

INVOLVEMENT OF INFLAMMATION IN TAU
PATHOLOGY AND MECHANISMS THAT DRIVE
NEURODEGENERATION
IN ALZHEIMER'S DISEASE

by

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A dissertation submitted to the Graduate Faculty in
Biochemistry in partial fulfillment of the requirements for
the degree of Doctor of Philosophy,
The City University of New York

UMI Number: 3311213

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ABSTRACT

Two of the hallmarks of neurodegenerative disorders, such as Alzheimer's disease (AD), are (1) the appearance of proteinaceous deposits in inclusion bodies containing aggregates of ubiquitinated proteins and (2) activated microglia and astrocytes surrounding the diseased neurons. In Alzheimer's disease, the intracellular inclusion bodies are known as neurofibrillary tangles (NFT). The mechanisms leading to inclusion body formation and their role in the progression of neurodegeneration are still largely unknown.

Many of the proteins that accumulate in inclusion bodies depend on the ubiquitin/proteasome pathway (UPP) for their degradation. This pathway is responsible for the bulk (~80%) of intracellular protein degradation. Because of its central role in the removal of mutated and misfolded proteins by degradation, disruption of the UPP is particularly relevant to the accumulation of aberrant proteins observed in aging-related neurodegenerative disorders, such as AD. Besides containing ubiquitinated proteins, one of the major components of NFTs is the microtubule associated protein "tau". Tau protein is abundant in neurons and is a highly soluble protein. Tau must be cleaved first to function as a seed for its self aggregation.

Our **main hypothesis** is that toxic inflammation factors released by microglia and astrocytes will damage proteins in neurons causing protein misfolding. An abrupt or chronic increase in damaged proteins will overwhelm the proteasome, particularly in old age when proteasome activity is clearly impaired. If not resolved, the ensuing accumulation of ubiquitinated proteins is potentially toxic and drives the cell to activate a death pathway, therefore launching apoptosis. Caspase activation associated with apoptosis leads to caspase-mediated proteolysis of a variety of

proteins including tau, which is a microtubule stabilizing protein. Tau cleavage will destabilize microtubules and cause the collapse of the cell structure. In addition, tau cleavage will promote protein aggregation. All of these events culminate in neurodegeneration.

We tested our hypothesis by incubating neuronal cells with the cytotoxic product of inflammation prostaglandin J2 (PGJ2). As we proposed, the initial event observed upon PGJ2 treatment was the accumulation of ubiquitinated proteins. This was followed by apoptosis coinciding with caspase activation and tau cleavage, culminating in protein aggregation and cell death. In other studies, we established a direct correlation between proteasome impairment (accomplished by a genetic manipulation of its chymotrypsin-like activity) with an increased vulnerability to stress conditions induced by the heavy metal cadmium. Finally, we identified a unique aging-dependent mechanism that contributes to proteasome dysfunction in *Drosophila melanogaster*. Our studies were the first to show that the major proteasome form in old flies is the weakly active 20S core particle, while in younger flies the fully assembled 26S holoenzyme is the preponderant proteasome form.

In conclusion, these studies support the view that maintaining proteasome activity is critical to cell

survival. If proteasome activity is disrupted by inflammation or oxidative stress or even by the build-up of mutant proteins, this will have a catastrophic effect on cell survival resulting in the induction of apoptosis. The ensuing activation of caspase-mediated proteolysis will lead to partial cleavage of a variety of proteins including tau that will in turn promote protein aggregation culminating in neurodegeneration. This neurodegenerative process is exacerbated at later stages in life, because proteasome function seems to decline abruptly at an old age. A better understanding of the mechanisms leading to the build-up of protein aggregates will open up new targets for treatment of neurodegenerative disorders, such as AD, that are associated with chronic inflammation and protein aggregation.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

Aβ Amyloid Beta fragment	FTDP-17 Hereditary
AD Alzheimer's disease	Frontotemporal dementia
ALS Amyotrophic lateral	with parkinsonism-17
sclerosis	GSK3β glycogen synthase
-AMC -7-amido-4-	kinase-3 beta
methylcoumarin (fluorogenic	HT4 Mouse neuroblastoma
substrate)	cells
ATP Adenosine triphosphate	IL Interleukin
BRCA1 Breast Cancer 1 gene	MAPK Mitogen activated
CHIP Chaperone dependant E3	protein kinase
ligase	MTOC Microtubule organizing
CNS Central nervous system	center
COX Cyclooxygenase	MTT assay (3-(4,5-
CSF Cerebrospinal fluid	Dimethylthiazol-2-yl)-2,5-
DAPI 4',6-diamidino-2-	diphenyltetrazolium
phenylindole	bromide, a tetrazole) cell
DNA Deoxyribonucleic acid	viability assay
DP1/DP2 PGD2 receptor 1 and	Mutaβ5 Beta 5 proteasome
2	subunit mutant. N-terminal
ECL Enhanced	threonine 1 to alanine
chemiluminescence	substitution.
ER Endoplasmic Reticulum	-NA -b-naphtylamide

NAC N-acetyl cystein
NF κ B nuclear factor Kappa B
NFT Neurofibrillary tangle
NSAID Non steroidal anti
inflammatory drug
PARP Poly ADP ribose
polymerase
PD Parkinson's disease
PGJ2 PGD2 PGE2
Prostaglandin J2-D2-E2
L-PGDS lipocalin-type
prostaglandin D2 synthase
H-PGDS Hematopoietic
prostaglandin D2 synthase
PHF Paired helical filament
-pNA -p-nitroanilide
PP1 PP2A PP2B Protein
phosphatase 1-2A-2B
PPAR γ Peroxisome
proliferator activated
receptor gamma
PVDF polyvinylidene
fluoride
RNA Ribonucleic acid
SK-N-SH Human caucasian
neuroblastoma cells
UPP Ubiquitin proteasome
pathway
Ub Ubiquitin
WT β 5 Wild type beta 5
proteasome subunit
Z benzyloxycarbonyl

CHAPTER I

INTRODUCTION

Portions of this introduction were published in the journal
Neurodegenerative Disorders 2006; 3(6):313-9

"It may take inflammation, phosphorylation and
ubiquitination to 'tangle' in Alzheimer's disease"

By

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Neurofibrillary tangles are one of the pathologic hallmarks of Alzheimer's disease (AD). Their major component is tau, a protein that becomes hyperphosphorylated and accumulates into insoluble paired helical filaments. During the course of the disease such filaments aggregate into bulky that get ubiquitinated. What triggers the formation is not known, but neuroinflammation could play a role. Neuroinflammation is an active process detectable in the earliest stages of AD. The neuronal toxicity associated with inflammation makes it a potential risk factor in the pathogenesis of chronic neurodegenerative diseases, such as AD. Determining the sequence of events that lead to this devastating disease has become one of the most important goals for AD prevention and treatment. In this introduction we focus on three topics relevant to AD pathology and to formation: (1) what triggers CNS inflammation resulting in glia activation and neuronal toxicity; (2) how products of inflammation might change the substrate specificity of kinases/phosphatases leading to tau phosphorylation at pathological sites; (3) the relationship between the ubiquitin/proteasome pathway and tau ubiquitination and accumulation in .

The overall aim of this introduction is to provide a summary of important contributions supporting the view that CNS inflammation might be a critical contributor to AD pathology. Neuronal cell death resulting from neuroinflammatory processes may have devastating effects as, in the vast majority of cases neurons lost to disease cannot be replaced. In order to design therapies that will prevent endangered neurons from dying, it is critical that we learn more about the effects of neuroinflammation and its products.

1.1. ALZHEIMER'S DISEASE AND INFLAMMATION

Alzheimer's disease is a chronic, age-related neurodegenerative disorder. This dementia affects mostly people 65 years or older. Its pathological characteristics are the presence of senile plaques and neurofibrillary tangles in the brain. The senile plaques are composed of aberrant aggregates of amyloid β peptide while the consist of hyperphosphorylated tau. Over the last decade it has become clear that chronic neuroinflammation is another constant feature of AD ¹. Indeed neuroinflammation, long considered to be peripheral to the disease, is being increasingly accepted as being associated with the onset of this neurodegenerative disorder ²⁻⁴.

As its peripheral counterpart, neuroinflammation is widely regarded as a double-edged sword: it can exert both neuroprotective and neurotoxic functions. The mechanisms that switch between neuroprotection and neurotoxicity are poorly understood. In the case of neuroinflammation is labeled chronic because of its extended time course. Exposing the brain to such a prolonged process over months even years will inevitably lead to tissue damage. Glia and neuronal interactions during inflammation are thus particularly relevant to neurodegeneration (reviewed in ⁵). At the histological level, the main actors of CNS

inflammation are the activated microglia ⁶, the astrocytes and, to a lesser extent, the neurons themselves ⁷. In addition, numerous studies have provided evidence for an increased activation of the complement system in areas of AD pathology ^{8;9}. Studies are still trying to assess the degree of inflammation in the brain of AD patients and the CSF markers fail to reflect exactly the situation of the isolated brain, highlighting the difficulty of assessing the real degree of inflammation in the brain ¹⁰⁻¹³.

The question persists regarding the role of inflammation in AD: Is it a driving force, a bystander or a beneficial response ¹⁴? If inflammation cannot be ruled as the initiator of the disease, its systematic presence in the pathological areas of the brain and the decrease of pathology after treatment with anti-inflammatory drugs links it to the development of the disease. If the acute activation of the microglia might be beneficial for the brain after injury, its chronic activation might not. Chronic inflammation may induce the release of toxic inflammatory products suspected to worsen the pathology and prevent the proper removal of waste products, emphasizing the necessity of a close control of inflammation. Microglia activation into phagocytic glial cells might be beneficial for the removal of toxic

aggregates or dying cells. Neurotoxic glial cells release inflammatory factors fueling the toxic course of events leading to cell death. A β aggregates seem to activate an inflammation response at the level of gene expression in microglia. A β triggers neurotoxic glial activation and release of inflammatory factors, preventing microglia from phagocytosing the A β aggregates, thus contributing to neuronal toxicity. This would support the hypothesis that A β accumulation activates chronically microglia. Since there is no A β phagocytosis, A β remains leading to chronic microglia activation and inducing the release of inflammation factors toxic for the neurons ¹⁵.

