 3 and the microtubule associated protein TAU. Caspase 3 was cleaved to a fragment (29kDa) that is not associated with its activation. Calpain-mediated truncation of caspase 3 to a non-active form could be a means to prevent execution of the apoptotic pathway under conditions of ATP-depletion, as apoptosis is an energy-dependent death pathway (Zong and Thompson, 2006). TAU is a major component of neurofibrillary tangles in AD and other tauopathies. TAU is reported to be cleaved by caspases and/or calpain depending on the pathological condition (Liu et al., 2011). The dual vulnerability of TAU to calpain and caspases needs to be taken into consideration if preserving TAU integrity is considered a therapeutic goal for AD and the other tauopathies.

In the third place, we establish that the three mitochondrial toxins elevate the activity and level of 20S proteasomes in neurons. 20S proteasomes in concert with immunoproteasomes degrade 70% to 80% of all oxidized proteins that are not aggregated (Pacifici et al., 1989;Pickering et al., 2010). Most oxidized proteins seem not to be ubiquitinated, supporting the notion that they are degraded in a ubiquitin- and ATP-independent manner (Grune et al., 2004;Kastle and Grune, 2011). In contrast, under mild conditions of oxidative stress, the UPP is up-regulated (Shang and Taylor, 2011). Based on these

studies, it seems that recruitment of 26S or 20S proteasomes for degradation of oxidatively modified proteins depends on the level of oxidative stress: under mild conditions, the 26S proteasome is the prevalent degradation machinery, while under harsh conditions the 20S proteasome takes over (Aiken et al., 2011). This difference can be explained by 26S proteasomes and the ubiquitination machinery being more vulnerable to oxidative damage than 20S proteasomes (Reinheckel et al., 1998).

In summary, our findings support an adaptive response of the ubiquitin/proteasome pathway to a decline in ATP due to mitochondrial dysfunction in neurons: the steps of the UPP pathway that are energy-dependent, i.e. the ubiquitination cascade and 26S proteasome function, are down-regulated. Concomitantly, 20S proteasome activity and levels are increased to promote protein degradation in a ubiquitin- and ATP-independent manner. One of the factors that contributes to the switch from 26S to 20S proteasomes is calpain activation as it cleaves Rpn10, a subunit of the 19S particle. Rpn10 could act as a 26S proteasome gatekeeper to promote its disassembly when ATP is deficient. Under these conditions, upgrading 20S proteasome levels and activity would ensure efficient protein degradation without energy expenditure. Reduction in ubiquitinated proteins and 26S proteasomes as well as the increase in 20S proteasomes in neurons was observed upon acute and long-term mitochondrial

impairment. Under acute stress conditions, unregulated protein degradation as carried-out by 20S proteasomes may be advantageous to promote degradation of randomly unfolded oxidized proteins. However, regulated protein degradation by the ubiquitin/proteasome pathway is essential for long-term neuronal survival.

Figure legends

Figure 1 – Effect of three mitochondrial inhibitors, oligomycin, antimycin and rotenone on ATP levels and viability of rat cerebral cortical neurons. Neurons were treated with oligomycin (*panels 1 and 2*), antimycin (*panels 3*) and rotenone (*panels 4*) for the times and concentrations indicated. **(A)** ATP steady state levels (pmoles/μg of protein) were assessed with the sensitive luciferin/luciferase system. Data represent the mean and s.e. from at least three experiments per group. **(B)** Cell viability was assessed with the MTT assay. Percentages represent the ratio between the data for each condition and control (100%). Values indicate means and s.e. from at least 7 determinations. Asterisks identify values that are significantly different from control (* $p<0.05$; ** $p<0.01$; *** *$p<0.001$*).

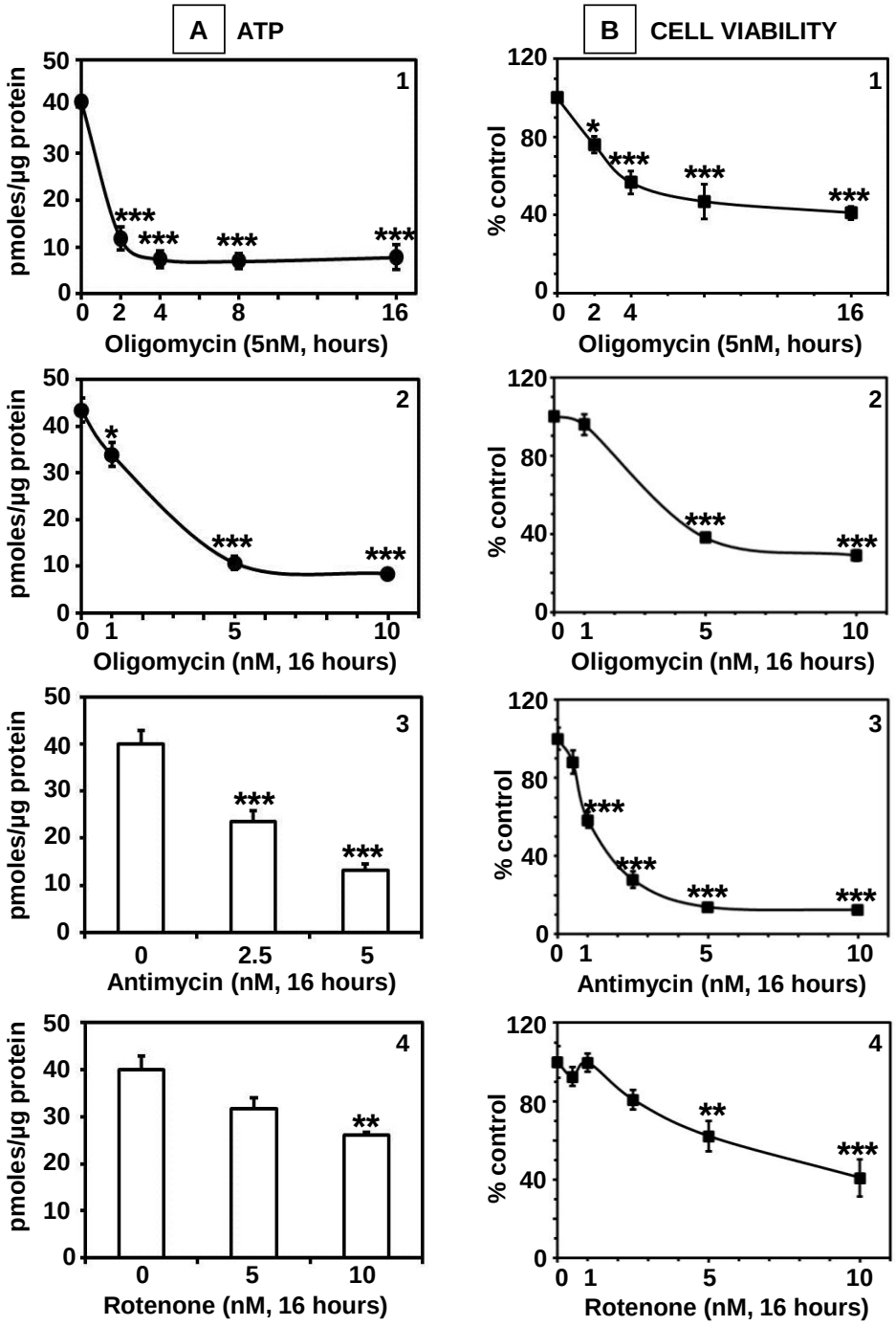
A
ATP
B
CELL VIABILITY
pmoles/µg protein
% control
Oligomycin (5nM, hours)
Oligomycin (nM, 16 hours)
Antimycin (nM, 16 hours)
Rotenone (nM, 16 hours)
1
2
3
4

Figure 1

Figure 2 – Effect of three mitochondrial inhibitors, oligomycin, antimycin and rotenone on ubiquitinated proteins and free ubiquitin in rat cerebral cortical neurons (A, B, and C). Effect of oligomycin on the levels of E1- and E2-ubiquitin thiol esters (D). Effect of epoxomicin (proteasome inhibitor) on ubiquitinated proteins (E). Total lysates from rat E18 cerebral cortical neurons treated as indicated were analyzed by western blotting (30 µg of protein/lane) probed with the respective antibodies to detect in: **(A, B and C)** - Ub-proteins (*panels 1*), free ubiquitin (*panels 2*), and βIII-tubulin (loading control, *panels 3*); **(D)** - E1-ubiquitin (Ub) and E2-Ub thiol esters (*upper bands*) and native E1 and E2 (*lower bands)* run under non-reducing conditions (*left side, panels 1 and 2*); E1 and E2 run under reducing conditions with β-mercaptoethanol (*right side, panels 1 and 2*); actin (loading control, *panels 3*); **(E)** - Ub-proteins and actin (loading control). Molecular mass markers in kDa are shown on the left (A) and right (C, D and E). In A, B and C the levels of ubiquitinated proteins (polyUb, *black squares*) and free ubiquitin (monoUb, *white squares*) were semi-quantified by densitometry (*panels 4*). Data represent the percentage of the pixel ratio for Ub-proteins and free ubiquitin (mono) over βIII-tubulin (βIII-tub) for each condition compared to control (100%). Values are means and s.e. from three experiments. Asterisks

identify values that are significantly different from control (*p<0.05, **p<0.01, ***p<0.001).

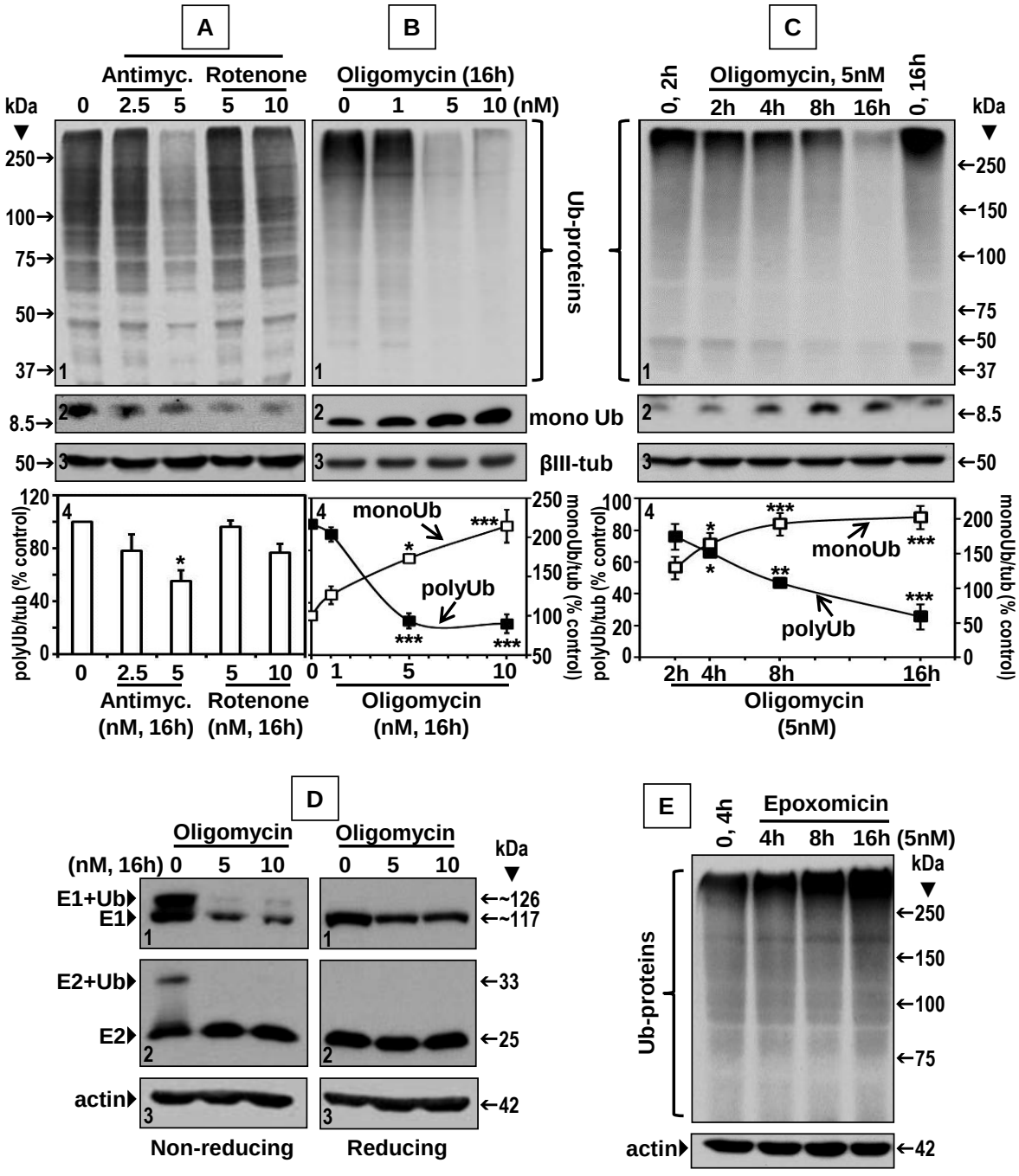

Figure 2

Figure 3 – Effect of three mitochondrial inhibitors, oligomycin, antimycin and rotenone on proteasome activity and levels in rat cerebral cortical neurons. To assess changes in proteasome activity, cell extracts were prepared from rat E18 cerebral cortical neurons treated for 16h with antimycin or rotenone **(A)** or oligomycin **(B)**, or with 5nM oligomycin for different time points **(C)**. Clear lysates (30µg/sample) were subjected to non-denaturing gel electrophoresis as described under "Experimental Procedures". 26S and 20S proteasomal (*indicated in the middle by arrows*) chymotrypsin-like activity was assessed with Suc-LLVY-AMC by the in gel assay (*panels 1*). Proteasome levels were detected by immunoblotting with anti-Rpt6 (*panels 2*) and anti-β5 antibodies (*panels 3*). Proteasome chymotrypsin-like activity (*panels 5*) and levels (*panels 6*) were semi-quantified by densitometry [26S(2), *black squares*, 26S (1), *gray squares* and 20S, *white circles*].Percentages represent the ratio between data for each condition and control (DMSO) considered to be 100%. Values in B and C are means and s.e. from three experiments, and in A from a representative experiment from three.

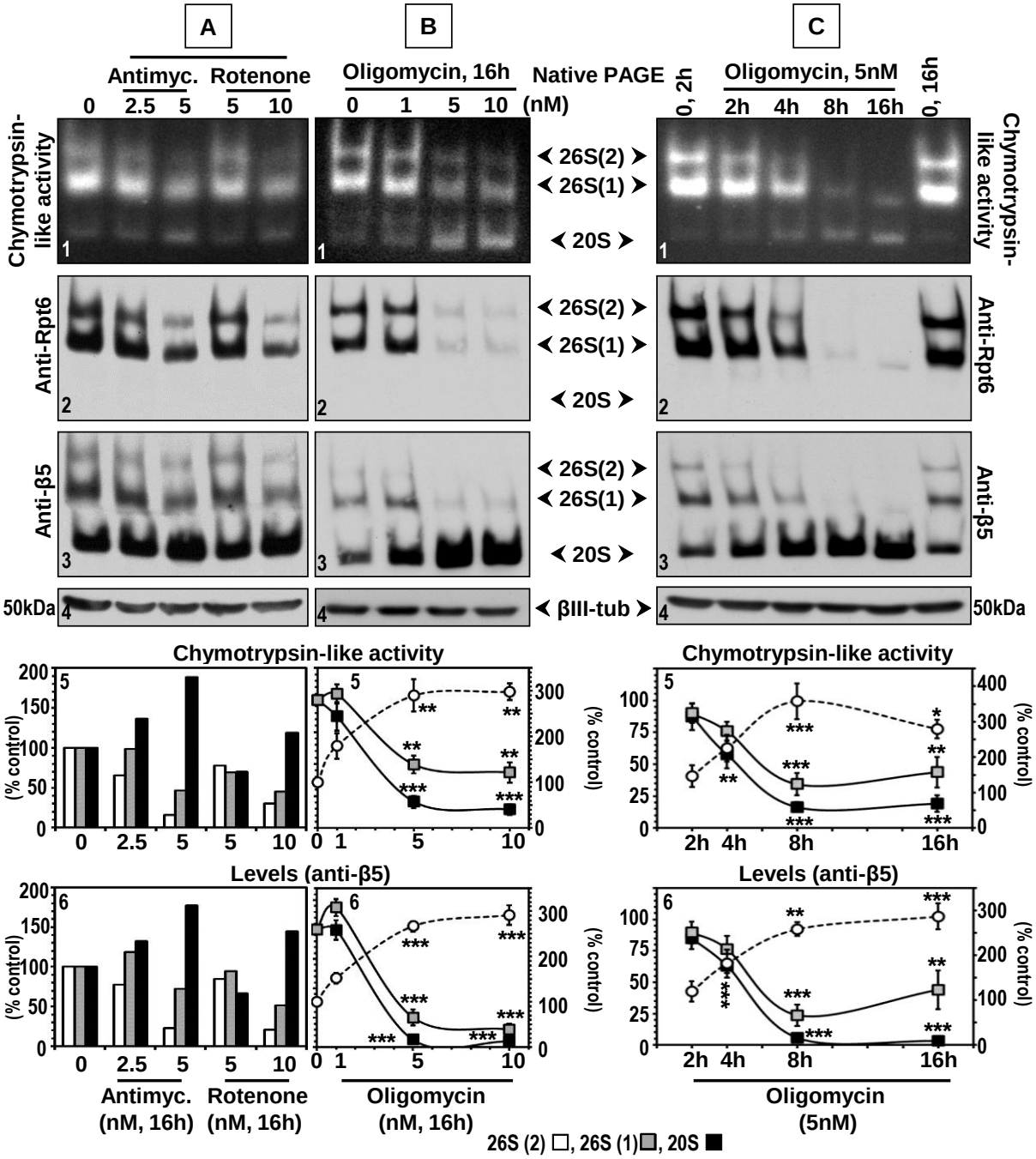
A
B
C
Antimyc.
Rotenone
Oligomycin, 16h
Native PAGE
(nM)
Oligomycin, 5nM
Chymotrypsin-like activity
Anti-Rpt6
Anti-β5
26S(2)
26S(1)
20S
βIII-tub
50kDa
Levels (anti-β5)
(% control)
Antimyc. (nM, 16h)
Rotenone (nM, 16h)
Oligomycin (nM, 16h)
Oligomycin (5nM)
26S (2) □, 26S (1)▒, 20S ■

Figure 3

Figure 4 – Effect of oligomycin on the sedimentation velocity of proteasomes in rat cerebral cortical neurons. Total lysates (one mg protein/sample) were fractionated by glycerol density gradient centrifugation (10-40% glycerol corresponding to fractions 1 to 24). Aliquots (50μl) of each fraction obtained from control (Ct, *black squares*), and oligomycin (5nM, 8h, *white squares*) treated cells were assayed for chymotrypsin-like activity with Suc-LLVY-AMC (*top graph*). Immunoblot analyses of each fraction were probed with antibodies that react with Rpt6 (19S regulatory particle, *panels 1 and 3*) or α5, (core particle, *panels 2 and 4*). Proteins were precipitated with acetone from 450μl of each fraction. Similar results were obtained in triplicate experiments.

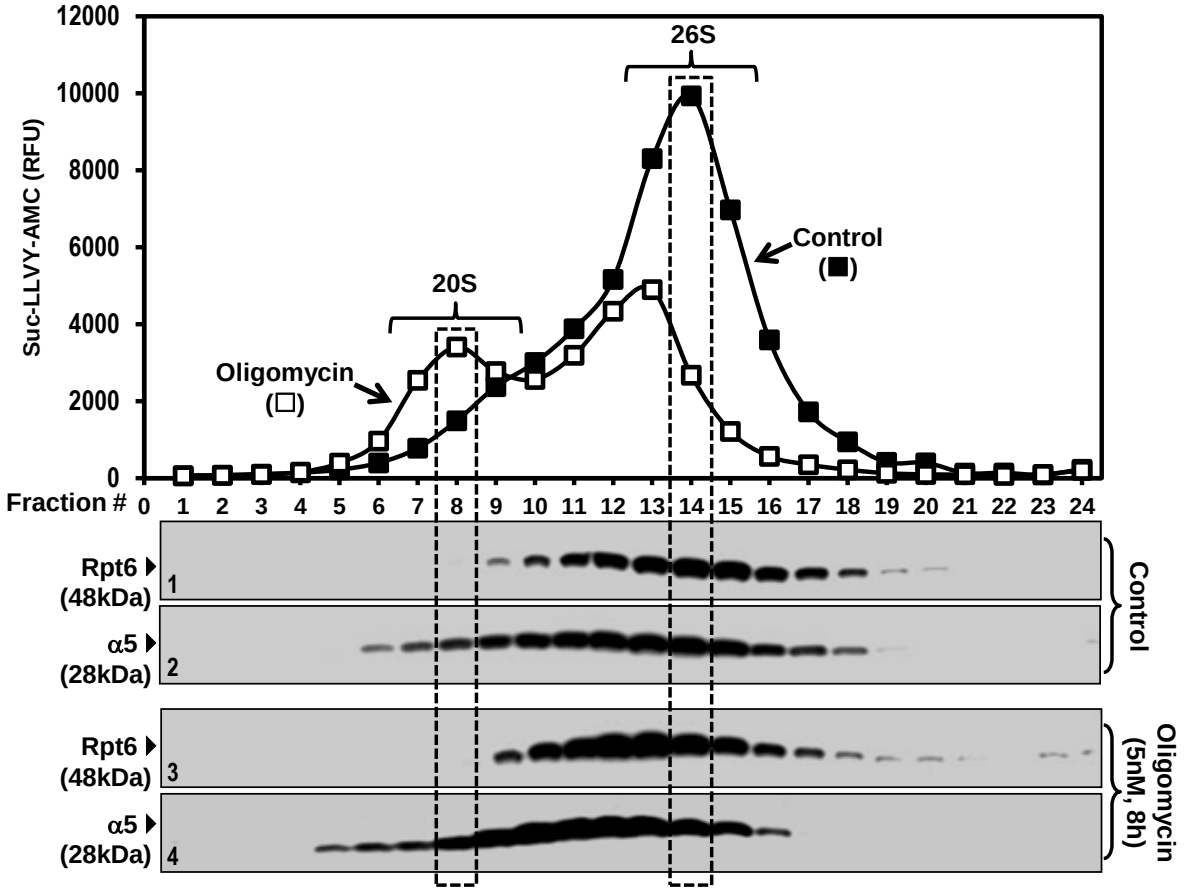
12000
10000
8000
6000
4000
2000
0
Suc-LLVY-AMC (RFU)
26S
20S
Control
(■)
Oligomycin
(□)
Fraction #
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Rpt6
(48kDa)
1
α5
(28kDa)
2
Control
Rpt6
(48kDa)
3
α5
(28kDa)
4
Oligomycin
(5nM, 8h)

Figure 4

Figure 5 – Effect of oligomycin on proteasome subunit levels (A and B), caspase 3 and α-spectrin (C), as well as TAU cleavage (D) in rat cerebral cortical neurons. Total lysates from rat E18 cerebral cortical neurons treated as indicated were analyzed by western blotting (30 µg of protein/lane) probed with the respective antibodies to detect in: **(A and B)** - proteasome subunits (19S particle: Rpn10, Rpn2, Rpt5 and Rpt6; 20S core: α5 and β5) and βIII-tubulin (loading control); **(C)** – caspase 3 (*panel 1*) and α-spectrin (*panel 2*); **(D)** – TAU (*panels 1 and 2*) and actin (loading control, *panel 3*). Molecular mass markers in kDa are shown in the center (A and B) and on the left (C and D). Similar data were obtained from at least duplicate experiments. Cas3, caspase 3; Tau FL, full length TAU.