In P301S Tg mice the activation of microglia precedes tau pathology generated by the transgene and the abrogation of tau-induced microglial activation could retard progression of these disorders ¹⁶.

Microglia are immunocompetent cells that take up residence in the developing brain as fetal macrophages. They are the first responsive element to brain damage or insult. Studies have shown that amyloid β plaques present in the AD brain act as chemoattractants for glial cells ¹⁷⁻²⁰. These extracellular aggregates will trigger the accumulation of activated microglia around their periphery, in turn inducing the release of pro-

inflammatory cytokines, chemokines, reactive oxygen species, complement proteins and deleterious pro-inflammatory mediators, such as some forms of prostaglandins²¹⁻²⁴. The functions and syntheses of all of these molecules are diverse, complex and beyond the scope of this introduction. For example, cytokines and chemokines regulate the intensity and duration of the immune response. Cytokine polymorphism is becoming a major focus of attention for determining its relationship to increased risk of developing AD²⁵. Prostaglandins (PGs), also produced by microglia, are active arachidonic acid metabolites that are rapidly degraded thus acting as local hormones. The key enzymes in the synthesis of s are cyclooxygenases (COX). The brain expresses COX-1 and COX-2 under normal physiological conditions but COX-2 levels are dynamically regulated by pro-inflammatory signals, such as cytokines, and by physiological neuronal plasticity²⁶.

Astrocytes are the most common cells in the brain and are involved in maintaining the functional integrity of synapses. Although astrocyte functions remain to be fully understood, it seems quite clear that they are co-localized with pro-inflammatory molecules and microglia at the sites of a β deposition^{27;28}. Astrocytes in the brain release chemokines that are able to modulate the

activation state of microglia through Nrf2-dependent antioxidant gene expression. As a consequence microglia synthesize more oxidant scavengers, such as glutathione. This will reduce the overall oxidative load disrupting the self propelled cycle of microglia activation, inflammation and its collateral damages ²⁹.

Neurons produce complement proteins, several cytokines (IL-1, IL-6, α and pentraxins, a family of acute phase proteins involved in activation of the complement system. Most interestingly, neurons were shown to activate microglia ^{30;31}. Indeed, $\text{a}\beta_{1-42}$ -treated neuronal cells have the ability to bind microglia via CD14 ³² inducing the release of IL-6 ³³. It is also remarkable that treatment of neurons with D2, the major CNS prostaglandin, results in a phenotypic change that induces microglia activation producing a buildup of pro-inflammatory signals ³⁴. In addition, studies with post mortem human brains show that COX-2 is overexpressed in neurons during the early stages of AD, prior to maximal activation of astrocytes and microglia ³⁵. These studies suggest that in AD pro-inflammatory responses might be initiated by neurons and followed by and enhanced by astrocytes and microglia. Indeed, different studies suggest that detection of inflammatory factors in cerebrospinal fluid is a good

indicator of the disease and a possible strategy for early diagnosis ^{36;37}.

The toxicity of inflammation is mostly mediated by generation of two forms of free radicals (and reactive nitrogen species) released by microglia as a mechanism for attacking a noxious agent ^{38;39}. Furthermore, the peroxidase activity of also produces free radicals including superoxide ⁴⁰. Free radicals are highly reactive species that induce deleterious alterations of protein structure and activate cell apoptosis ⁴¹. Unlike microglia, most cells are sensitive to oxidative stress and neurons are no exception. A means to avoid the formation of such radicals could involve a restraint of the inflammatory response. Interestingly, a highly toxic prostaglandin, 15d-PGJ2, inhibits NADH-ubiquinone reductase of the complex one of the mitochondrial respiratory chain, leading to the formation and accumulation of ROS ⁴², the release of cytochrome c from the mitochondria ⁴³and cellular death through apoptosis ^{44;45}.

The ability to halt inflammation seems to be impaired in AD. The use of nonsteroidal anti-inflammatory drugs (NSAID) to prevent or treat AD is a hotly debated strategy ^{45;45;46}. Most NSAIDs inhibit and ideally should decrease the generation of prostaglandins and . Recent clinical

trials with NSAIDs were based on the hypothesis that these drugs could be preventive in the early but not advanced stages of the disorder. However, the data suggest that NSAIDs failed to fulfill the expectations of delaying memory loss. It is possible that massive use of NSAIDs is deleterious for the neurons, as these drugs modulate γ -secretase, an enzyme involved in $a\beta$ formation⁴⁷. Furthermore, COX-2 activity could play an important role in memory, as it is involved in synaptic plasticity^{48;49}.

Arachidonic signaling through the pathways yields an enormous variety of products, some of them with pro-survival others with pro-death effects. For instance, prostaglandins are known to be pro-inflammatory under certain conditions and anti-inflammatory under others (reviewed in⁵⁰). The dual role of prostaglandins is quite complex as a single prostaglandin can bind to multiple receptors. Accordingly, PGE2 can bind to four different membrane-bound receptors (EP1--4), each triggering different responses inside the cell. While EP1 and EP3 activation is linked to neurotoxicity^{51;52}, EP4 activation has neuroprotective and anti-inflammatory effects^{53;54}. Likewise, two receptors (DP1 and DP2) were identified for PGD2. DP1 activation increases cAMP, activates protein kinase A and is linked to an anti-inflammatory response,

while DP2 activation increases the cellular influx of calcium and triggers a pro-inflammatory response (reviewed in ⁵⁵). PGD2-mediated activation of DP1 was found to be neuroprotective while DP2 activation was neurotoxic ⁵⁶. Besides binding to different receptors some prostaglandins, such as PGD2 and PGE2, are spontaneously metabolized by non-enzymatic dehydration to cyclopentenone prostaglandins, such as PGA2 and PGJ2, respectively. Cyclopentenone prostaglandins may have an anti-inflammatory effect by inhibiting the NFκB pathway or a neurotoxic effect by inducing apoptosis (reviewed in ⁵⁷). As suggested in the latter review 'formation of cyclopentenone eicosanoids in the brain may represent a novel pathogenic mechanism, which contributes to many neurodegenerative conditions ⁵⁸.

Clearly, the effects of different prostaglandins are variable and may depend on factors such as intracellular concentrations, receptor types, cell types and timing of activation of downstream targets that participate in the inflammatory response. Rather than inhibiting , modulating the effects of through more specific interference with their selective synthases and/or their receptors may be more effective, as prostaglandins can play different roles in recovery or degeneration. Much more needs to be learned

about the functions of inflammation in the normal and diseased CNS. The challenge resides in dissecting the dual nature of neuroinflammation as it has both positive and negative effects differing spatially and temporally in the CNS.

1.2. INFLAMMATION-DEPENDENT POST-TRANSLATIONAL MODIFICATIONS OF TAU

1.2.1 TAU HYPERPHOSPHORYLATION IN ALZHEIMER'S DISEASE

Tau is a microtubule-associated protein that stabilizes microtubules in a phosphorylation-dependent manner. Tau pathology appears to be closely related to the severity of dementia in AD. The pathological NFT in AD contain paired helical filaments (PHF) with hyperphosphorylated tau. PHF formation from tau molecules may follow several steps that involve tau phosphorylation, conformational changes and finally polymerization. Abnormal hyperphosphorylation of tau is responsible for its loss of biological activity, its resistance to degradation and is likely to be critical to NFT formation. Hyperphosphorylation of tau at pathologically relevant sites seems to precede NFT formation, to disrupt axonal integrity and to trigger neuronal loss ⁵⁹. If indeed abnormal hyperphosphorylation facilitates tau assembly into PHF, it is of the utmost importance to identify kinases and phosphatases that contribute to tau hyperphosphorylation and also to understand what triggers their enzymatic activities. Since pro-inflammatory

conditions in the brain have been implicated in AD we will discuss recent evidence linking inflammation to tau hyperphosphorylation and NFT formation.

A direct link between inflammation and tau phosphorylation was established in experiments showing that activation of microglia with or fragments induced neuronal tau phosphorylation in co-cultures with rat primary neocortical neurons ⁶⁰. The microglia effects on tau phosphorylation were mimicked by directly treating the primary neurons with IL-1 β . Microglia-induced tau phosphorylation appeared to be mediated by p38-MAPK, a proline-directed kinase. In other studies, treatment of rat hippocampal neurons with IL-6 induced anomalous tau hyperphosphorylation on epitopes dependent on proline-directed kinase activity, including Cdk5 and p38-MAPK ⁶¹. - induced inflammation in 3xTg-AD transgenic mice that harbor three mutant human genes (APP_{K670N; M671L}, PS1_{M146V}, and tau_{P301L}) was also shown to exacerbate tau pathology ⁶². In these mice, inflammation-induced tau hyperphosphorylation was mediated by Cdk5. The latter is a kinase activated by binding to its membrane cofactor p35. During inflammation, the calcium-dependent cysteine protease calpain is activated leading to cleavage of p35 to p25, a 208-residue carboxyl-terminal fragment of p35. The p25 product is more

stable and has a higher affinity for Cdk5 than p35. Generation of the p25 fragment prolongs activation and mislocalization of Cdk5 resulting in hyperphosphorylation of atypical substrates, including tau ⁶³. In the human brain, elevated levels of p25 correlate with AD. Together these studies support the view that one of the mechanisms inducing anomalous tau hyperphosphorylation may be initiated by inflammation and mediated by Cdk5 and/or p38-MAPK.