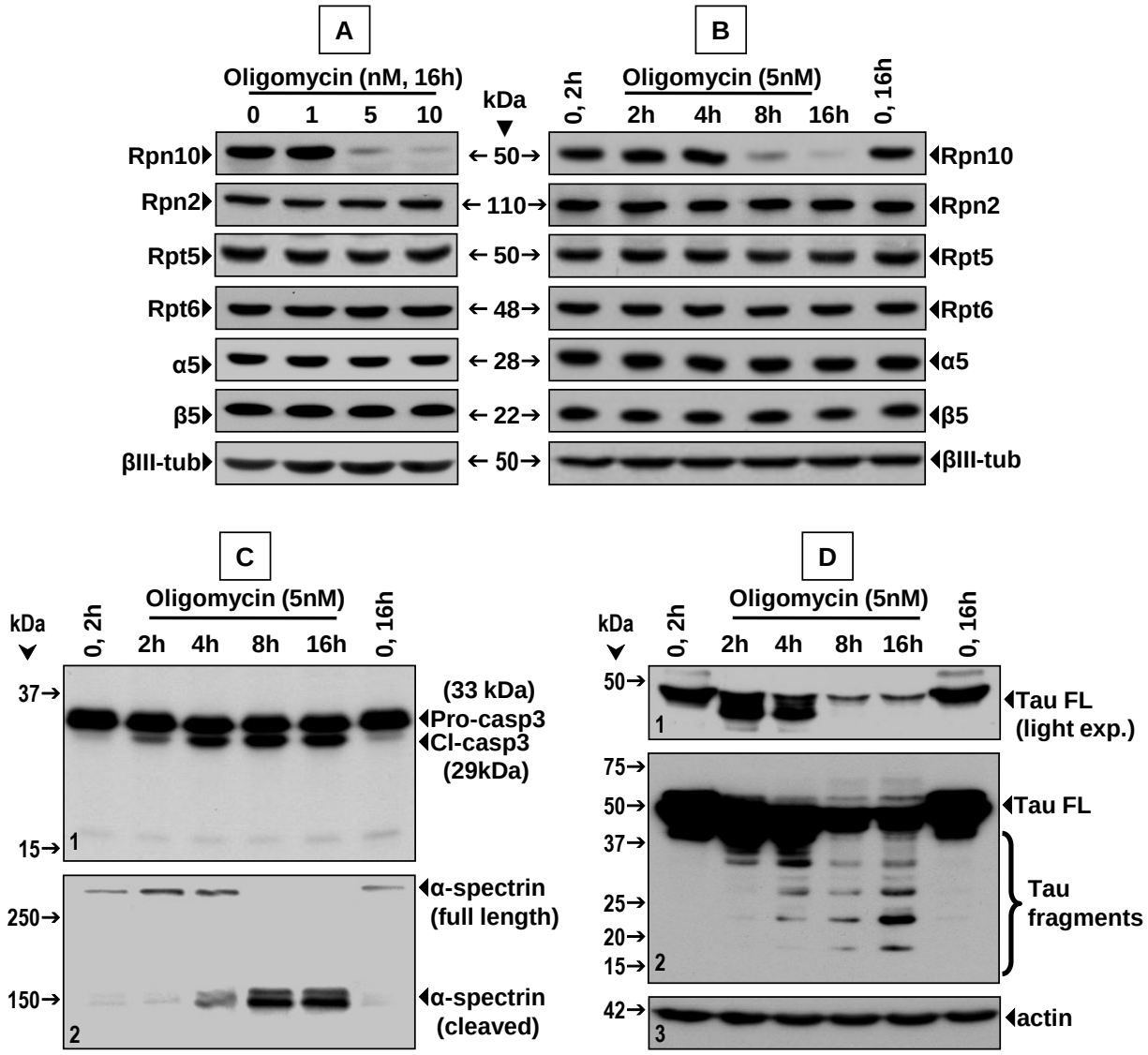
A
Oligomycin (nM, 16h)
0
1
5
10
kDa
Rpn10
Rpn2
Rpt5
Rpt6
α5
β5
βIII-tub
50
110
50
48
28
22
50
B
Oligomycin (5nM)
0, 2h
2h
4h
8h
16h
0, 16h
Rpn10
Rpn2
Rpt5
Rpt6
α5
β5
βIII-tub
C
Oligomycin (5nM)
kDa
0, 2h
2h
4h
8h
16h
0, 16h
37
15
(33 kDa)
Pro-casp3
Cl-casp3
(29kDa)
1
250
150
α-spectrin
(full length)
α-spectrin
(cleaved)
2
D
Oligomycin (5nM)
kDa
0, 2h
2h
4h
8h
16h
0, 16h
50
1
Tau FL
(light exp.)
75
50
37
25
20
15
Tau FL
Tau
fragments
2
42
actin
3

Figure 5

Figure 6 – Calpain inhibitors but not proteasome or lysosomal inhibitors prevent/diminish the effects of oligomycin on Rpn10, caspase 3, α-spectrin, TAU, as well as proteasome activity and levels in rat cerebral cortical neurons. Total lysates from rat E18 cerebral cortical neurons were analyzed in: **(A)** - by western blotting (30 µg of protein/lane) probed with the respective antibodies to detect Rpn10 (*panel 1*), caspase 3 (casp3, *panel 2*), α-spectrin (*panel 3*), TAU (*panel 4*) and actin (loading control, *panel 5*). Molecular mass markers in kDa are shown on the right. **(B)** – by the in gel assay (30µg/sample) to assess 26S and 20S proteasome (*indicated in the middle by arrows*) chymotrypsin-like activity (*panel 1*) and levels detected by immunoblotting with anti-Rpt6 (*panel 2*) and anti-β5 antibodies (*panel 3*). Neurons were pre-treated for 30min with epoxomicin (10nM and 20nM ep, proteasome inhibitor), chloroquine (10µM CQ, lysosomal inhibitor), calpain inhibitor III (20µM Cp3, Z-Val-Phe-CHO), calpeptin (20µM Cpt, calpain inhibitor Z-Leu-Nle-CHO), and then with oligomycin (5nM, 8h). Similar data were obtained in duplicate experiments. Tau FL, full length TAU. The numbers at the bottom of panels 1, 2 and 3 represent 26S and 20S proteasomal chymotrypsin-like activity and levels, respectively, under each treatment condition. Percentages represent the ratio between data for each condition and control (DMSO) considered to be 100%. Values are from a representative experiment of three.

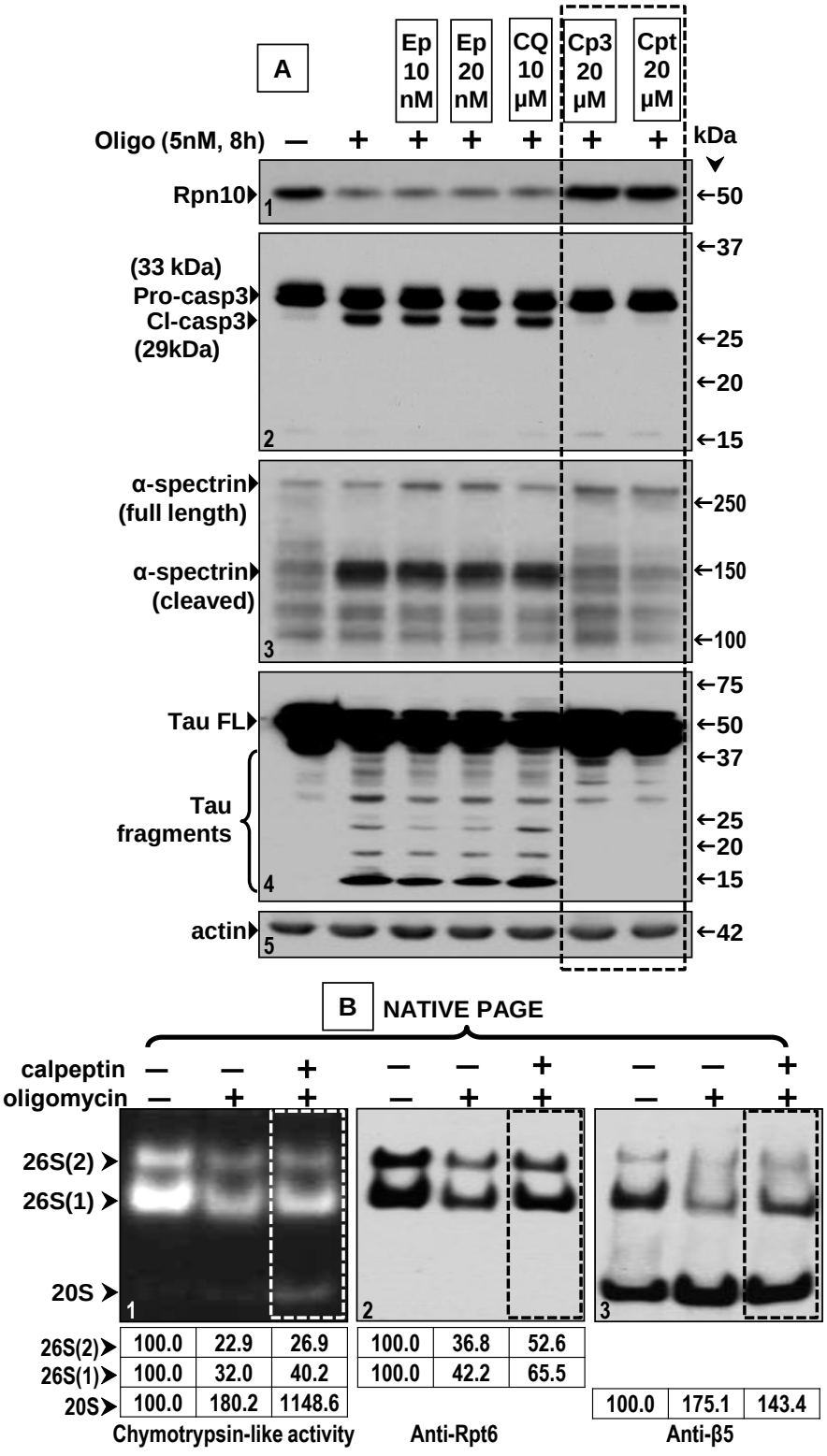
A
Ep 10 nM
Ep 20 nM
CQ 10 μM
Cp3 20 μM
Cpt 20 μM
Oligo (5nM, 8h) — + + + + + +
kDa
Rpn10
1
←50
(33 kDa)
Pro-casp3
Cl-casp3
(29kDa)
2
←37
←25
←20
←15
α-spectrin
(full length)
α-spectrin
(cleaved)
3
←250
←150
←100
Tau FL
Tau
fragments
4
←75
←50
←37
←25
←20
←15
actin
5
←42
B
NATIVE PAGE
calpeptin — — + — — + — — +
oligomycin — + + — + + — + +
26S(2)
26S(1)
20S
1
2
3
26S(2) 100.0 22.9 26.9 | 100.0 36.8 52.6
26S(1) 100.0 32.0 40.2 | 100.0 42.2 65.5
20S 100.0 180.2 1148.6 | 100.0 175.1 143.4
Chymotrypsin-like activity
Anti-Rpt6
Anti-β5

Figure 6

Figure 7 – Effect of long-term incubations (up to 7 days) with oligomycin on rat cerebral cortical neurons. Rat E18 cerebral cortical neurons were treated with oligomycin (1nM) up to 7 days). Total lysates were analyzed by the in gel assay (30µg/sample) to assess 26S and 20S proteasome **(A)** chymotrypsin-like activity (*panels 1 and 2*), and **(B)** levels detected by immunoblotting with anti-Rpt6 (*panel 1*), anti-β5 (*panel 2*) and anti-βIII-tubulin (βIII-tub, loading control, *panel 3*) antibodies. 26S and 20S proteasomes are indicated in the middle by arrows. In **(C)** the lysates were analyzed by western blotting (30 µg of protein/lane) to assess ubiquitinated proteins (Ub-proteins). Molecular mass markers in kDa are shown on the left. In A, B and C, similar data were obtained in duplicate experiments. In **(D)** ATP steady state levels (pmoles/µg of protein) were assessed with the sensitive luciferin/luciferase system. Data represent the mean and s.d. from two experiments per group. Asterisks identify values that are significantly different from control (* $p<0.05$; ** $p<0.01$).

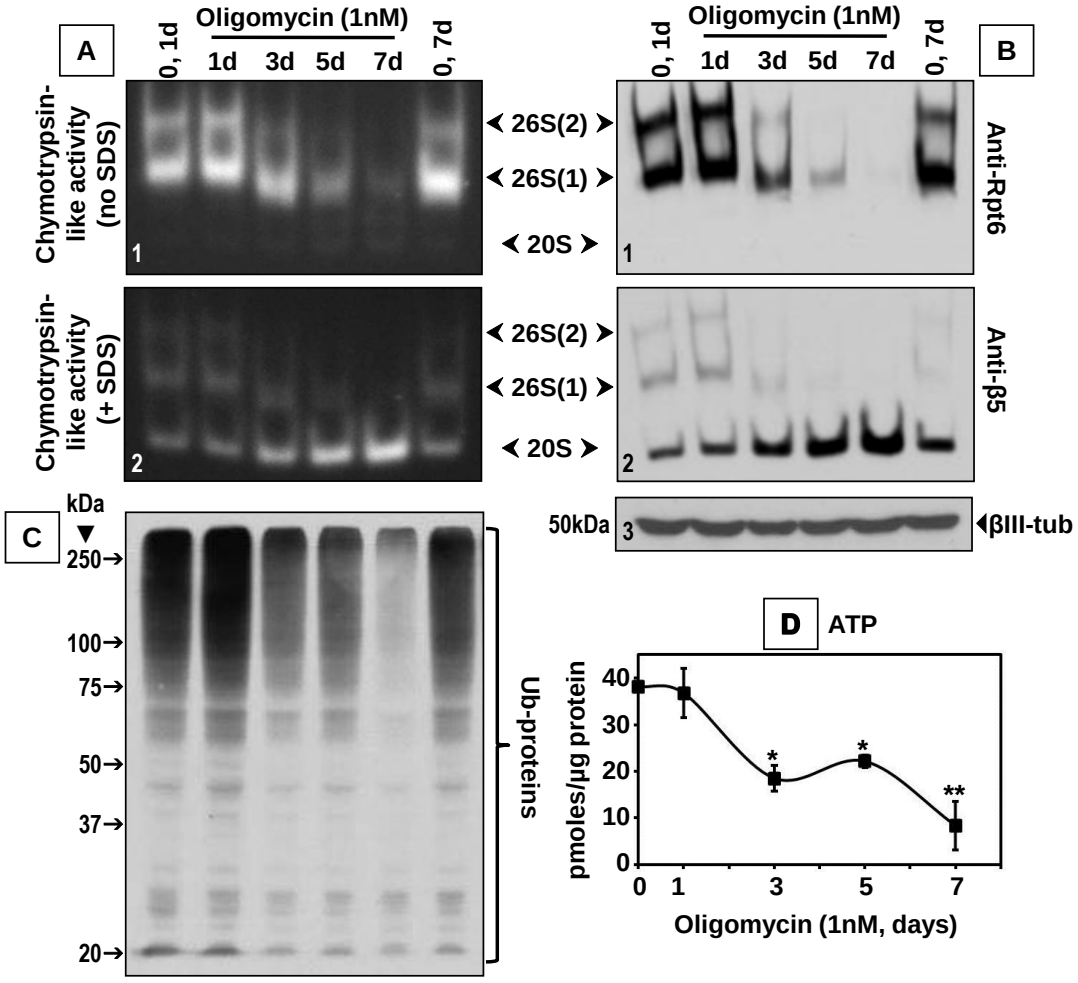
A
Oligomycin (1nM)
0, 1d
1d
3d
5d
7d
0, 7d
Chymotrypsin-like activity (no SDS)
Chymotrypsin-like activity (+ SDS)
26S(2)
26S(1)
20S
1
2
B
Anti-Rpt6
Anti-β5
50kDa
3
βIII-tub
C
kDa
250
100
75
50
37
20
Ub-proteins
D
ATP
pmoles/μg protein
40
30
20
10
0
Oligomycin (1nM, days)

Figure 7

CHAPTER III

COORDINATION BETWEEN PROTEASOME IMPAIRMENT AND CASPASE ACTIVATION LEADING TO TAU PATHOLOGY: NEUROPROTECTION BY cAMP

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Hunter College of City University of New York,

New York, NY 10065

FROM

Cell Death and Disease (in press)

Qian Huang conducted the Epoxomicin experiments and triple administrations of db-cAMP and PACAP27 against PGJ2 toxicity

3.1. ABSTRACT

Neurofibrillary tangles (NFTs) are hallmarks of Alzheimer disease (AD). The main component of NFTs is TAU, a highly soluble microtubule-associated protein. However, when TAU is cleaved at Asp421 by caspases it becomes prone to aggregation leading to NFTs. What triggers caspase-activation resulting in TAU cleavage remains unclear. We investigated in rat cortical neurons a potential coordination between proteasome impairment and caspase-activation. We demonstrate that upon proteasome inhibition the early accumulation of detergent-soluble ubiquitinated (SUb)-proteins paves the way to caspase-activation and TAU pathology. This occurs with two drugs that inhibit the proteasome by different means: the product of inflammation prostaglandin J2 (PGJ2) and epoxomicin. Our results pinpoint a critical early event, i.e. the build-up of SUb-proteins that contributes to caspase-activation, TAU cleavage, TAU/Ub-protein aggregation, and neuronal death. Furthermore, to our knowledge we are the first to demonstrate that elevating cAMP in neurons with dibutyryl-cAMP or the lipophilic peptide PACAP27, prevents/diminishes caspase-activation, TAU-cleavage and neuronal death induced by PGJ2, as long as these PGJ2-induced changes are moderate. Dibutyryl-cAMP also stimulated proteasomes, and mitigated proteasome inhibition induced by PGJ2. We propose that targeting cAMP/PKA

to boost proteasome activity in a sustainable manner, could offer an effective approach to avoid early accumulation of SUb-proteins and later caspase-activation and TAU cleavage, possibly preventing/delaying AD neurodegeneration.

3.2. INTRODUCTION

Alzheimer disease (AD) is an age-related neurodegenerative disorder. Little is known about the initial pathology, and when symptoms are detected, neurodegeneration is so advanced that it is seldom reversible. Neurofibrillary tangles (NFTs) are pathological hallmarks of AD. The major component of NFTs is TAU, a microtubule associated protein that is abundant in neurons and highly soluble; yet TAU aggregates abnormally in AD (Mandelkow et al., 2007). Truncation of TAU at Asp421 (ΔTAU) by caspases is an early event in AD tangle pathology (Gamblin et al., 2003;Rissman et al., 2004;de Calignon et al., 2010). In addition, ΔTAU is detected in NFTs indicating that the apoptotic cascade is involved in NFT formation (Cotman et al., 2005).

The initial events leading to caspase-activation and ΔTAU are poorly defined. We propose that proteasome impairment could be one of the initial critical events that contributes and leads to caspase-activation concurring with ΔTAU, protein aggregation, and neuronal death. There is a general agreement that proteasome

impairment is involved in the pathogenesis of AD. Defective proteasome activity is connected to the early phase of AD characterized by synaptic dysfunction, as well as to late AD stages linked to accumulation and aggregation of ubiquitinated (Ub) proteins in both senile plaques and NFTs (Upadhya and Hegde, 2007;Oddo, 2008).

To investigate a potential coordination between proteasome impairment and caspase-activation leading to TAU pathology, we treated rat cerebral cortical neurons with two drugs that inhibit the proteasome by different means: prostaglandin J2 (PGJ2) and epoxomicin. PGJ2 is an endogenous product of inflammation that inhibits the proteasome by inducing oxidation of its subunit S6 ATPase (Rpt5) (Ishii et al., 2005), and/or by promoting dissociation of the 26S proteasomes (Wang et al., 2006). By promoting 26S proteasome disassembly, PGJ2 resembles the effects of agents that induce oxidative stress (Aiken et al., 2011). We recently demonstrated in rat cortical neurons, that PGJ2 induces accumulation of Ub-proteins, caspase-activation, ΔTAU and its aggregation as well as neuritic dystrophy (Arnaud et al., 2009). Epoxomicin is a specific and irreversible inhibitor of the proteasome that forms a covalent adduct with the amino terminal Thr of the 20S proteasome catalytic subunits, generating irreversible morpholino adducts (Groll et al., 2000). Other proteasome inhibitors, i.e. MG132 and lactacystin, were

shown to induce apoptosis via caspase-activation in rat cortical neurons, but Ub-protein accumulation/aggregation and TAU pathology were not addressed (Qiu et al., 2000).

We report now that in rat cortical neurons, the build-up of detergent (NP40)-soluble ubiquitinated (SUb)-proteins induced by PGJ2 or epoxomicin was detected significantly earlier than caspase-activation, ΔTAU and TAU/Ub-protein aggregation. In addition, to our knowledge we are the first to report that elevating cAMP via treatment with a single dose of dibutyryl-cAMP (db-cAMP) or the lipophilic peptide PACAP27, prevents caspase-activation, ΔTAU, and protein aggregation induced by short-term incubations (up to 8h) with PGJ2. In the cortical neurons, db-cAMP alone also increased 26S proteasome activity significantly, and reduced 26S proteasome inhibition by PGJ2. Furthermore, db-cAMP and PACAP27 offered neuroprotection against short-term incubations with PGJ2. In long-term studies (24h), three consecutive doses of the cAMP-elevating drugs administered 4h apart, reduced changes induced by PGJ2. Altogether, these data support the notion that targeting the cAMP/PKA pathway to stimulate 26S proteasome activity in a robust and sustainable manner, could prevent the early accumulation of SUb-proteins, and later avoid caspase-activation leading to TAU cleavage and TAU pathology. If applied early before neurons reach a point of no return, elevating cAMP could be an effective therapeutic

strategy to prevent/delay neurodegeneration associated with protein aggregation in AD.

3.3. MATERIALS AND METHODS

3.3.1. Materials - Prostaglandin J2 was from Cayman Chemical (Ann Arbor, MI, USA) and epoxomicin from Peptides International Inc. (Louisville, KY). Adenosine 3′, 5′-cyclic monophosphate dibutyryl sodium salt (db-cAMP), was from Calbiochem/EMD Bioscience (Gibbstown, NJ). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was from Sigma-Aldrich (St. Louis, MO). The substrate Suc-LLVY-AMC and the peptide PACAP27 (pituitary adenylate cyclase activating polypeptide-27) were from BACHEM Bioscience Inc. (King of Prussia, PA). *Antibodies:* rabbit polyclonal anti-Ub-proteins (1:1,500, cat# Z0458, Dako North America, Carpinteria, CA); rabbit polyclonal anti-β5 (1:1,000, cat# PW8895) from BIOMOL, Plymouth Meeting, PA; mouse monoclonal anti-β-actin (1:10,000, cat# A2228, Sigma, Saint Louis, MO); mouse monoclonal TAU C3 (1:5,000; detects TAU cleaved at Asp421; ep: a.a. 412–421) and mouse monoclonal TAU C5 (1:50,000; detects all TAU isoforms and ΔTAU; ep: a.a. 210–241) were courtesy of Dr. L. Binder (Northwestern University, Chicago, IL, USA); rabbit polyclonal anti-caspase 3 (1:1000, cat# 9662, Cell Signaling Technology, Danvers, MA). The respective secondary antibodies with HRP

conjugate (1:10,000) were from Bio-Rad Laboratories (Hercules, CA).

3.3.2. Cell cultures - *Dissociated* cultures from Sprague Dawley rat embryonic (E18, both sexes) cerebral *cortical neurons* were prepared as follows: the isolated cortices free of meninges were digested with papain (0.5mg/ml from Worthington Biochemical Corp., Lakewood, NJ) in Hibernate E without calcium (BrainBits LLC., Springfield, IL) at 37°C for 30min in a humidified atmosphere containing 5% CO2. After removal of the enzymatic solution, the tissues were gently dissociated in Neurobasal media (Invitrogen, Carlsbad, CA). Dissociated tissues were centrifuged at 300Xg for 2min. The pellet was resuspended in Neurobasal media without antibiotics and plated on 10cm dishes pre-coated with 50µg/mL poly-D-lysine (Sigma, Saint Louis, MO). *Cells* were plated at a density of $6X10^6$ cells per 10cm dish, or $2.5X10^5$ cells per well on 24-well plates (cell viability only). Cultures were maintained in Neurobasal media supplemented with 2% B27 and 0.5mM glutamine (all from Invitrogen) at 37°C in a humidified atmosphere containing 5% CO2. Half of the medium was changed every 4 days.

3.3.3. Culture treatments – Cortical neurons were treated for 4h, 8h, 16h, 24h or 48h with DMSO or ultra pure filtered

water (controls) or with different drugs: PGJ2 and epoxomicin in DMSO; db-cAMP and PACAP27 in ultra pure filtered water added directly to DMEM without serum supplemented with 0.5mM glutamine and 1mM sodium pyruvate (all from Invitrogen). The final DMSO concentration in the medium was 0.5%. For a single administration, db-cAMP was added to the cultures 30min prior to PGJ2, while PACAP27 was added in conjunction with PGJ2. For triple (3X) administrations, each of the cAMP-elevating drugs was added firstly as for the single administration, and then 4h and 8h after that. PGJ2 was added only once at the beginning of the treatment. At the end of the incubations, all cultures were washed twice with phosphate buffered saline (PBS) and processed for the different assays as described below.

3.3.4. Cell viability assay - Cells were treated with DMSO (0, control, vehicle for PGJ2), or PGJ2 (5µM or 10µM) in conjunction with three consecutive doses of water (control, vehicle for db-cAMP and PACAP27), db-cAMP (1mM, db-cAMP) or PACAP27 (100nM) over a period of 24h. Cell viability was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described in (Mosmann, 1983).