Inflammation was also shown to alter the activity of GSK3 β , another proline-directed kinase postulated to play a role in the pathological hyperphosphorylation of tau. Treatment of HEK293/tau441 cells with nitric oxide donors induces tau phosphorylation at the S262 and S396/404 pathological sites in a GSK3 β -dependent manner ⁶⁴. GSK3 β dependency was corroborated by an observed decrease in its Ser9 phosphorylation, which is required for kinase activation, and by preventing tau hyperphosphorylation by pretreating the cells with LiCl, a known GSK3 β inhibitor ⁶⁵. Co-expression of a triple FTDP-17 tau mutation with GSK3 β in transgenic mice induced an earlier formation of PHF-like structures and a more rapid atrophy of hippocampal gyrus than in transgenic mice overexpressing GSK3 β alone. These results support a synergy between the

two tau modifications, i.e. point mutations and hyperphosphorylation, and link tau hyperphosphorylation to GSK3 β activity ⁶⁶. Based on the studies described above, Cdk5, p38-MAPK and GSK3 β are likely candidates for mediating the effects of inflammation on the pathological hyperphosphorylation of tau in AD.

Since tau activity is regulated by its degree of phosphorylation and dephosphorylation it is also reasonable to investigate if deregulation of tau phosphorylation under pro-inflammatory conditions is caused by decreases in phosphatase activity. Phosphatases such as PP1, PP2A, PP2B and PP2C are known to reverse tau phosphorylation, with PP2A postulated to be the phosphatase that dephosphorylates most tau phosphorylation sites. A study addressing the activity of the different phosphatases demonstrated that PP2A accounts for 71% of tau dephosphorylation activity ⁶⁷. Decreased methylation of the catalytic subunit of the PP2A complex leading to its inhibition was observed in the affected areas of AD brains ⁶⁸. Interestingly, PP2A was shown to be a key regulator of JNK activity known to drive the expression of cytokines, such as IL-1 β ⁶⁹. A decrease in PP2A activity, such as identified in AD brains, may thus exacerbate the deleterious effects of inflammation on the brain, by

increasing cytokine synthesis. Overall, the studies discussed above support a role for inflammation in the process of NFT formation associated with AD, through activation of proline-directed kinases or inhibition of phosphatases and thus promoting the pathological hyperphosphorylation of tau.

1.2.2 TAU CLEAVAGE IN ALZHEIMER'S DISEASE

a) Caspases

Since 1988⁷⁰ it is well established that the core of insoluble PHFs contained proteolytically cleaved tau. It is now known that tau truncation is indeed an early event in the disease state that triggers the appearance of filamentous tau in NFTs in AD.⁷¹ Even though A β and the senile plaques are an early hallmark of AD, cognitive impairment is directly tied to the amount of neurofibrillary tangles inside neurons. AD brain filaments show a core of the microtubule-binding domain of tau and a small number of flanking sequences while the surface of the filament is coated with full length tau.

The current hypothesis is that intracellular (in the ER, or endosomes) and extracellular A β deposits (protofibrils and oligomers) cause cellular apoptosis. Although the apoptosis characteristics of DNA cleavage

have not been demonstrated, the activation of cysteine aspartyl proteases (caspases) has been well established. The membrane (spectrin), cytosolic (intermediate filaments), and cytoskeletal proteins (tau) are all *in vitro* caspase substrates. Even though apoptosis *per se* is incomplete in neurodegeneration, apoptotic mechanisms are probably playing a role in neurodegeneration ⁷².

In vitro studies demonstrated that after arachidonic stimulation, polymerization of full length and C-terminal cleaved tau forms filaments that are morphologically similar. In both cases the polymers resemble the unpaired straight filaments present in NFTs. Interestingly, treatment of E18 rat primary cortical and hippocampal neurons with fibrillar A β peptides in the medium activates caspases and tau cleaved at Asp421 (Tau Δ 421) is detected by western blot analysis. This caspase cleavage of tau can partially be prevented when the cultures are pre-incubated with a potent caspase inhibitor ⁷³. Treatment of hippocampal neurons with fibrillar A β induces neurite degeneration and complete collapse of microtubules only when tau is expressed in the cells. Tau depleted neurons show no signs of degeneration in the presence of A β , supporting a role for Tau in A β -induced neurodegeneration ⁷⁴. This spectrum of *in vitro* and *in vivo* models strongly

asserts a direct link between the extra-cellular amyloid aggregation and the intra-cellular neurofibrillary tangle formation.

The mechanism of tau aggregation and its toxicity is the source of an extensive array of studies. Wang *et al.* demonstrated in N2a cells that the sequentially cleaved N-terminal and C-terminal tau protein is the seed for full length tau aggregation. N-terminal cleavage of tau induces a change of conformation (going from unfolded protein to β -sheet structure), making the N-terminal cleaved tau more susceptible to C-terminal cleavage. The consequence of the last cleavage induces the aggregation of full length tau into filamentous structures. In relation to aggregation, the phosphorylation state of tau seems secondary to its cleavage. Also interesting is the direct link between the aggregate buildup and the level of cell toxicity. Although the C-terminal proteases of tau are still unidentified, they are of particular interest to alleviate the generation of amyloidogenic fragments and their deadly aggregation ⁷⁵.

In vitro cleavage assays of full length tau in the presence of various caspases show that tau is more susceptible to the protease activity of executioner caspases (-3, -7) than initiator caspases (-1, -4, -5, -8,

-10). The resulting C-terminal product is 45.9kDa and a small 2kDa fragment. Interestingly Tau Δ 421 seems more prone to adopt MC1 conformation than full length tau. The MC1 conformation is the result of the folding of the N-terminal segment of Tau which interacts with the microtubule binding region. MC1 is a distinct conformation of Tau in AD, that precedes PHFs formation and is detected in Braak stage I and II⁷⁶. Its detection in structurally intact axons hints toward a prepathological cleavage event. *In vitro* aggregation assays indicate a faster rate of oligomerization for Tau Δ 421 as well as a seeding effect for the full length Tau nucleation. In AD hippocampus, immunohistochemical staining for Tau Δ 421 showed widespread labeling within the CA1 region. This distribution correlates inversely with cognitive function and colocalizes with intraneuronal and extraneuronal A β deposits. The treatment of E18 rat primary cortical neurons with A β 1-42 triggers the cleavage of tau detectable within 6 hrs post incubation suggesting that caspase cleavage of tau was initiated by A β 1-42 accumulation.⁷⁷

Double labeling of AD and control hippocampal sections for cleaved fodrin (a cytoskeletal protein and calpain-caspase substrate) and PHF showed a clear co-

localization of these markers. Quantitative analysis showed that as the extent of NFT formation increased, there was a significant corresponding increase in cleaved fodrin immunolabeling. This provides evidence that there is an association between NFT formation and activation of apoptotic pathways in AD⁷⁸. Furthermore tau was shown to be an *in vitro* substrate for Caspase-3. It was also demonstrated in rat hippocampal cells that Tau Δ 421 is a potent effector of apoptosis, implying that tau may be involved in the self-propagating processes suggested to drive neurodegeneration^{79;80}.

Tau Δ 421 is also a common feature of other Tauopathies. Indeed cleaved tau is detected not only in AD but also in Pick's disease, supranuclear palsy, corticobasal degeneration, dementia with Lewy bodies. Notably, these neurodegenerative diseases are not characterized by extracellular A β accumulation.

The presence of hyperphosphorylated and cleaved tau together suggest that common initiating events other than extra-cellular A β accumulation leads to the activation of caspase and cleavage of cytoskeletal proteins⁸¹. Phosphorylation precedes cleavage in tangle evolution and it seems that phosphorylation at S422 prevents caspase cleavage some time during the progression of AD. Indeed *in*

vitro pseudophosphorylation of tau at S422 makes tau more resistant to caspase-3 proteolysis and to other tau cleavages as the pathology advances, suggesting a potential protective mechanism that could inhibit cleavage *in vivo*.⁸²

The two main activation pathways for apoptosis are (1) via death-receptor and caspase 8 initiation and (2) through the mitochondrial pathway and caspase 9 initiation. The two pathways collude with the pro-caspase 3 cleavage and triggering of the caspase cascade. Immunohistochemistry of AD and control brains showed a colocalization of Casp-8 and NFT. Rohn *et al*⁸³ however demonstrated, with a caspase-9 cleaved specific antibody, the "labeling of neurons in the hippocampus and enthorinal cortex from severe AD brains, as well as oxidized DNA/RNA damages". The colocalization of activated casp-8 and casp-9 suggests that both apoptotic pathways occur within the same neurons of AD brains. Remarkably there is a negative correlation between casp-9 activation and tangle formation suggesting that casp-9 precedes tangle formation. A β could lead to death-receptor apoptosis, while oxidative stress, due to aging or disease, induces mitochondrial damage and casp-9 activation. These two pathways result in over-

activation of casp-3 leading to tau cleavage as well as to its pathological aggregation.

b) Calpain

Normal tau isolated from human brains as well as recombinant tau isoforms are *in vitro* substrates for calpain⁸⁴ and in AD calpain I is highly activated.⁸⁵ *In vitro* experiments with tau isolated from human tissues demonstrated that the degree of tau phosphorylation has no impact on its affinity for calpain. In addition, the tau fragments generated were similar to those isolated from AD brain extracts. However, the aggregation of tau into PHF makes tau resistant to calpain digestion suggesting that the cleavage of tau by calpain precedes its aggregation.⁸⁶

For a long time it has been speculated that following injuries, neuronal cell death was the result of necrosis, characterized by massive ion influx, mitochondrial swelling, cell swelling, non-specific DNA breakage in the nuclei and plasma membrane rupture. More recent data showed that in chronic neurodegenerative diseases such as Huntington's disease, AD and ALS, apoptosis and necrosis are both occurring. The key enzyme of the necrotic neuronal death pathway is calpain, a pro-enzyme

heterodimer activated autocatalytically and by intracellular Ca^{2+} .