3.3.5. Western blotting - After treatment, cells were rinsed twice with PBS and harvested by gently scraping into ice-

cold lysis buffer [20mM Tris-HCl, pH 7.5, 137mM NaCl, 1mM EGTA, 2.5mM $Na_4P_2O_7$,1mM β-glycerophosphate, 50mM NaF, 1mM phenylmethylsulfonyl fluoride, 1% NP40, 1mM Na_3VO_4, 1% Glycerol and protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO)]. Following lysis (at least 30min, -80°C), cell extracts were centrifuged (19,000xg for 10min) at 4°C for separation into two fractions: NP40-soluble (supernatant) and NP40-insoluble (pellet). The pellet was resuspended in a buffer containing 1% SDS and 10mM Tris-EDTA pH 7.5. Protein concentration of both fractions was determined (BCA kit, Pierce, Rockf., IL). Note that we consider soluble ubiquitinated (SUb)-proteins those that are NP40-soluble, and Ub-aggregates those that are NP40-insoluble as well as detected by the filter trap assay described below. Western blot analysis was carried out following SDS-PAGE. Normalized samples were boiled for 5min in Laemmli buffer and loaded onto gels (40µg of protein/lane). Following electrophoresis, proteins were transferred onto an Immobilon-P membrane (Millipore, Bedford, MA, USA). The membrane was probed with the respective antibodies, and antigens were visualized by a standard chemiluminescent horseradish peroxidase method with the ECL reagent. Semi-quantitative analysis of protein detection was done by image analysis with the ImageJ program (Rasband, W.S., ImageJ, U.S. NIH, Maryland, http://rsb.info.nih.gov/ij/, 1997-2006).

3.3.6. Filter trap assay - After treatment, media was removed and cells were lysed as for western blotting. Samples were normalized to 0.5μg/μl using a buffer containing 2% SDS and 10mM Tris-EDTA pH 7.5. From the different extracts, 50μg of proteins were filtered through a 0.2μm nitrocellulose membrane (BIO-RAD, Hercules, CA) using a 96-well dot blot apparatus (Schleicher & Scheull Inc.). Each well was washed twice with washing buffer containing 0.1% SDS and 10mM Tris-EDTA, pH 7.5. Captured aggregates were detected by immunoblotting using the anti-ubiquitin and the TAU C3 antibodies.

3.3.7. In-gel proteasome activity and levels - Upon treatment with vehicle (control, DMSO or water) or the respective drugs, cells were washed twice with PBS and harvested for the in-gel assay as described in (Wang et al., 2006). The native gels loaded with 30μg protein/lane, were run at 150V for 120min. The in-gel proteasome activity was detected by incubating the native gel on a rocker for 10min at 37^{o}C with 15ml of 300μM Suc-LLVY-AMC followed by exposure to UV light (360nm). Gels were photographed with a NIKON Cool Pix 8700 camera with a 3-4219 fluorescent green filter (Peca Products, Inc). Proteins on the native gels were transferred (110mA) for 2h onto PVDF membranes. Immunoblotting was carried-out for detection of the

20S and 26S proteasomes with the anti-β5 subunit antibody, which reacts with a subunit of the 20S core particle, therefore detects 26S and 20S proteasomes. The antigen was visualized by a chemiluminescent horseradish peroxidase method with the ECL reagent.

3.3.8. Statistical analysis - Statistical significance was estimated using one-way ANOVA (Tukey-Kramer multiple comparison test) with the Instat 2.0, Graphpad Software (San Diego, Ca).

3.4. RESULTS

3.4.1. Detergent (NP40)-soluble ubiquitinated proteins accumulate early upon proteasome inhibition, and prior to caspase-activation as well as ΔTAU formation – We investigated a temporal correlation between proteasome inhibition and caspase-activation by treating rat cortical neurons with PGJ2 (Fig.1A) or epoxomicin (Fig. 1B). The time-course studies clearly demonstrate that detergent (NP40) soluble ubiquitinated (SUb)-proteins are detected upon a 4h treatment with 20μM PGJ2 or 20nM epoxomicin (Fig. 1A and 1B, panels 1), the earliest time point analyzed. Robust aggregates of Ub-proteins as well as caspase 3-activation, ΔTAU formation, and TAU aggregates (Fig. 1A & 1B, panels 2 to 6, respectively) were detected much later, i.e. after at least 16h of treatment. Large (>0.2μm), SDS-insoluble

Ub- and TAU-aggregates were assessed with the filter trap assay. Remarkably, the decline in SUb-proteins observed at 16h and 24h, corresponds to a robust increase in Ub-aggregates. SUb-proteins are considered here to be those that are NP40-soluble, and Ub-aggregates those that are NP40-insoluble as well as those that are retained with the filter trap assay.

It is important to clarify that the TAU C3 antibody, which specifically detects TAU cleaved at Asp421 (ΔTAU, epitope a.a. 412–421), reacts with two bands. The upper band is often detected under control conditions, i.e. in cells treated with DMSO (vehicle) alone. The lower band concurs with TAU aggregates and appears only under conditions of robust caspase-activation (see Fig. 1A & 1B, panels 4 and 5). We thus consider the lower band to be the major product of caspase-cleavage of TAU at Asp421. Probing the western blots with the TAU C5 antibody (epitope a.a. 210–241, Fig. 1A & 1B, panels 7), detected all full length TAU isoforms (Tau FL) as well as ΔTAU, the latter with a pattern similar to the one obtained with the TAU C3 antibody.

Notably, proteasome inhibition for 48h with epoxomicin did not increase the level of full length TAU nor did it generate high molecular weight forms of TAU corresponding to Ub-TAU (Fig. 1B, panels 7). Instead, epoxomicin caused an accumulation of various TAU fragments ranging in size between 37kDa and 15kDa,

thus smaller than ΔTAU. These data indicate that upon TAU cleavage at Asp421 by caspases, TAU is further cleaved by unidentified proteases that generate smaller fragments detected upon proteasome inhibition. Actin levels (Fig. 1A & 1B, panels 8) were not altered by the treatments.

We assessed with the native in gel assay, the effects of PGJ2 and epoxomicin on proteasome activity and levels in the cortical neurons. This assay detects the three native proteasome forms: 26S with two regulatory caps [26S (2)] or one cap [26S (1)], and the 20S core particle alone (20S). Proteasome chymotrypsin-like activity was determined with the substrate Suc-LLVY-AMC (Fig. 1A & 1B, panels 10). Under control conditions (first lanes), the activity of the 20S proteasome is substantially lower than the 26S, because the 20S is a latent form of the proteasome (Goldberg, 2007). Proteasome levels were established by immunoblotting with an anti-β5 antibody (Fig. 1A & 1B, panels 11). The β5 subunit is a component of the 20S core, thus the antibody detects both 26S and the 20S proteasomes. PGJ2 and epoxomicin inhibited the 26S proteasome in a manner that parallels the accumulation of Ub-proteins induced by both drugs. Furthermore, PGJ2 caused 26S proteasome disassembly while 20S proteasome levels increased (Fig. 1A, panel 11). We used lower epoxomicin concentrations in this assay to be able to determine

the gradual time-dependent decrease in proteasome activity, which by 16h was low (Fig. 1B, panel 10).

3.4.2. Three doses of db-cAMP or PACAP27 reduce the changes induced by long-term (24h) incubations with PGJ2 – To overcome the long-term effects of PGJ2 we increased the number of treatments with the cAMP-elevating drugs as described under "Materials and Methods". As shown in Fig. 2, three doses of db-cAMP (1mM) or PACAP27 (100nM) clearly diminished the levels of ΔTAU (panel 1), ΔTAU aggregates (panel 2), caspase-3 cleavage (panel 3), soluble Ub-proteins (panel 4), Ub-protein aggregates (panel 5), and loss of cell viability (panel 7) induced by 24h treatment with 5μM PGJ2. Incubations with 10μM PGJ2 caused more severe changes than 5μM PGJ2, and thus were harder to overcome. In the absence of PGJ2, three doses of the cAMP-elevating drugs did not alter the levels of the proteins tested (not shown). In addition, treatment with just two doses of the cAMP-elevating drugs was ineffective against PGJ2 (not shown).

3.5. DISCUSSION

Our current data with rat cerebral cortical neurons demonstrate a temporal correlation between proteasome inhibition and caspase-activation that leads to TAU cleavage at Asp421 associated with TAU pathology, and cell death. The temporal

correlation depicts the accumulation of detergent (NP40) soluble ubiquitinated (SUb)-proteins occurring early upon proteasome impairment. Caspase-activation, TAU cleavage at Asp421, and the aggregation of TAU and Ub-proteins, occur significantly later. Large aggregates (detected with the filter trap assay) also appear late in this toxic cascade. The filter trap assay captures large (>0.2µm) and SDS-insoluble aggregates (Wanker et al., 1999). The sequence of proteolysis-related events was triggered by the product of inflammation PGJ2 and the specific proteasome inhibitor epoxomicin. While PGJ2 mimics the effect of some oxidative stressors by causing dissociation of 26S proteasomes (Wang et al., 2006;Aiken et al., 2011), epoxomicin forms covalent adducts with the 20S proteasome active sites (Groll et al., 2000). The finding that both drugs induce a similar temporal response to proteasome impairment, suggests that these proteolysis-related events could be shared by various proteotoxic conditions that induce a decline in proteasome activity in neurons. This temporal response to proteasome inhibition strongly supports the notion that the accumulation of SUb-proteins, if not resolved, could be one of the critical events triggering caspase-activation that mediates TAU cleavage and generates aggregation-prone fragments of TAU. Other studies support this notion. For example, 26S proteasome dysfunction was sufficient to trigger neurodegenerative disease in a transgenic

mouse model developed by conditionally depleting a 26S proteasome subunit in forebrain neurons (Bedford et al., 2008). The mutant mice exhibited diffuse accumulation of ubiquitinated proteins in forebrain neurons at two weeks of age. Caspase-activation and intraneuronal Ub-positive inclusions were observed later, at 4 weeks of age, indicating extensive neurodegeneration in the targeted neurons (Bedford et al., 2008). In another study, proteasome impairment was found to occur early in the progression of the pathological events detected in 3xTg-AD mice, leading to Aβ and TAU accumulation (Tseng et al., 2008). Based on these studies we propose that elevating proteasome activity to prevent the accumulation of SUb-proteins early in the neurodegenerative process, could be an effective approach to prevent caspase-activation and TAU pathology.

We also demonstrate, for the first time to our knowledge, that elevating cAMP via db-cAMP or PACAP27 prevents caspase-activation and generation of ΔTAU induced by PGJ2. TAU proteolysis is recognized as playing an important role in TAU aggregation and neurodegeneration in AD (Gamblin et al., 2003;Rissman et al., 2004). Thus, blocking TAU cleavage at Asp421 could be a potential therapeutic approach against TAU pathology. We show that only a single dose of db-cAMP or PACAP27 blocked caspase-activation and ΔTAU upon short-term (up to 8h) treatment with PGJ2. To attempt to overcome the harsh effects of

long-term (24h) incubations with PGJ2, we decided to increase the number of treatments with the cAMP-elevating drugs. Administration of three sequential doses of the cAMP-elevating drugs was necessary to diminish ΔTAU, caspase-activation, and loss of cell viability promoted by long-term (24h) incubations with 5μM PGJ2. It is notable that PACAP27 mimics the protective effects of db-cAMP at considerably lower concentrations (nanomolar for PACAP27 vs. millimolar for db-cAMP). The difference in effectiveness could be due to some properties of db-cAMP, such as that it remains inactive until endogenous esterases/amylases remove the butyrate (Schwede et al., 2000). Furthermore butyrate, by itself, affects gene transcription and PKC, thus interfering with several cAMP-dependent pathways (Schwede et al., 2000). As discussed above in relation to PACAP27, treatment with a single dose of the peptide is unlikely to be optimal and/or maximized for long-term neuroprotection, because its action depends on the production of hydrolysable cAMP. This notion is supported by our data showing that three consecutive doses of the cAMP-elevating drugs diminish long-term (24h) effects of PGJ2. Interestingly, PACAP was shown to enhance α-secretase activity (Kojro et al., 2006) and improve memory in rats (Sacchetti et al., 2001). That PACAP27 shows promise in delaying *AD* is corroborated by a recent study showing that long-term daily intranasal administration of PACAP slowed down AD-

like pathology in APP[V717I] AD transgenic mice (Rat et al., 2011). In the latter studies, TAU pathology was not addressed. Together these results support the view that, due to its beneficial properties, PACAP27 could be a very interesting agent for long-term treatment of AD [reviewed in (Reglodi et al., 2011)]. The challenge is to identify which mechanisms PACAP27 is triggering to bring about its protective effects.