The key enzyme in the caspase cascade of apoptotic neuronal death is caspase 3, existing as a pro-enzyme and being activated by caspase 8- or caspase 9-mediated catalysis. Calpain and caspase 3 mediate only partial cleavage and not complete cleavage of their substrates. It is interesting to notice that the two death pathways, necrosis involving calpain and apoptosis involving caspase 3, are in many instances interconnected. For example in certain cell types, such as in cerebellar granule neurons, calpain is activated during apoptosis. Calpain and caspase 3 share the same substrates which are mostly cytoskeleton related proteins. Although the cleavage sites of the substrates differ, the resulting fragments are very close in size. Considering that the two cell deaths pathways are both activated in the case of neurodegenerative disorders, it might be of interest to try to prevent both phenomena to preserve the cells ⁸⁷. Interestingly, testosterone prevents the calpain cleavage of tau but not its caspase cleavage, while estrogen does the reverse in rat hippocampal neurons treated with $\text{A}\beta$ fragments. This reinforces the parallel activation of both pathways that include the two proteases, calpain and caspase 3 ⁸⁸.

c) Cathepsin D

In 1997 Kennessey et al ⁸⁹ demonstrated that different isoforms of human tau are degraded by cathepsin D *in vitro*, and that cleavage by cathepsin D could occur at neutral pH. In AD, cathepsin D level is elevated in disease vulnerable neurons, and amyloid deposition as well as endosomal-lysosomal changes are early events in late-onset AD ⁹⁰. Gallyas-Braak silver staining of AD neurons and normal neurons, reveals intracellular granules containing altered tau ⁹¹. This suggests that lysosomes and their aspartic-protease cathepsin D may normally degrade altered tau ⁹². Disruption of lysosomes induces release of cathepsins to the cytoplasm. In rat hippocampal slices, lysosomal disruption results in the hyperphosphorylation and fragmentation of tau that assembles into structures having the appearance, size, and epitope of early-stage neurofibrillary tangles ⁹³. These events could be partially prevented by cathepsin D inhibitors.

Cathepsin D is rapidly translocated from the lysosome to the cytoplasm during apoptosis linked to ROS, supposedly the consequence of lipid peroxidation in intracellular membranes ⁹⁴. Once the lysosomal membrane is damaged cathepsin D leaks into the cytoplasm. Since there is no known endogenous cytoplasmic inhibitor, cathepsin D

that is very stable at cytoplasmic pH, activates the effector caspase-3⁹⁵. Furthermore, cathepsin D release into the cytoplasm precedes the relocation of cytochrome c during apoptosis induced by oxidative stress, suggesting once again the potentiating apoptotic activity of cathepsin D⁹⁶.

d) Controversy surrounding tau ubiquitination and degradation by the proteasome.

There is little doubt that hyperphosphorylated tau is a major component of NFT. Whether NFT tau is ubiquitinated and degraded *in vivo* by the ubiquitin-proteasome pathway remains controversial. One could speculate that the decrease in proteasome activity observed in AD brain regions with severe degeneration⁹⁷ may contribute to the accumulation of ubiquitinated tau in NFT.

At least two mechanisms relevant to AD were postulated to inhibit proteasome activity. One of the mechanisms is associated with frameshift mutants of ubiquitin B (UbB+1) found to be co-localized with NFT and senile plaques in the cerebral cortex of patients with sporadic AD⁹⁸. A single dinucleotide deletion (GA) in the first GAGAG motif of UbB mRNA produces UbB+1, which lacks the C-terminal glycine, an amino acid critical for ubiquitination. UbB+1

molecules may impair degradation of ubiquitinated proteins by competing with wildtype ubiquitin for the interaction with the 26S proteasome and thus lead to neuronal cell death^{99;100}. The other mechanism leading to proteasome inhibition involves the direct binding of PHFs to the proteasome thus impairing its activity¹⁰¹.

The finding that proteasome activity is decreased in AD brains does not necessarily imply that the proteasome is involved in the *in vivo* degradation of tau. A tau/proteasome link remains questionable. On the one hand, tau was shown to be degraded by the proteasome *in vitro*^{102;103}, in cell cultures¹⁰⁴⁻¹⁰⁷, in rat brain cortex extracts¹⁰⁸ and in 3Tg-AD transgenic mice¹⁰⁹. Tau degradation by the proteasome is postulated to require its K63-polyubiquitinated form to interact with the sequestosome 1/p62, a putative 'shuttling' partner for some proteasome substrates¹¹⁰. On the other hand, tau degradation was shown to be independent of proteasome activity in rat primary hippocampal neurons¹¹¹, human neuroblastoma SH-SY5Y cells^{112;113} and in *Drosophila* harboring a temperature-sensitive mutant allele of the 20S proteasome¹¹⁴. The reasons for this discrepancy are unclear but might be related to the different experimental protocols and model systems involved in the various studies.

While the controversy surrounding the *in vivo* degradation of tau by the proteasome remains unresolved, other studies focused on the pathologic aspect of a putative tau/proteasome link. It is clear that tau is hyperphosphorylated prior to its aggregation in PHFs, which then lead to NFT formation. Hyperphosphorylated tau is recognized and ubiquitinated by the Ubox protein CHIP¹¹⁵⁻¹¹⁷. Interestingly, CHIP levels in human AD brains were found to be inversely proportional to insoluble tau accumulation¹¹⁸. In a JNPL3 mouse tauopathy model, CHIP levels were low in the spinal cord, the most prominent region exhibiting tau inclusions and neuronal loss in these mice. In addition, mice lacking CHIP exhibited the highest levels of insoluble tau in the brain¹¹⁹. These results suggest that CHIP delays the formation of tau aggregates. CHIP is a co-chaperone with intrinsic ubiquitin ligase activity, a property that allows its chaperone function to switch from protein folding to protein degradation¹²⁰. CHIP is activated after binding with Hsc/Hsp70, forming a complex that selectively ubiquitinates hyperphosphorylated tau in conjunction with UbcH5B¹²¹. Interestingly, PHF tau was found to be ubiquitinated at its microtubule binding domain,

suggesting that tau ubiquitination may be an early pathological event in the AD cascade ¹²².

Sumolation may be another tau modification relevant to its pathogenic role. Sumolation is a post-translational modification consisting of the addition of 'small ubiquitin-like modifiers' to lysine residues of target proteins, which under certain conditions leads to a partial proteasome digestion and to a change in protein function ¹²³. Tau sumolation was found to be up-regulated in cells treated with phosphatase inhibitors or microtubule depolymerizing agents ¹²⁴.

Overall these studies highlight challenges for future research to understand the requirements for achieving the proper balance between tau stability and degradation to prevent its pathological aggregation into PHFs leading to NFT formation.

1.3. OVERALL RELEVANCE

There is no doubt that chronic inflammation is a critical factor in the pathogenesis of neurodegenerative disorders, such as Alzheimer's disease (AD). Increases in cyclooxygenase-2 (COX-2) levels in neurons and glia (astrocytes and microglia) are observed in these disorders associated with chronic inflammation. However, there is a profound gap in our understanding of how pro-inflammatory cyclooxygenases and their prostaglandin products redirect cellular events to promote neurodegeneration. The **main goal** of our studies is to address this crucial omission. We elucidate some of the downstream mechanisms by which COX-2 products, in particular cyclopentenone prostaglandin J₂, cause neuronal injury.

Our studies underscore a synergy between proteasome deficiency and inflammation in triggering neurodegeneration. Cells must constantly rely on proteasome activity to remove abnormal proteins produced under pro-inflammatory and other stress conditions. Impairment of proteasome function will exacerbate the deleterious effects of inflammation, and activate pathways leading to neurodegeneration. Elucidation of mechanisms that cause pathological protein aggregation will be

opening up new and important possible targets for treatment of neurodegenerative disorders, such as AD, that are associated with chronic inflammation and protein degradation.

CHAPTER II

INVOLVEMENT OF INFLAMMATION IN TAU PATHOLOGY IN NEURONAL CELLS THROUGH PROSTAGLANDIN J2

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Submitted for publication, March 2008

2.1. ABSTRACT

Neurofibrillary tangles (NFT) are a hallmark of Alzheimer's disease (AD). The major NFT component is tau, which is truncated at Asp421 (Δ tau), hyperphosphorylated, and aggregates into insoluble paired helical filaments. AD brains also exhibit inflammation manifested by activated astrocytes and microglia, which produce cytotoxic agents among them prostaglandins. We treated human neuroblastoma cells with prostaglandin J2 (PGJ2), a highly reactive lipid electrophile that covalently binds free sulfhydryls of glutathione and cysteine residues in cellular proteins forming Michael adducts. Clearly, the initial event observed upon PGJ2-treatment was the build-up of ubiquitinated proteins. The onset of apoptosis followed manifested by caspase activation and caspase-mediated proteolysis of tau, generating Δ tau (Asp421). Cathepsin inhibition stabilized Δ tau suggesting its lysosomal clearance. Upon PGJ2-treatment tau accumulated in a large perinuclear aggregate. Δ Tau was also observed in PGJ2-treated rat primary neuronal cultures. Overall, our data suggest that products of inflammation such as PGJ2 promote an abnormal build-up of ubiquitinated proteins that, if not cleared, launches caspase-mediated proteolysis that

generates $\Delta\tau$. This may indicate a new inflammation-mediated pathway for tau pathology in AD.