In conclusion, our data clearly demonstrate that the accumulation of SUb-proteins is an early event that occurs after treating cortical neurons with two drugs, i.e. PGJ2 and epoxomicin, that inhibit the proteasome by different means. Since there is compelling evidence for impairment of proteasome activity in AD (Keller et al., 2000) and aging (Keller et al., 2002), it is possible that the sequence of proteolysis-related events that we established here and that are triggered by PGJ2 and epoxomicin, is similar in the early stages of neurodegeneration in AD (modeled in Fig. 9). Initially, low levels of SUb-proteins that escape degradation, accumulate in the affected areas of the AD brain. These SUb-proteins could further exacerbate proteasome malfunction. Several studies demonstrate that increasing the levels of polyubiquitin chains (Piotrowski et al., 1997) and/or aggregation prone proteins, such as PHF-TAU (Keck et al., 2003), decreases proteasome activity. When these proteins accumulate in the cell, they can

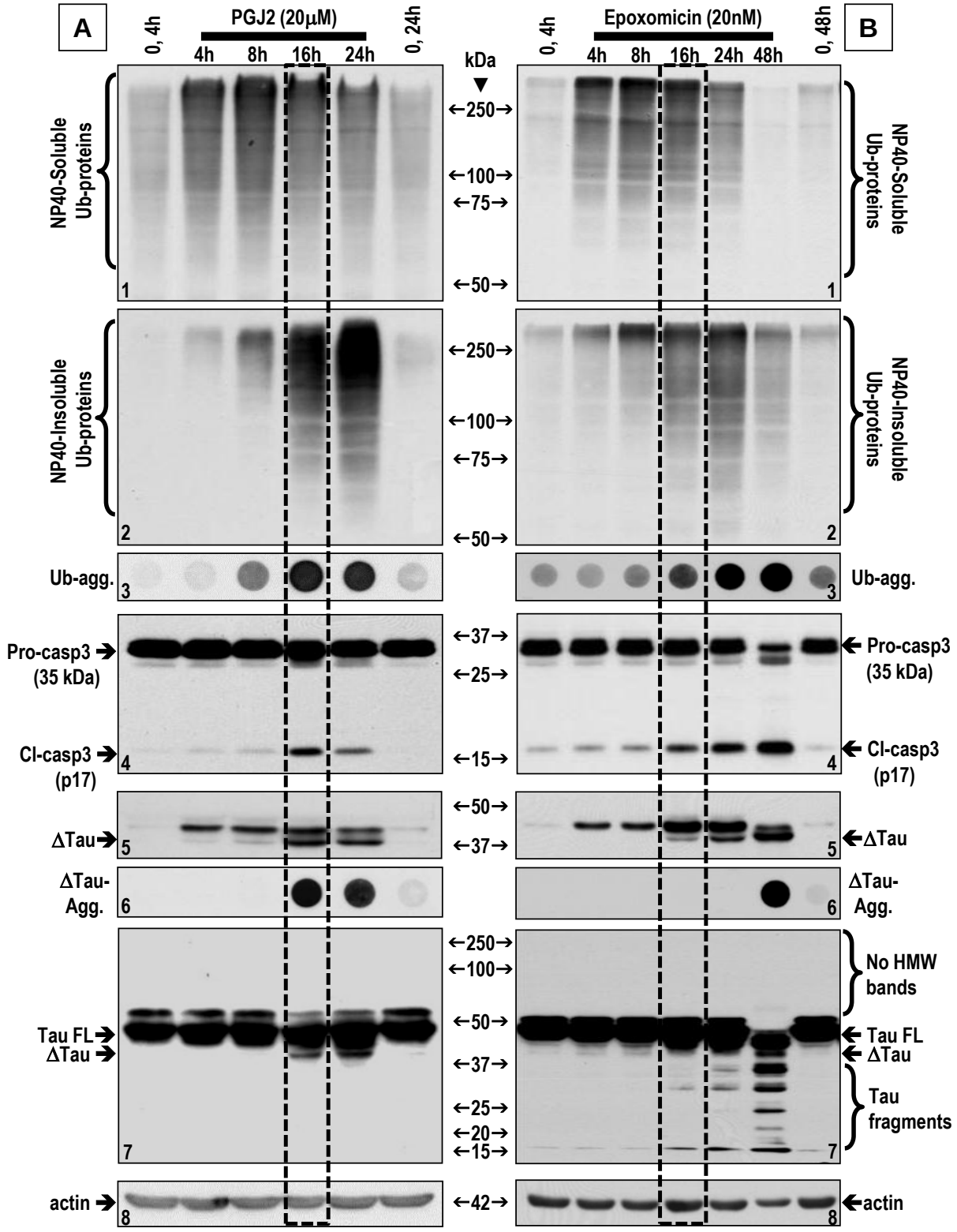
bind to proteasomes and block access of other substrates to the proteasomal degradation machinery, thus further aggravating the proteotoxic situation. With time, proteasome activity continues to deteriorate to a point that the neurons can no longer cope with the proteotoxic stress and reach a point of no return. This feed-forward incremental proteasome inhibition could be a major factor in neurodegeneration. We propose (modeled in Fig. 9) that targeting the proteasome to enhance its activity in a robust and sustainable manner via cAMP/PKA signaling, could avoid the early accumulation of SUb-proteins, followed later on, by caspase-activation and protein aggregation. An early intervention strategy could prevent neurons from reaching a point of no return, and provide an effective therapy to avoid/delay neurodegeneration in AD.

Figure legends

Fig. 1 – Accumulation of NP40-soluble ubiquitinated proteins paves the way for caspase 3 activation, caspase-mediated TAU cleavage (ΔTAU), and aggregation of Ub-proteins as well as TAU, induced by PGJ2 (A) or epoxomicin (B). Western blot analyses to detect Ub-proteins (NP40-soluble and NP40-insoluble), caspase 3, TAU cleaved at Asp421 (ΔTau), full length TAU (Tau FL), and actin (loading control) in cell extracts of rat E18 cerebral cortical neurons (40 µg of protein/lane). The cortical neurons were treated with 20µM PGJ2 (A) or 20nM epoxomicin (B) for different time points. Cell extracts were subjected to centrifugation to separate the NP40-soluble and NP40-insoluble fractions as described under "Material and Methods". The blots were probed with anti-Ub-proteins antibody (panels 1, 2 & 3), caspase 3 (panels 4), TAU C3 antibody (TAU cleaved at Asp421, epitope a.a. 412-421, panels 5 & 6), TAU C5 antibody (detects all TAU isoforms and ΔTAU; ep: a.a. 210–241, panel 7) and anti-actin antibody (panel 8). Molecular mass markers in kDa are shown in the middle. Ub-aggregates (Ub-agg.) and ΔTAU aggregates (ΔTau-agg.) were analyzed with the filter trap assay (50 µg of protein/dot, panels 3 & 6). The levels of NP40-soluble Ub-proteins (solid squares), cleaved caspase 3 (crosses), and ΔTAU

(open squares) were semi-quantified by densitometry (panels 9). Data represent the percentage of the pixel ratio for soluble Ub-proteins, cleaved caspase 3, or ΔTAU, over actin for each condition compared to control (100%). Values are means and s.d. from at least two experiments. Asterisks identify values that are significantly different from control (**p<0.01, ***p<0.001). Ub-proteins, ubiquitinated proteins; ΔTau, TAU cleaved at Asp421; Tau FL, full length TAU.

To assess changes in proteasome activity, cell extracts were prepared from rat E18 cerebral cortical neurons treated with 20μM PGJ2 (A) or 5nM epoxomicin (B) for different time points. Clear lysates (30μg/sample) were subjected to non-denaturing gel electrophoresis as described under "Material and Methods". 26S and 20S proteasomal (indicated in the middle by arrows) chymotrypsin-like activity was assessed with Suc-LLVY-AMC by the in gel assay (panels 10). Proteasome levels were detected by immunoblotting with the anti-β5 antibody (panels 11). The numbers at the bottom of panels 10 and 11 represent 26S and 20S proteasomal chymotrypsin-like activity and levels, respectively, under each treatment condition. Percentages represent the ratio between data for each condition and control (DMSO) considered to be 100%. Values are means from duplicate experiments.


A
PGJ2 (20μM)
0, 4h
4h
8h
16h
24h
0, 24h
B
Epoxomicin (20nM)
0, 4h
4h
8h
16h
24h
48h
0, 48h
kDa
NP40-Soluble Ub-proteins
NP40-Insoluble Ub-proteins
Ub-agg.
Pro-casp3 (35 kDa)
Cl-casp3 (p17)
ΔTau
ΔTau-Agg.
Tau FL
ΔTau
actin
No HMW bands
Tau fragments
250
100
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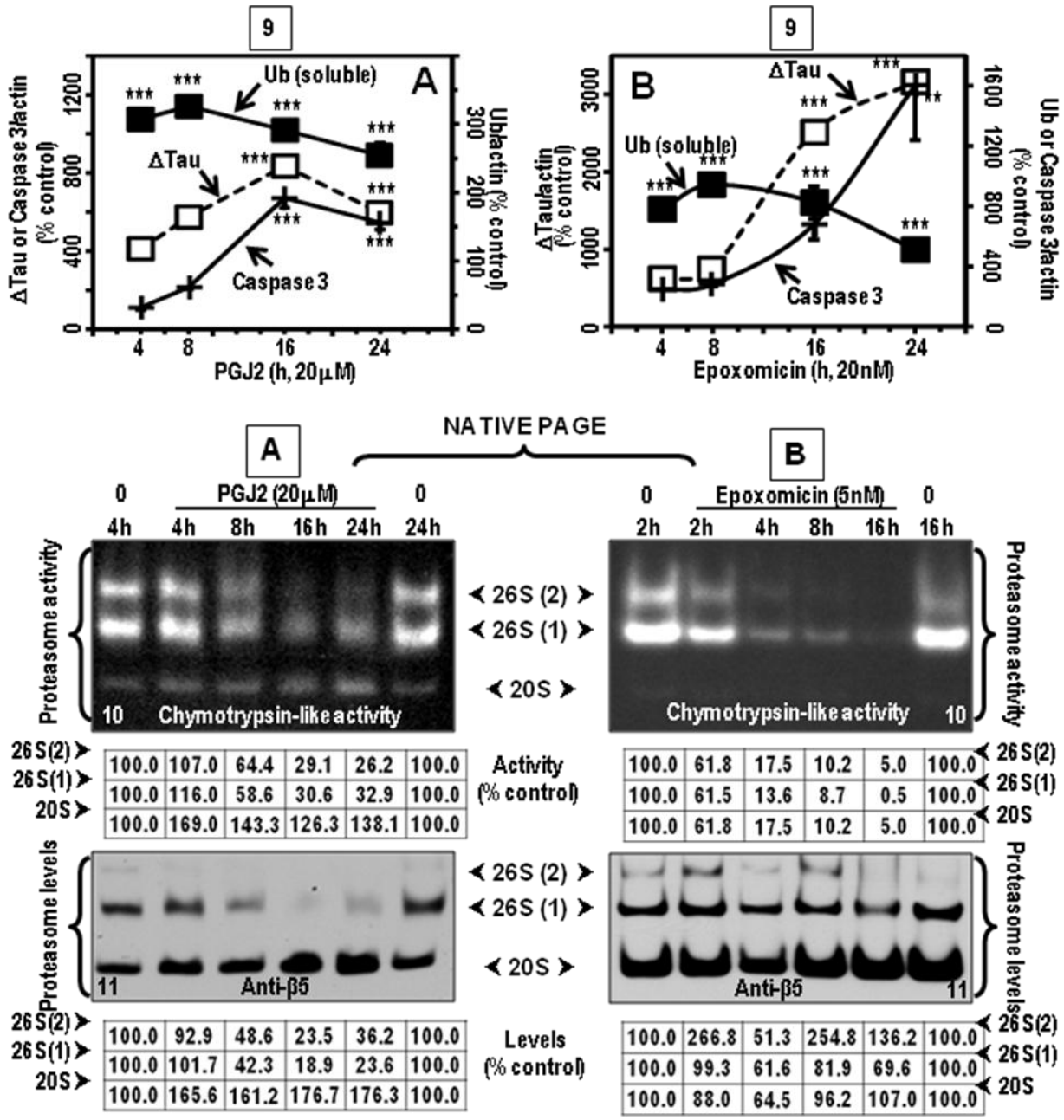
1
2
3
4
5
6
7
8

Figure 1



26S(2)	100.0	107.0	64.4	29.1	26.2	100.0
26S(1)	100.0	116.0	58.6	30.6	32.9	100.0
20S	100.0	169.0	143.3	126.3	138.1	100.0

26S(2)	100.0	61.8	17.5	10.2	5.0	100.0
26S(1)	100.0	61.5	13.6	8.7	0.5	100.0
20S	100.0	61.8	17.5	10.2	5.0	100.0

26S(2)	100.0	92.9	48.6	23.5	36.2	100.0
26S(1)	100.0	101.7	42.3	18.9	23.6	100.0
20S	100.0	165.6	161.2	176.7	176.3	100.0

26S(2)	100.0	266.8	51.3	254.8	136.2	100.0
26S(1)	100.0	99.3	61.6	81.9	69.6	100.0
20S	100.0	88.0	64.5	96.2	107.0	100.0

Figure 1 (cont.)