2.2. INTRODUCTION

Inflammation is implicated in Alzheimer's disease (AD)¹²⁵. A recent study with P301S mutant human tau transgenic mice established that hippocampal synaptic pathology and microgliosis could be the earliest manifestations of neurodegeneration related to tauopathies¹²⁶. Prominent microglial activation was shown to precede tangle formation and immunosuppression of young P301S Tg mice diminished tau pathology and increased lifespan. It was proposed that neuroinflammation is linked to early progression of tauopathies¹²⁷.

Activated microglia and astrocytes produce a variety of agents, among them prostaglandins¹²⁸. The major prostaglandin produced in the CNS is prostaglandin D2 (PGD2)^{129;130}. PGD2 levels were found to be significantly increased in the frontal cortex of AD patients compared to age matched controls¹³¹. PGD2 is produced by two distinct types of prostaglandin D2 synthases (PGDS): (1) the lipocalin enzyme (L-PGDS) and (2) the hematopoietic enzyme (H-PGDS)¹³². In addition, PGD2 binds to G protein-coupled seven transmembrane receptors, DP1 and DP2¹³³, which are robustly expressed in the hippocampus and cerebral cortex¹³⁴. In AD patients and in Tg2576 mice, a well established AD model, the levels of H-PGDS and DP1 were found to be

selectively up-regulated in microglia and astrocytes within senile plaques ¹³⁵. Based on these results it was suggested that PGD2 acts as a mediator of plaque associated inflammation in the AD brain ¹³⁶. Similarly, L-PGDS which is one of the most abundant CSF proteins produced in the brain, was localized in amyloid plaques in both AD patients and Tg2576 mice ¹³⁷. Secreted L-PGDS in the CSF has a dual function: it increases CSF-PGD2 levels ¹³⁸ and also acts as a lipophilic-ligand carrier ¹³⁹. L-PGDS was found to bind A β monomers and prevent A β aggregation, suggesting that L-PGDS is a major A β chaperone and disruption of this function could be related to the onset and progression of AD ¹⁴⁰.

PGD2 exerts both neuroprotective and neurotoxic effects through its binding to DP1 and DP2 receptors, respectively ¹⁴¹. PGD2 is very short lived and readily undergoes *in vivo* and *in vitro* non-enzymatic dehydration to generate the biologically active cyclopentenone J2 prostaglandins, which include PGJ2, Δ 12-PGJ2 and 15-deoxy- Δ 12,14-PGJ2 (15d-PGJ2) ¹⁴².

Unlike most other classes of prostaglandins, cyclopentenone prostaglandins like PGJ2 have a cyclopentenone ring with reactive α,β -unsaturated carbonyl

groups that form covalent Michael adducts with nucleophiles such as free sulfhydryls in cysteine residues of glutathione and cellular proteins ¹⁴³. Just like serine, threonine and tyrosine phosphorylation is crucial for various signal transduction pathways, it has become recently clear that a highly conserved redox reaction of cysteine thiols in proteins provides post-translational means for regulating redox signaling ¹⁴⁴. *S*-nitrosylation of cysteine thiols by nitric oxide was first discovered, followed by the more recently revealed *S*-alkylation by electrophiles (electron deficient carbon centers). Electrophile binding by endogenous metabolites such as PGJ2 is currently regarded as playing a crucial role in determining whether neurons will live or die ¹⁴⁵.

We focus on PGJ2 because it is potently neurotoxic and a highly reactive product of inflammation. A recent review suggested that "formation of cyclopentenone eicosanoids [such as PGJ2] in the brain may represent a novel pathogenic mechanism that contributes to many neurodegenerative conditions" ¹⁴⁶. Because of their high reactivity with thiol-containing intracellular compounds like glutathione or thiol-containing proteins via Michael addition, any attempt to measure PGJ2 levels in human tissues or fluids will be highly inaccurate and will not

reflect its biological activity ¹⁴⁷. Similar to PGJ2, nitric oxide has a half life of just a few seconds, and yet is well accepted as a major signaling molecule in neurons and in the immune system ¹⁴⁸. The same can be said for PGJ2, i.e. that it is a major signaling molecule/oxidative stress agent.

Here we show that the lipid electrophile PGJ2 induces tau cleavage at Asp421 in a time- and dose-dependent manner in human neuroblastoma SK-N-SH cells. PGJ2-induced tau cleavage is mediated by caspases, which are activated upon cell treatment with PGJ2. In addition, we demonstrate that the abnormal build-up of ubiquitinated proteins induced by PGJ2 clearly preceded caspase activation, caspase-mediated tau cleavage and apoptosis. PGJ2 modulation of tau cleavage was also observed in rat primary neuronal cultures. These results demonstrate that products of inflammation such as PGJ2 have a bifunctional effect on intracellular protein turnover. They impair the ubiquitin/proteasome pathway leading to the abnormal build-up of ubiquitinated proteins that, if not cleared, activates caspase-mediated proteolysis responsible for generating Δ tau that could serve as a seed for cytotoxic protein aggregation. This abnormal and sequential build-up of intracellular ubiquitinated proteins followed by the

formation of aggregation prone truncated proteins, such as Δ tau, could be a major factor in the progression of AD neurodegeneration.

2.3 MATERIALS AND METHODS

2.3.1 MATERIALS

PGJ2, ciglitazone and BADGE were from Cayman Chemical (Ann Arbor, MI). Protease inhibitors: caspase irreversible inhibitors (2 μ M) for individual caspases 1 through 6, 8 through 10 and 13, the pan caspase inhibitor (Z-VAD-FMK), which inhibits all known caspases, and the negative control Z-FA-FMK (FMK) were from BioVision, CA. Caspase inhibitor I, which inhibits all caspases and is the same as the pan caspase inhibitor, calpeptin (Z-Leu-Nleu-CHO), calpain inhibitor I (N-Acetyl-Leu-Leu-Nle-CHO), calpain inhibitor III (Z-Val-Phe-CHO), and Pepstatin A (Iva-Val-Val-Sta-Ala-Sta) were from Calbiochem (San Diego, CA). Calpain inhibitor (Z-Leu-Leu-CHO) was from BioMol (Plymouth Meeting, PA), PSI [Z-Ile-Glu(OtBu)-Ala-Leu-CHO] from Peptides International Inc. (Louisville, KY), and NAC (N-Acetyl-Cysteine) from Sigma-Aldrich, St. Louis, MO. Primary antibodies: Tau C3 (mouse monoclonal, tau cleaved at Asp421) 1:500 from Covance (Emeryville, CA), Tau clone 5 (mouse monoclonal) 1:1000 courtesy Dr. L. Binder (Northwestern University, Chicago, Il), TauY9 (rabbit polyclonal) 1:1000 from BioMol (Plymouth Meeting, PA), pan tau clone 13 (mouse monoclonal) 1:1000 from Santa-Cruz

(Santa Cruz, CA), mouse monoclonal anti- γ -tubulin (1:2,000, clone GTU-88) from Sigma-Aldrich, rabbit polyclonal anti-ubiquitinated proteins (1:1,500) from Dako Cytomation (Carpinteria, CA), anti-cleaved PARP (1:1000 clone 19F4) from Cell Signaling Tech (Danvers, MA), anti-hsp90(86) (1:2000) from Affinity Bioreagent Inc., Golden, CO. The respective secondary antibodies with fluorophores (1:50) were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). TO-PRO-3 iodide (nuclear staining) was from Invitrogen (Carlsbad, CA).

2.3.2 CELL CULTURES

SK-N-SH cells are a human neuroblastoma cell line derived from peripheral tissue ¹⁴⁹. The cells are maintained at 37°C in MEM with Earle's salts containing 5% normal fetal bovine serum, 2mM L-glutamine, 1mM sodium pyruvate, 0.4% MEM vitamins, 0.4% MEM nonessential amino acids, and 100 units/ml penicillin, 100 μ g/ml streptomycin in 5% CO₂.

Rat E18 cortical neuronal cultures were from Neuromics (Edina, MN). The cells were maintained at 37°C, in 5% CO₂, in neurobasal media supplemented with 2% B27 and 0.5mM glutamine (all from Invitrogen) and half of the medium was changed every 3 days.

2.3.3 CELL TREATMENTS

Cell cultures were treated for the indicated times with vehicle (DMSO, dimethyl sulfoxide) or with different concentrations of PGJ2 in DMSO added directly to serum-containing medium. The final DMSO concentration in the medium was 0.5%. At the end of the incubation, all cultures were washed twice with phosphate buffered saline (PBS) and processed for the different assays as described below. Cell washes removed unattached cells, therefore subsequent assays were performed on adherent cells only.

2.3.4 WESTERN BLOTTING

Western blot analysis was carried out by SDS-PAGE on 8% or 10% polyacrylamide gels. After treatment cells were rinsed twice with PBS and were harvested by gently scraping into ice-cold homogenization buffer [20mM Tris-HCl, pH 7.5, 137mM NaCl, 1mM EGTA, 2.5mM Na₄P₂O₇, 1mM βglycerophosphate, 50mM NaF, 1mM PMSF, 1% NP40, 1mM Na₃VO₄, 1% Glycerol and protease inhibitor cocktail (Sigma-Aldrich)]. Samples were boiled for 5-min in Laemmli buffer and loaded onto gels (30-50μg of protein/lane). Following electrophoresis, proteins were transferred to an Immobilon-P membrane (Millipore, Bedford, MA). The membrane was probed with the respective antibodies and

antigens were visualized by a chemiluminescent horseradish peroxidase method with the ECL reagent. As a control for protein loading the western blots were probed for actin [mouse monoclonal anti-actin (1:2,500, clone AC-20) from Sigma-Aldrich]. 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).

2.3.5 CELL VIABILITY

Cell survival was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described in ¹⁵⁰.