Fig. 2 – Three doses of db-cAMP (1mM) or PACAP (100nM) diminish the effects of long-term (24h) incubations with PGJ2. Rat E18 cerebral cortical neurons were treated with DMSO (0, control, vehicle for PGJ2), or PGJ2 (5μM or 10μM) in conjunction with three consecutive doses of water (control, vehicle for db-cAMP and PACAP27), db-cAMP (1mM, db-cAMP) or PACAP27 (100nM) over a period of 24h. The cAMP-elevating drugs were added as described under "Materials and Methods". Western blots of the NP-40 soluble fractions (30 μg of protein/lane) were probed for TAU cleaved at Asp421 (ΔTAU, panel 1), caspase 3 (panel 3), soluble Ub-proteins (panel 4), and actin (panel 6, loading control). ΔTAU- and Ub-aggregates (panels 2 and 5) were assessed with the filter trap assay (30 μg of protein/dot). Molecular mass markers in kDa are shown on the right. Similar results were obtained in duplicate experiments. ΔTau, TAU cleaved at Asp421; Pro, zymogenic, and Cl, cleaved forms of caspase 3. Cell viability (panel 7) was assessed with the MTT assay. Percentages represent the ratio between the data for each condition and control (100%). Values indicate means and s.e. from six determinations. Asterisks identify values that are significantly different from treatment with water alone or db-cAMP alone (white bars, respectively) within each group, with ***$p<0.001$. Dashed lines compare conditions that are not significantly different.

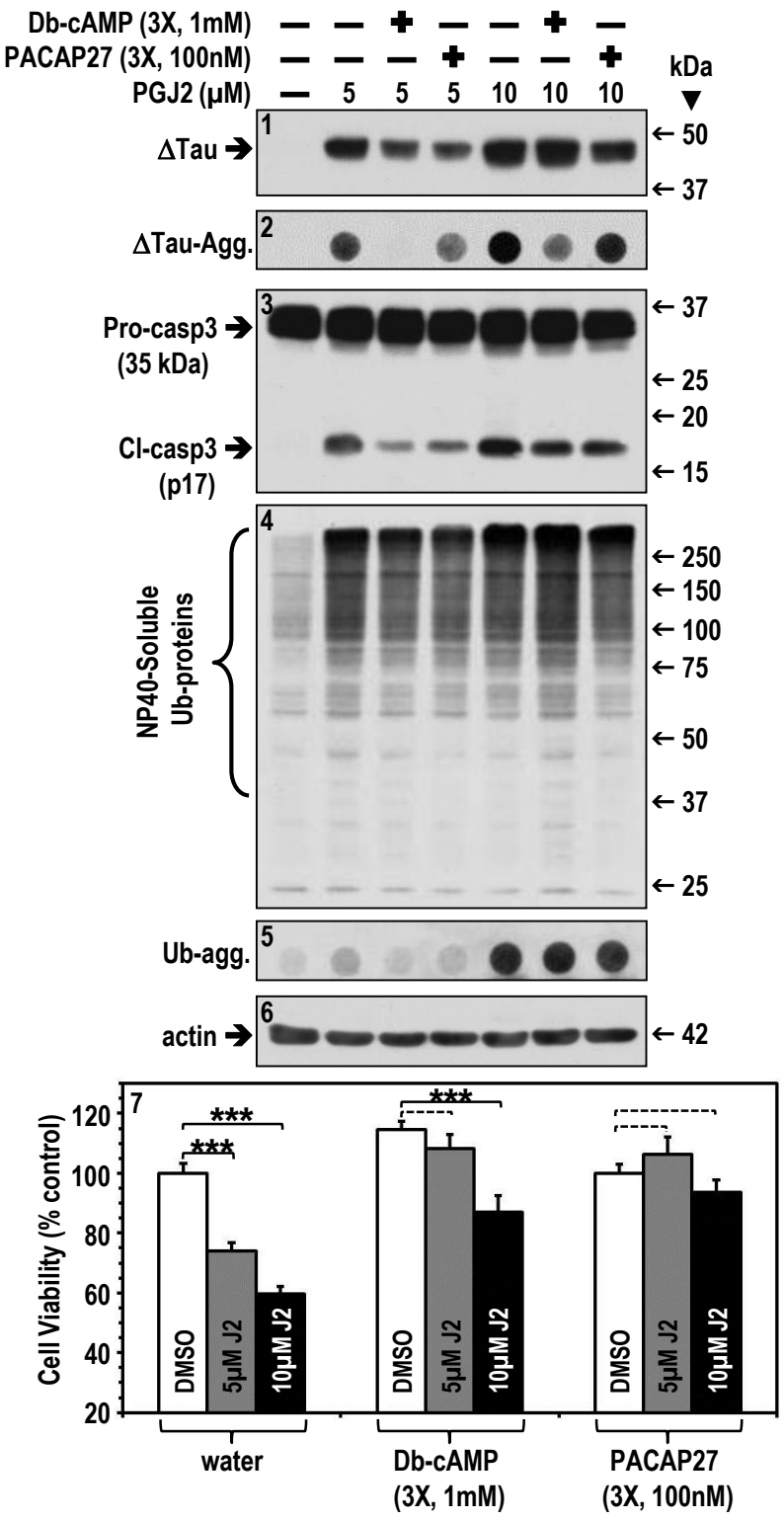

Db-cAMP (3X, 1mM)
PACAP27 (3X, 100nM)
PGJ2 (μM)
— — + — — + —
— — — + — — +
— 5 5 5 10 10 10
kDa
1
ΔTau
← 50
← 37
2
ΔTau-Agg.
3
Pro-casp3
(35 kDa)
Cl-casp3
(p17)
← 37
← 25
← 20
← 15
4
NP40-Soluble
Ub-proteins
← 250
← 150
← 100
← 75
← 50
← 37
← 25
5
Ub-agg.
6
actin
← 42
7
Cell Viability (% control)
120
100
80
60
40
20
***
***
DMSO
5μM J2
10μM J2
water
Db-cAMP
(3X, 1mM)
PACAP27
(3X, 100nM)

Figure 2

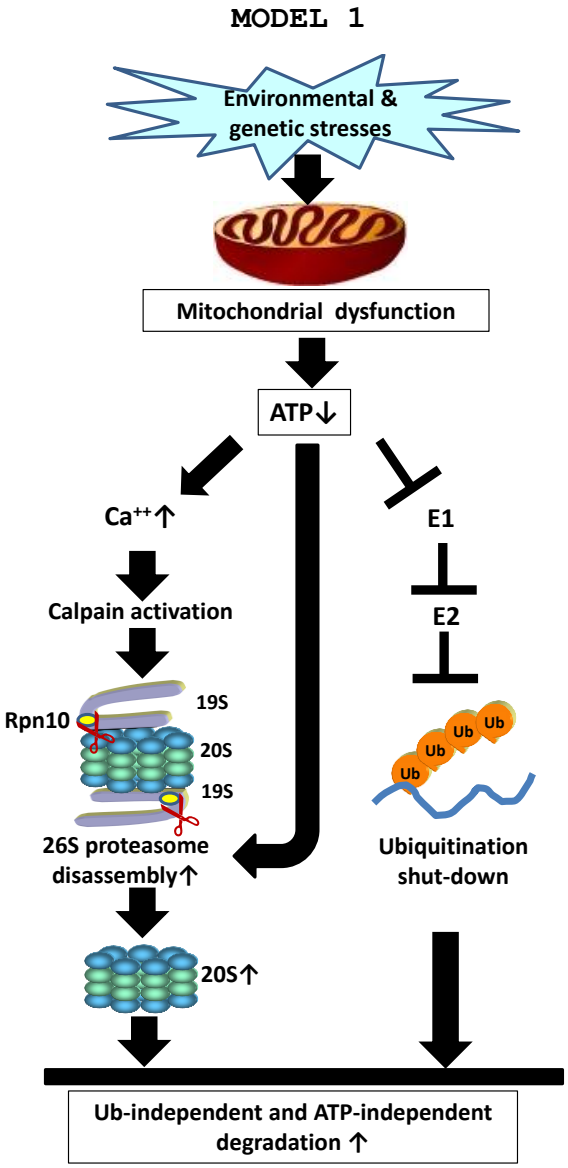
CHAPTER IV

MODELS AND CONCLUSIONS

Impairment of the ubiquitin/proteasome pathway (UPP) and mitochondrial dysfunction are implicated in the etiology of Alzheimer disease (AD). The UPP is highly regulated and energy dependent for the initial step of the ubiquitination cascade, the assembly of 26S proteasomes, and degradation of ubiquitinated proteins, which include some mitochondrial proteins. Mitochondria are the main source of energy production in neurons. The UPP and mitochondria are thus mutually dependent. In terms of etiology, impairment of the UPP or mitochondria as an early or upstream event is still elusive. It is thus critical to investigate the relationship between these two processes, i.e. UPP and mitochondria dysfunction.

MODEL 1 - Our data show that mitochondria impairment leads to dramatic changes in the UPP. Due to ATP depletion, the ubiquitin activation step by E1 enzyme is inhibited, causing subsequent inhibition of E2-dependent conjugation and of the whole ubiquitination cascade. In addition, energy deficit and subsequent calcium influx trigger calpain activation. Both ATP depletion and calpain activation promote 26S proteasome disassembly, thus up-regulating 20S proteasomes. Based on this data, we postulate that shutting-down ubiquitination and up-regulating 20S proteasomes are cellular responses to energy deficits. These responses are of significance, since ubiquitin-

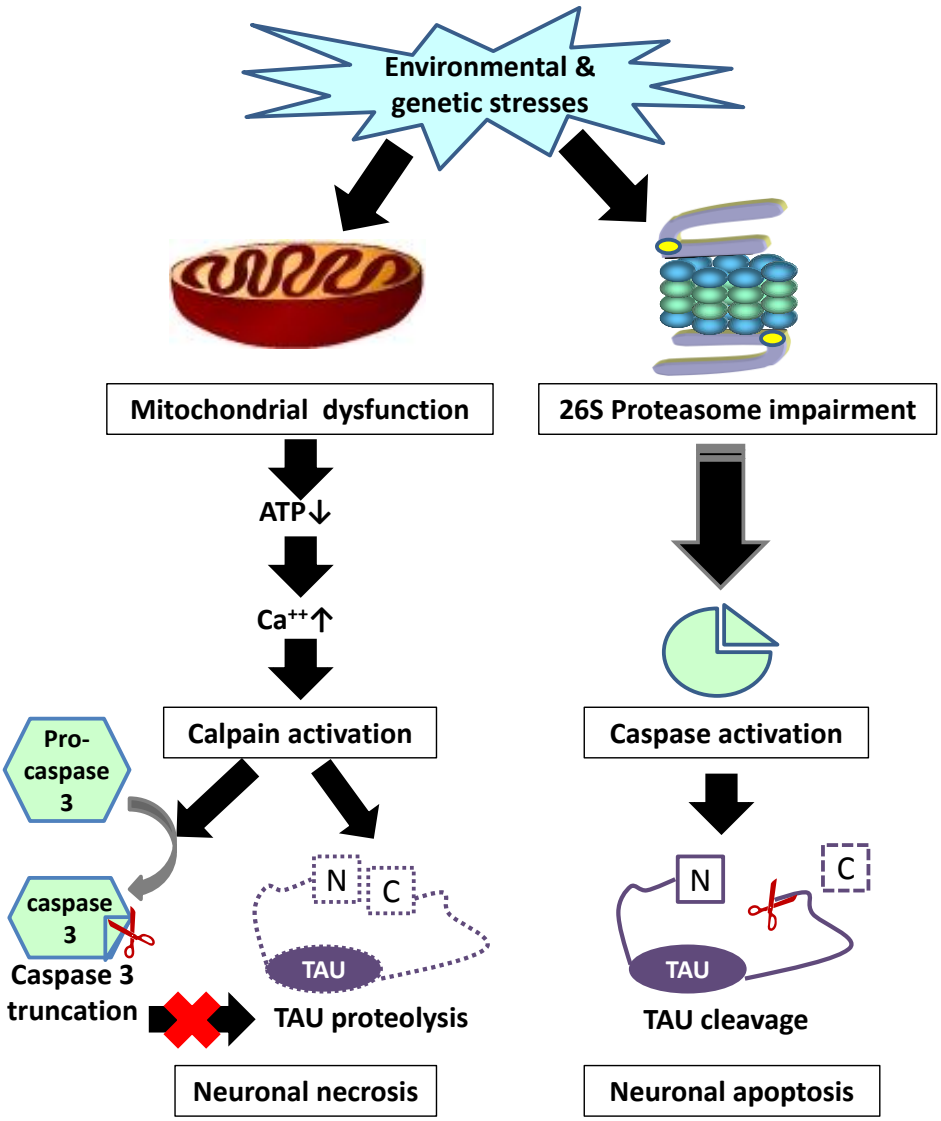
and ATP-independent degradation by 20S proteasomes seems to be a major turnover pathway for oxidatively modified proteins generated upon mitochondrial impairment (Davies, 2001). It is interesting that neurons adjust to an environment of limited energy by shifting protein degradation from the normal pattern by the UPP to an energy- and ubiquitin-independent pattern by 20S proteasomes, to adapt to these environmental changes.