2.3.6 CASPASE ACTIVITY ASSAYS

Caspase screening assays (for caspases 2, 3, 8 and 9) and caspase 3 assays were carried out with ApoAlert caspase kits (plates) from ClonTech (Mountain View, CA) following manufacturer's specifications.

2.3.7 SUBCELLULAR FRACTIONATION

After treatment SK-N-SH cells in 10cm dishes (4.5×10^5 cells/ml) were rinsed twice with PBS. Cells were harvested by gently rocking the dishes with 2ml of pre-warm (37°C) cell dissociation solution (Sigma-Aldrich) for 10min and packing the cells by centrifugation at 500Xg for three

min. The cell pellet was then lysed and the separation of enriched nuclear and cytoplasmic proteins was performed with a nuclear and cytoplasmic kit (NE-PER from PIERCE, Rockford, IL). After removing the soluble cytoplasmic and enriched nuclear extracts, the remaining pellet fraction was re-suspended in denaturing buffer (0.01M Tris-EDTA pH 7.5, 2% SDS).

2.3.8 IMMUNOFLUORESCENCE

After treatment cells were rinsed with PBS, fixed for 15min at 37°C in 2% formaldehyde in media, quenched with 50mM NH₄Cl/PBS for 20min, permeabilized with 0.1% saponin/PBS, blocked with 2% BSA/PBS, and incubated with the antibodies listed in each figure. Slides were mounted with Vectashield mounting medium hard set for immunofluorescence (Vector Laboratories, Inc., Burlingame, CA). Cell staining was visualized with Leica TCS SP2 confocal microscope (Leica microsystems, Exton, PA). TO-PRO-3 iodide (Invitrogen, Carlsbad, CA) was used for nuclear staining.

2.3.9 PROTEIN CONCENTRATION

was determined with the bicinchoninic acid assay kit (Pierce, Rockford, IL) or the Bradford Assay (Bio-Rad, Hercules, CA).

2.3.10 STATISTICAL ANALYSIS

statistical significance was estimated using one-way ANOVA (Tukey-Kramer multiple comparison test) or the unpaired "t" test with the InStat 2.0, Graphpad Software (San Diego, Ca).

2.4. RESULTS

2.4.1 PGJ2 INDUCES ACCUMULATION OF UBIQUITINATED PROTEINS, TAU CLEAVAGE AND APOPTOSIS IN A DOSE DEPENDENT MANNER - Tau cleavage is an early event in AD tangle pathology ¹⁵¹. Furthermore, microglia activation was shown to precede tangle formation in the P301S tauopathy mouse model ¹⁵². We thus determined the effect of the product of inflammation PGJ2 on tau cleavage in human SK-N-SH neuroblastoma cells. We assessed tau immunoreactivity in human neuroblastoma SK-N-SH cells with two monoclonal antibodies: tauC3 (epitope a.a. 412-421), which is specific for tau cleaved at Asp421 (Fig. 1A), and tau13 (Fig. 1E, pan tau, epitope a.a. 2-18), which detects all tau isoforms of human origin. Tau C3 detected two major bands with an approximately molecular mass of ~60kDa (tau FL, full length) and ~44kDa (Δ tau, tau cleaved at Asp421), while pan tau reacted with all tau isoforms as well as with Δ tau. Equal protein loading was demonstrated by probing the blots with the anti-actin antibody (Fig. 1D).

It is clear that the levels of Δ tau increased in a concentration-dependent manner (Fig. 1A, E and G) reaching a peak at 20 μ M PGJ2 after 16h of treatment (Fig. 1A, boxed). The Δ tau peak coincided with a peak in apoptosis

assessed by PARP [poly (ADP-ribose) polymerase] cleavage (Fig. 1B and G) and with a ~53% decrease in cell survival assessed with the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (Fig. 1F). Notably, the highest levels of ubiquitinated proteins were observed in cells treated with lower concentrations of PGJ2 (10 μ M). These results indicate that the production of Δ tau is synchronized with the cell death pathway but trails the build-up of ubiquitinated proteins.

2.4.2 PGJ2 INDUCES ACCUMULATION OF UBIQUITINATED PROTEINS, TAU CLEAVAGE AND APOPTOSIS IN A TIME DEPENDENT MANNER - To establish the time-dependency of the PGJ2 effect on Δ tau we incubated SK-N-SH cells with 20 μ M PGJ2 for 2h, 4h, 8h, 16h and 20h. Just like PARP cleavage (Fig. 2B and G), an indicator of apoptosis, Δ tau was detected 16h post PGJ2- treatment (Fig. 2A, E and G). At this time point a significant decrease (~33%) in cell survival assessed with the MTT assay was also observed (Fig. 2F). This remarkable coincidence of tau and PARP cleavage indicates that Δ tau is associated with the onset of apoptosis.

The accumulation of ubiquitinated proteins was detected 2h after the PGJ2 treatment and reached a peak at 4h post-

treatment (Fig. 2C and G). Clearly, the abnormal accumulation of ubiquitinated proteins precedes the onset of apoptosis and the generation of the stable Δ tau and cleaved PARP, suggesting that if the cell cannot clear the overwhelming build-up of ubiquitinated proteins induced by PGJ2 it launches caspase-mediated proteolysis.

2.4.3 IN SK-N-SH CELLS PGJ2-TREATMENT ACTIVATES CASPASES WHICH IN TURN MEDIATE TAU CLEAVAGE - Since

Δ tau coincided with the onset of apoptosis we investigated if PGJ2 activates intracellular caspases. Initially, we run a caspase screening assay and established that treatment of SK-N-SH cells with 20 μ M PGJ2 for 16h, significantly ($p \leq 0.014$, T test) activated all caspases tested, i.e. initiator caspases (CASP2, CASP8 and CASP9) as well as the effector caspase 3, albeit different levels of activation were observed (Fig. 3A). Not surprisingly, caspase 3 showed the greatest activation (~20-fold) upon PGJ2-treatment, since it is an effector caspase. Following 8h of treatment with 20 μ M PGJ2, caspase 3 activity was significantly higher than in control ($p < 0.001$, asterisks) and reached a peak (24-fold increase) by 16h post-treatment. The peak in caspase 3 activity coincides with the peak in PARP cleavage, a known substrate for this

caspase, and Δ tau peak supporting the view that tau is also a substrate for caspase 3.

In SK-N-SH cells tau cleavage induced by 16h of treatment with 20 μ M PGJ2 (Fig. 4A, *boxed lanes 2 and 10*) was attenuated by pre-treatment with specific irreversible inhibitors (2 μ M) for individual caspases 1 through 6, 8 through 10 and 13 (Fig. 4A, *lanes 3-8 and 11-14*). The pan caspase inhibitor, which inhibits all known caspases, also lessened the levels of Δ tau (Fig. 4A, *lane 15*). The negative control Z-FA-FMK (FMK) did not affect full length tau (Fig. 4A, *lane 16*). Overall, these results clearly indicate that PGJ2-induced tau cleavage is caspase-mediated.

2.4.4 PHARMACOLOGICAL MANIPULATIONS OF TAU

CLEAVAGE IN SK-N-SH CELLS - There are no specific prostaglandin synthases leading to PGJ2 production and no specific PGJ2 receptors have yet been identified ¹⁵³. However, PGJ2 can act via the intranuclear receptor PPAR γ (peroxisome proliferator-activated receptor γ) ¹⁵⁴. To test if Δ tau is induced by PPAR γ activation, we treated SK-N-SH cells with ciglitazone a *bone fide* PPAR γ ligand. As shown in Fig. 5A (*lane 7*) ciglitazone did not induce tau cleavage and the PPAR γ antagonist BADGE (bisphenol A

diglycidyl ether) failed to prevent PGJ2-induced Δ tau (Fig. 5A, compare boxed lane 2 with lane 3) showing that PPAR γ does not mediate this PGJ2 effect on tau.

Besides acting through receptors, PGJ2 can exert its action by covalently binding to free sulfhydryl groups on cysteines in glutathione and cellular proteins¹⁵⁵. N-acetyl-cysteine (NAC) exhibits direct and indirect antioxidant properties and binds to electrophilic groups¹⁵⁶. We thus tested if NAC prevented PGJ2 cleavage of tau. As seen in Fig. 5B (lane 6), NAC totally prevented the formation of Δ tau.

Since PGJ2 is known to inhibit proteasome activity in cells¹⁵⁷ we investigated whether a proteasome inhibitor [PSI, N-benzyloxycarbonyl-Ile-Glu(O-t-butyl)-Ala-leucinal] would mimic the PGJ2 effect on Δ tau. As shown in Fig. 5B (lanes 4 and 5) PSI did not increase the levels of Δ tau. Neither did the calpain inhibitor Z-LL-CHO (Fig. 5B, lane 3).

We also investigated if inhibitors of other intracellular proteases prevented tau cleavage by PGJ2 in SK-N-SH cells. Four different calpain inhibitors (Fig. 6A) and the general cathepsin inhibitor pepstatin (Fig. 6B) failed to abrogate PGJ2-induced Δ tau. In fact, we observed

the highest levels of Δ tau in cells co-treated with caspase inhibitor 1 and pepstatin prior to PGJ2 (Fig. 6B, *boxed lane 3*). These results mimic what was observed, to a lesser extent, with PGJ2 in conjunction with 20 μ M caspase inhibitor 1 (Fig. 5A, *lane 4*). Caspase inhibitor 1, Z-VAD-FMK, inhibits all caspases. However, it was previously demonstrated that caspase inhibitors with fluoromethyl ketones (FMK) also reduce the activity of lysosomal cathepsins ¹⁵⁸. These data indicate that caspase inhibitors exert a bifunctional, concentration-dependent effect on tau cleavage: low concentrations (2 μ M) diminish Δ tau, while high concentrations (20 μ M) increase Δ tau. The latter effect may indicate that once formed, Δ tau is removed by lysosomal cathepsins, which are also inhibited by the higher (20 μ M) caspase inhibitor 1 concentrations. A similar effect was not observed on PARP, as 20 μ M caspase inhibitor 1 prevented PARP cleavage (Fig. 5A, *middle panel, lane 4*).