MODEL 2 – Necrotic and apoptotic neuronal death are both associated with neurodegenerative disorders, such as AD. Two major proteases, calpain and caspase 3, play distinct roles in the two forms of neuronal death. While calpain is activated mostly in necrosis, caspase 3 is only activated in neuronal apoptosis. Our data address the two different neuronal death pathways. Mitochondrial-linked energy deficits lead to increased membrane permeability, and thus calcium influx, which activates calpain, causing cleavage of selective proteasome subunits (Rpn10), cytoskeletal substrates, such as **α**-spectrin and TAU, as well as truncation of pro-caspase 3, blocking its activation. All of these events are associated with necrotic neuronal death. In contrast, with impairment of 26S proteasome by PGJ2 or epoxomicin, caspase 3 is activated, which cleaves TAU at Asp 421, generating aggregation-prone ΔTAU thus promoting TAU aggregation. All of these events are associated with apoptotic neuronal death. Generation of distinct TAU fragments depends on which of the two proteases is activated. Caspase 3-induced TAU cleavage at Asp 421, namely ΔTAU, has a high tendency for aggregation (Rissman et al., 2004). Calpain-induced TAU fragments, including a typical “17 kDa” fragment, represents a marker for enhanced calpain activity in AD (Garg et al., 2011). We detected this typical 17kDa fragment in oligomycin-treated neurons, and observed preventive effects of calpain inhibitors

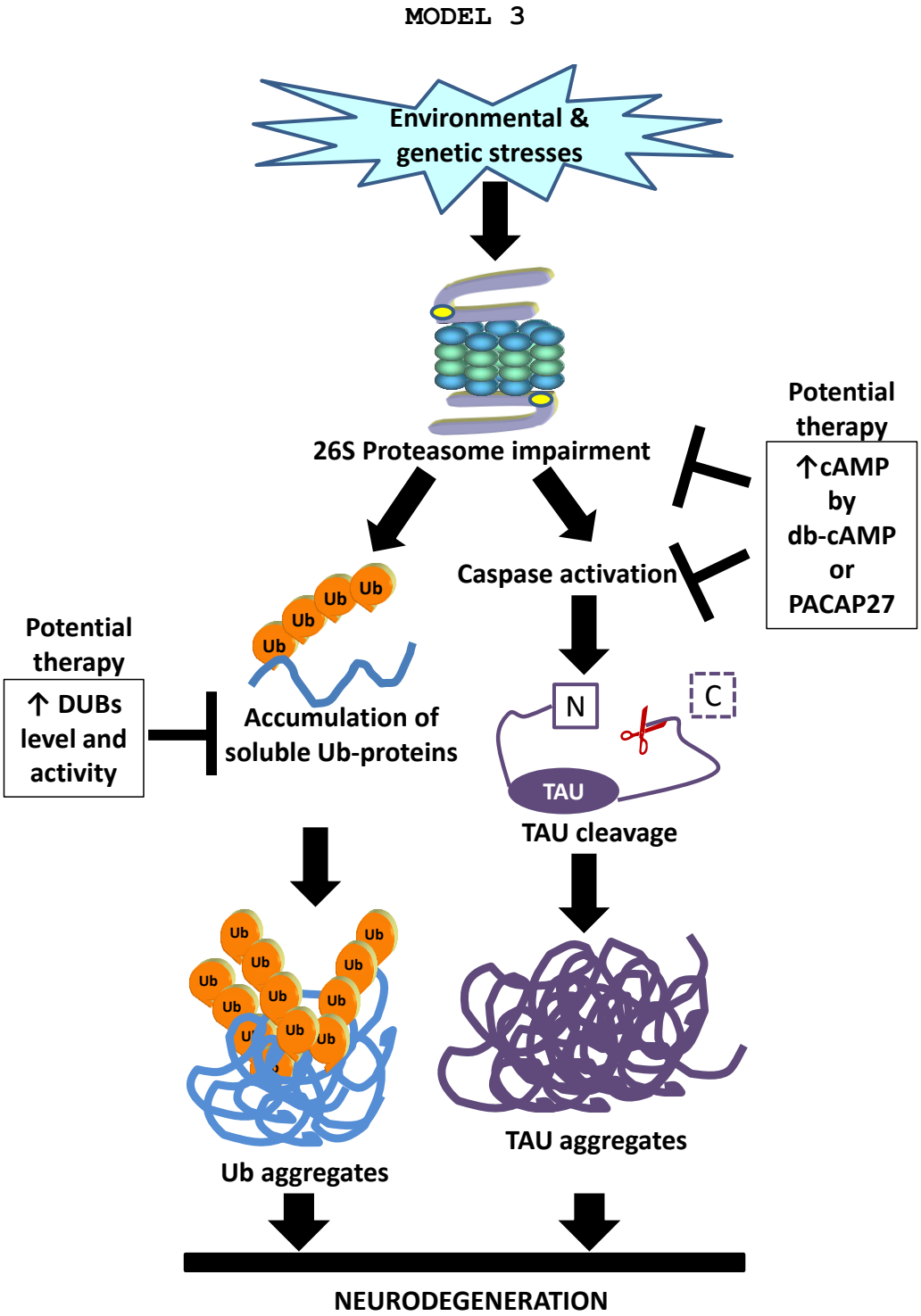
on the generation of this fragment. Calpain and caspase 3 activation are both implicated in the progression of AD (Saito et al., 1993;Grynspan et al., 1997;Tsuji et al., 1998;Gervais et al., 1999). For therapeutic purposes, it would be important to test if combined treatments with calpain and caspase 3 inhibitors enhance neuroprotection relevant to AD.

MODEL 2



MODEL 3 - We have demonstrated that 26S proteasome impairment with two different drugs, PGJ2 and epoxomicin, leads to accumulation of soluble ubiquitinated proteins, caspase activation, generation of ΔTAU, and formation of ubiquitin and TAU aggregates. Since proteasome impairment is the upstream event leading to these subsequent pathological changes, we have tried two cAMP-enhancing drugs, db-cAMP and PACAP27, to prevent: (1) reduction of 26S proteasome activity induced by PGJ2, (2) hinder activation of caspase 3, and (3) prevent formation of TAU aggregates. We tested these drugs under mild toxic conditions, i.e. high dosage of PGJ2 (10μM) for short-term (8h) incubations, or low dosage of PGJ2 (5μM) for long-term (24h) treatments. In both conditions, the cAMP elevating drugs diminished caspase 3 activation, generation of ΔTAU, and loss of neuronal viability. The decline in soluble and aggregated ubiquitinated proteins was less apparent, suggesting that they could be a major factor causing neuronal toxicity. The accumulation of ubiquitinated proteins can also be induced by inhibition of deubiquitinating enzymes (DUBs). Notably, besides inhibiting the proteasome, PGJ2 was shown to inhibit ubiquitin hydrolases, such as UCH-L1 (Li et al., 2004). Based on these findings, we postulate that simultaneously enhancing the activities of 26S proteasomes and deubiquitinating enzymes, such

as UCH-L1, may improve the chances of preventing/halting PGJ2 toxicity.



CONCLUSION: Overall our hypothesis is that during the processes of aging and/or neurodegeneration, different environmental and/or genetic stresses gradually affect the function of mitochondria and/or proteasomes in neurons. This will cause individual or joint activation of the two death pathways necrosis and apoptosis, including stimulation of calpain and/or caspases that lead to generation of TAU fragments and formation of aggregates. Since the initiating stimuli may vary, so will the corresponding death pathways that are turned-on. It is thus important to attempt combination therapeutic strategies aiming at multiple targets in order to achieve maximal protection against the onset and/or progression of AD.

CHAPTER V

FUTURE DIRECTIONS

The ubiquitin/proteasome pathway (UPP) is crucial for degradation of most intracellular proteins in cells. It is of great significance to study the regulatory mechanisms of this pathway especially in neurodegenerative disorders, since these diseases share the common pathological feature of ubiquitinated protein aggregation. Elucidation of the underlying molecular mechanism by which UPP is affected in neurodegeneration, and attempts to elevate UPP function, will be helpful for development of therapeutic strategies that prevent/slow the progression of neurodegeneration.

We have also demonstrated in a neuronal cell model that mitochondrial-linked energy deficits affect UPP and TAU degradation. Calpain might be a key player in these processes. To test this aspect of neurodegeneration *in vivo* and to select calpain as a therapeutic target for prevention of AD needs further investigation.

We propose that future studies should focus on:

1) Establish the mechanism by which oligomycin reduces Rpn10 levels and destabilizes 26S proteasome structure.

Our data reveal that oligomycin, a mitochondrial ATP synthase inhibitor, selectively reduces Rpn10 protein level in a concentration- and time-dependent manner in cortical neurons.

Other proteasome subunits tested, such as Rpn2, Rpt5 and Rpt6 in 19S regulatory particle or α5 and β5 in 20S core particle, were not affected. It is unclear why, among all proteasome subunits tested, Rpn10 is susceptible to calpain cleavage upon mitochondrial dysfunction. Based on the finding that calpain inhibitors preserved Rpn10 levels, we anticipate that Rpn10 is a calpain substrate. *In vitro* studies with purified Rpn10 incubated with calpain for different times, should test calpain-mediated Rpn10 proteolysis assessed by western blotting. To address the impact of Rpn10 in preserving 26S proteasome assembly, *RNAi* experiments should be conducted to establish if knocking down Rpn10 impairs 26S proteasome assembly assessed with the in gel assay. These results will further substantiate our hypothesis that Rpn10 is vitally important for preserving 26S proteasome assembly and is susceptible to calpain-mediated proteolysis.

2) Investigate if mitochondria dysfunction leads to TAU pathology *in vivo*.

Our data with primary neuronal cultures indicate that mitochondria inhibitors lead to ATP depletion, calpain activation, and TAU cleavage. It is unknown if this series of events occurs *in vivo*. To test this hypothesis, mice should be treated with mitochondrial inhibitors, such as oligomycin, to

assess if the neuronal changes that we detected in the cell culture model are observed in an *in vivo* setting.

3) Determine the neuroprotective effect of calpain inhibitors *in vivo*.

Our data demonstrate that calpain inhibitors prevent cleavage of several proteins upon mitochondria impairment, including Rpn10, pro-caspase 3, α-spectrin, Rpn10, and TAU. In terms of the impact of Rpn10 on 26S proteasome assembly, we showed that calpain inhibitors diminished 26S proteasome disassembly and inhibition. In regards to pro-caspase 3, calpain inhibitors prevented truncation of pro-caspase 3. Together, these results suggest that calpain is a promising target for prevention of neurodegeneration linked to mitochondria impairment. To test this hypothesis *in vivo*, TAU transgenic mice or mitochondria inhibitor-treated mice, should be administered calpain inhibitors, such as calpeptin, to evaluate their neuroprotective effects. Alternatively, co-administration of calpain and caspase inhibitors should be tested to enhance the chances of neuroprotection.

4) **Evaluate if enhancing cAMP levels together with increasing deubiquitinating enzyme (DUB) activity will prevent ubiquitinated protein accumulation and aggregation.**

We established in primary neuronal cultures that cAMP-elevating drugs, i.e. db-cAMP or PACAP27, prevent the decline in 26S proteasome activity and caspase 3 activation induced by prostaglandin J2 (PGJ2), provided that these changes are mild. The cAMP-elevating drugs were less effective in reducing ubiquitinated protein accumulation and aggregation. It is well established that PGJ2 affects 26S proteasomes and deubiquitinating enzymes (DUBs) (Li et al., 2004). Attempts to enhance for example, ubiquitin C-terminal hydrolase UCH-L1 activity, along with cAMP-elevating drugs could be an optimal strategy to prevent toxicity of conditions that mimic the deleterious effect of PGJ2. Enhancement of DUB activity has to be within a moderate range, so as not to prevent ubiquitin-dependent protein degradation by 26S proteasomes.

5) OVERALL CONCLUSION

Further elucidation of the mechanisms by which mitochondria impairment leads to 26S proteasome disassembly via calpain activation, and determining if similar mechanisms occur *in vivo* are critical to the understanding of AD etiology and development of therapeutic strategies to prevent/slow the progression of neurodegeneration.

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