2.4.5 UPON TREATMENT OF SK-N-SH CELLS WITH PGJ2

Δ TAU IS ASSOCIATED WITH AN ENRICHED NUCLEAR FRACTION – Upon PGJ2 treatment (20 μ m, 16h) proteins from total lysates from control (C) and treated (T) cells were

separated into cytoplasmic (CYTOP.) and enriched nuclear fractions as well as into a pellet fraction. The samples were then analyzed by western blotting. In PGJ2-treated cells Δ tau was detected mostly in the enriched nuclear fraction where full length tau (TAU FL) was also apparent (FIG. 7A AND B, *lane 4*). Truncated tau (Δ tau) was detected with monoclonal and polyclonal antibodies (FIG. 8) that react with different epitopes: Tau C3 (monoclonal, epitope a.a. 412-421) from Covance, tau clone 5 (monoclonal, epitope a.a. 210-241) courtesy from Dr. Binder, TauY9 (polyclonal, epitope a.a. 12-27) from BioMol, and pan tau clone 13 (monoclonal, epitope a.a. 2-18) from Santa-Cruz. these results confirm that the PGJ2-induced Δ tau detected in the enriched nuclear fraction is indeed truncated tau.

As expected, most of PARP is localized to the enriched nuclear fraction (Fig. 7C, *lanes 3 and 4*) and the heat shock chaperone HSP90 is predominantly found in the cytoplasm (Fig. 7D, *lanes 1 and 2*), attesting to the efficacy of the subcellular fractionation protocol (it is predicted that less than 10% contamination between enriched nuclear and cytoplasmic fractions is obtained with this protocol). However, probing a parallel gel with an anti- γ -tubulin antibody demonstrated that this protein,

which is a component of the microtubule organizing center (MTOC) was distributed to approximately the same extent in all subcellular fractions (Fig. 7E, lanes 1-6). This finding suggests that tau (FL and Δ tau) detected in the enriched nuclear fraction may be associated with the MTOC, since this intracellular structure co-localizes with the enriched nuclear fraction as well. Interestingly, two γ -tubulin bands were immunodetected in the pellet fraction (Fig. 7E, lanes 5 and 6). The heavier band may correspond to monoubiquitinated γ -tubulin¹⁵⁹.

2.4.6 PGJ2 INDUCES THE FORMATION OF A LARGE PERINUCLEAR TAU AGGREGATE NEXT TO A NUCLEAR INDENTATION - The distribution of tau was altered by PGJ2 treatment. In control cells tau (*green*) exhibits a uniform distribution that extends from the nuclear envelope out to the periphery of the cell (Fig. 9A, larger in D). The nucleus is stained red with a carbocyanine monomer (TO-PRO-3 iodide, Invitrogen). Upon treatment with 20 μ M PGJ2 for 16h tau immunoreactivity (Fig. 9B, larger in C) emerged as a large perinuclear aggregate (*white arrows*) next to a nuclear indentation. The large perinuclear aggregate detected in PGJ2-treated cells resembles

aggresomes, first described by Kopito's group¹⁶⁰. Aggresomes are thought to be deposition sites for proteins that escape degradation by the ubiquitin/proteasome pathway and to be co-localized with centrosome/MTOC markers such as γ -tubulin [reviewed in¹⁶¹].

2.4.7 TAU IS CLEAVED AT ASP421 IN RAT PRIMARY CORTICAL NEURONAL CULTURES TREATED WITH PGJ2 - We incubated rat E18 primary cortical neuronal cultures with 20 μ M PGJ2 for 16h. As shown in Fig. 10A (*lanes 3 and 5, designated C*) the TauC3 antibody did not react with any major tau bands in control cultures. In cells treated with PGJ2 (*boxed lanes 4 and 6, designated T*) TauC3 immunoreactivity detected truncated tau (Δ tau) with a molecular mass similar to Δ tau identified in human SK-N-SH cells treated with PGJ2 (*lane 2*). The pan tau C5 antibody (Fig. 10B) detected full length tau in control cells (*lanes 7 and 9*) and full length as well as Δ tau in PGJ2-treated cells (*boxed lanes 8 and 10*). The blots were probed with anti-actin as protein loading control (Fig. 10C).

Upon immunofluorescent analysis with the TauC3 antibody, dystrophic-like neurites were observed in the cortical neuronal cultures treated with PGJ2 (Fig. 10D and E).

Sites of neuritic dystrophy are suggested by bulb-like accumulations of tau (*arrowheads*) and punctuate appearance (*arrows*) in the PGJ2-treated neurons. Control cultures did not exhibit any immunofluorescence with the TauC3 antibody (*not shown*). This is not surprising, since as shown in Fig. 10A (*lanes 3 and 5*), no immunoreactivity by western blot analysis with the same antibody was detected under control conditions.

2.5. DISCUSSION

2.5.1 PGJ2 MODULATES TAU CLEAVAGE (ASP421)

THROUGH CASPASE MEDIATED PROTEOLYSIS - Our study provides the first evidence that a product of inflammation, PGJ2, modulates tau cleavage at Asp421 through caspase-mediated proteolysis in neuronal cells. This is important in view of the fact that studies from other groups indicate that caspase-cleavage of tau at Asp421 is an early event in AD tangle pathology^{162;163}. We also demonstrate for the first time that PGJ2-induced tau cleavage at Asp421 (Δ tau) coincides with the onset of apoptosis indicated by caspase activation as well as by the concurrence of tau and PARP cleavage. Only caspase inhibitors attenuated Δ tau levels induced by PGJ2. Neither calpain nor cathepsin inhibitors diminished Δ tau levels. Our data also indicate that Δ tau must be cleared by lysosomal proteases, since cathepsin inhibition by pepstatin or by high concentrations (20 μ M) of caspase inhibitors that also affect cathepsins¹⁶⁴, caused an increase in PGJ2-induced Δ tau levels.

A similar tau cleavage at Asp421 was observed in rat cortical^{165;166} and hippocampal¹⁶⁷ neurons as well as organotypic hippocampal slice cultures¹⁶⁸ treated with A β 42

(β amyloid 1-42), suggesting a link between amyloid and NFTs in AD. Other studies demonstrated that prostaglandins (PGA1, PGE1, PGE2) increase $A\beta$ levels in neuroblastoma cells ^{169;170} and that deletion of the PGE2 receptor EP2 reduces amyloid burden in an AD transgenic mouse model ¹⁷¹. It is tempting to speculate that as a result of inflammation, prostaglandins increase $A\beta$ levels which in turn trigger tau cleavage at Asp421. This cascade of events would suggest a proinflammatory pathway linking amyloid and tau pathologies in AD.

2.5.2 PGJ2-INDUCED TAU CLEAVAGE IS PPAR γ -INDEPENDENT BUT OXIDATION-DEPENDENT - Our data show that PGJ2-induced tau cleavage is PPAR γ -independent. A PPAR γ activator (ciglitazone) failed to mimic the PGJ2 effect on tau and a PPAR γ -antagonist (BADGE) did not prevent tau cleavage induced by PGJ2. This is not surprising, since PPAR γ -agonists have been linked to neuroprotection rather than neurotoxicity ¹⁷². In fact PPAR γ agonists are being considered as AD therapeutic agents because they modulate inflammatory responses ¹⁷³. However, the protective effect of PPAR γ -agonists seems to be mediated by an anti-oxidant effect ¹⁷⁴. The finding that

the anti-oxidant NAC prevents PGJ2-induced tau cleavage as well as other PGJ2-dependent toxic effects ¹⁷⁵, supports the view that PGJ2 neurotoxicity is indeed mediated by its pro-oxidant nature.

2.5.3 BUILD-UP OF UBIQUITINATED PROTEINS PRECEDES CASPASE ACTIVATION AND Δ TAU FORMATION - AD

and many other neurodegenerative disorders, such as Parkinson's disease and amyotrophic lateral sclerosis, share an intriguing morphological feature that is the intracellular deposition of aggregated and ubiquitinated proteins in neurons of the affected CNS areas [reviewed in ¹⁷⁶]. The relationship between the accumulation of ubiquitinated proteins, which is a general phenomenon in these disorders, and more disease-specific pathology such as NFT in AD remains to be elucidated.

Our studies clearly demonstrate that the abnormal build-up of ubiquitinated proteins is an early event in the cytotoxic response to PGJ2. The accumulation of ubiquitinated proteins observed upon treatment with prostaglandins of the J2 series is a result of their inhibitory effect both on deubiquitination ¹⁷⁷⁻¹⁷⁹ and proteasome activity ¹⁸⁰⁻¹⁸². On the other hand, the reaction between cysteine thiol groups in proteins and the J2

prostaglandins through Michael addition provokes changes in protein tertiary structure ¹⁸³ that could impede protein degradation and lead to their accumulation. Overall, the chemical properties of J2 prostaglandins and their pro-oxidant and UPP disrupting effects render them extremely capable of bringing about the build-up of pro-apoptotic and detrimental proteins, such as p53 and ubiquitinated proteins ¹⁸⁴.

Our finding that the build-up of ubiquitinated proteins upon PGJ2-treatment precedes the onset of apoptosis suggests that cells launch the pro-death pathway if they do not succeed in removing the overwhelming levels of ubiquitinated proteins. Once apoptosis is activated caspase-mediated proteolysis is responsible for inducing partial protein cleavage, leading to the generation of, for example, truncated tau. Caspase-mediated proteolysis seems to be a more general phenomenon that cleaves not only PARP and tau but many other substrates recently identified by a proteome-wide effort to establish the caspase substrate repertoire ¹⁸⁵. Full-length tau is a highly soluble protein, yet in AD it appears as abnormal aggregates. Previous studies propose that truncated tau induces the aggregation of full-length tau and that tau

aggregates are toxic to cells¹⁸⁶⁻¹⁸⁸. Therefore, Δ tau may serve as a seed for cytotoxic protein aggregation.

2.5.4 PERINUCLEAR TAU AGGREGATES AND DYSTROPHIC

NEURITES - We found that Δ tau is detected in an enriched nuclear fraction of PGJ2-treated SK-N-SH cells. It is clear that the enriched nuclear fraction also contains γ -tubulin. The latter is a protein found in the cytoplasm but is concentrated at the microtubule organizing center (MTOC/centrosome) where it regulates microtubule nucleation. MTOCs are located near the nucleus, therefore it is not surprising that they co-fractionate in the enriched nuclear fraction. In our previous studies we demonstrated that PGJ2 induces the collapse of the cytoskeleton/ER¹⁸⁹. The PGJ2-dependent cytoskeletal rearrangement paralleled the development of a large MTOC aggregate co-localized with γ -tubulin and also containing ubiquitinated proteins. We now demonstrate that this perinuclear aggregate that resembles an aggresome¹⁹⁰ is also enriched in tau. These data suggest that tau is driven to the perinuclear aggregate by the microtubule collapse. We also established that PGJ2 caused neurite dystrophy in rat E18 primary cortical neurons indicating

that neuritic pathology is not always linked to amyloid plaques.

Notably, upon subcellular fractionation, we detected two γ -tubulin forms (~52kDa and 60kDa) in the pellet fraction of SK-N-SH cells. In addition, upon PGJ2-treatment, the 52kDa form became the predominant one. These two γ -tubulin forms differ in molecular mass by ~8kDa, suggesting that the ~60kDa form corresponds to monoubiquitinated γ -tubulin. Monoubiquitination of γ -tubulin at K48 by the ubiquitin ligase BRCA1 was shown to regulate centrosome numbers in breast cancer cells ¹⁹¹. BRCA1 was recently postulated to modulate neuronal cell cycle re-entry in AD ¹⁹². Furthermore, reexpression of cell cycle proteins and DNA synthesis in mice expressing non-mutant human tau support the hypothesis that tau pathology is linked to neurodegeneration via abnormal incomplete cell-cycle reentry ¹⁹³. Cell cycle suppression in neurons seems to be crucial to neuron survival ¹⁹⁴. These studies together with our observation that PGJ2 treatment potentially attenuates γ -tubulin monoubiquitination, may indicate a possible link between BRCA1 and its γ -tubulin substrate with cell cycle reentry in AD neurodegeneration.

In conclusion, it is clear that PGJ2 is not the only cytotoxic agent produced by activated glia (microglia and astrocytes) as a result of the chronic inflammatory process. Other factors, such as nitric oxide, IL1 β , IL6, TNF α and reactive oxygen species (e.g. superoxide anion) are also produced under conditions of inflammation. All of these cytotoxic agents must work in concert to induce synergistic neurotoxicity leading to neurodegeneration^{195;196}. Based on the data obtained with PGJ2 we propose a model in which any stimulus (physical, chemical or infectious) capable of inducing inflammation in a particular brain area, activates microglia and astrocytes. The activated glia release toxic products (such as PGJ2) onto neighboring neurons that cause UPP impairment or overwhelm proteasome activity causing the accumulation of ubiquitinated proteins. If these Ub-proteins fail to be cleared, apoptosis is triggered. During apoptosis caspase-mediated proteolysis generates aggregation prone partial protein digests such as Δ tau. These cleavage products of caspase-mediated proteolysis may serve as seeds for cytotoxic protein aggregation. Protein aggregation in conjunction with caspase activation would thus promote neurodegeneration. This sequence of events could explain

many pathological features of the AD neurodegenerative process.

CHAPTER III

A SINGLE AMINO ACID SUBSTITUTION IN A PROTEASOME SUBUNIT TRIGGERS AGGREGATION OF UBIQUITINATED PROTEINS IN STRESSED NEURONAL CELLS

Journal of Neurochemistry 2004 vol 90(1) pages: 19-28

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3.1. ABSTRACT

Accumulation of ubiquitinated proteins in inclusions is common to various neurodegenerative disorders, such as Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis, although it occurs in selective neurons in each disease. The mechanisms generating such abnormal aggregates and their role in neurodegeneration remain unclear. Inclusions appear in familial and non-familial cases of neurodegenerative disorders suggesting that factors other than particular mutations contribute to protein accumulation and aggregation.

Proteasome impairment triggered by aging or conditions such as oxidative stress, may contribute to protein accumulation and aggregation in neurodegeneration. To test this hypothesis in mouse neuronal cells, we over-expressed a 20S proteasome $\beta 5$ subunit with an active site mutation. The N-terminal threonine to alanine substitution resulted in impairment of the chymotrypsin-like activity, which is a rate-limiting step in protein degradation by the proteasome. The Thr1Ala mutation was not lethal under homeostatic conditions. However, this single amino acid substitution significantly hypersensitized the cells to oxidative stress triggering not only the accumulation and aggregation of ubiquitinated proteins including synuclein,

but also cell death. Our results demonstrate that this genetic manipulation of proteasome activity involving a single amino acid substitution causes the formation of protein aggregates in stressed neuronal cells independently of the occurrence of mutations in other cellular proteins. These results support the notion that proteasome disruption may be central to the development of familial as well as sporadic cases of neurodegeneration.

3.2. INTRODUCTION

In a wide variety of neurodegenerative disorders, such as AD, Parkinson's diseases (PD) and amyotrophic lateral sclerosis (ALS), aggregates of ubiquitinated proteins are detected in neuronal inclusions indicating that the ubiquitin/proteasome pathway may be deficient [reviewed in ¹⁹⁷]. The relationship between the formation of the intracellular inclusions and the selective neuronal cell death that occurs in the various neurodegenerative disorders remains undefined.

The ubiquitin/proteasome pathway is responsible for the turnover of many regulatory and misfolded proteins and is essential for cell growth as well as cell viability ¹⁹⁸. Proteolysis of polyubiquitinated proteins in this pathway is carried out by the 26S proteasome ¹⁹⁹, which includes a regulatory component, the 19S particle, and a proteolytic component, the 20S proteasome ²⁰⁰. The latter is composed of 28 subunits arranged in four heptameric-stacked rings ²⁰¹ and has a broad specificity hydrolyzing peptide bonds on the carboxyl side of hydrophobic (chymotrypsin-like), basic (trypsin-like) and acidic (caspase-like) amino acids ²⁰².

Under homeostatic conditions, polyubiquitinated proteins do not accumulate in cells since they undergo selective

proteolysis by the ubiquitin/proteasome pathway. The neuronal inability to eliminate polyubiquitinated proteins may result from a malfunction or overload of the ubiquitin/proteasome pathway or from structural changes in the protein substrates halting their degradation [reviewed in ²⁰³].

Our studies investigate the possibility that impairment of proteasome activity may be an important contributor to neurodegeneration. We previously established that incubations of mouse neuronal HT4 cells with proteasome inhibitors led to an accumulation of ubiquitinated proteins and to a decrease in cell viability ²⁰⁴. However, proteasome inhibitors may exert nonspecific effects as bound molecules or through interactions with other cellular proteins. To overcome this difficulty we decided to genetically manipulate proteasome activity.

In the present study, disruption of proteasome activity was achieved by generating a mutation on the active site of its constitutive $\beta 5$ catalytic subunit. The latter accounts for the chymotrypsin-like activity, which carries-out the rate-limiting step in protein degradation by the proteasome ²⁰⁵. The N-terminal Thr of $\beta 5$ was mutated to Ala because the 20S proteasome is a threonine protease in which the nucleophilic attack is mediated by the N-

terminal Thr of processed, catalytically active β subunits
206.

The results from our studies clearly demonstrate that in mouse neuronal HT4 cells, expression of a 20S proteasome β 5 subunit mutant (T1A) that impairs its chymotrypsin-like activity, is not dominant lethal under homeostatic conditions. However, this single amino acid substitution causes accumulation of ubiquitinated proteins in aggregates and cell death in stressed mammalian cells, under conditions in which stable transfectants expressing the WT β 5 construct or vector control are not affected. These findings underscore the requirement for an optimally functional proteasome for protein turnover under stress conditions and support the view that proteasome impairment may be a key player in sporadic cases of neurodegenerative disorders.

3.3. MATERIALS AND METHODS

(only those performed by Lisette T. ARNAUD)

3.3.1 GLYCEROL DENSITY GRADIENT CENTRIFUGATION

Cells were lysed in 0.01M Tris-EDTA, pH 7.5 by sonication and the lysates were centrifuged for 10-min at 19,000g at 4°C. The cleared supernatants were subjected to centrifugation at 83,000Xg for 24h in a Beckman SW41 rotor in a 10-40% glycerol gradient made in the same lysis buffer. Following centrifugation 14 fractions (500µl each) were collected and analyzed.

3.3.2 PEPTIDASE ACTIVITIES

Peptidase activities were assayed colorimetrically after 24h incubations at 37°C as described in ²⁰⁷. The chymotrypsin-like activity was measured with the substrates Suc-LLVY-AMC and Z-GGL-pNA and the caspase-like activity with Z-LLE-NA. All substrates were purchased from Bachem Bioscience Inc.

3.3.3 IMMUNOCYTOCHEMISTRY

For immunofluorescence, cells were fixed in ice cold methanol:acetone (1:1) at -20°C and co-incubated with anti-ub-conjugates (1:250) and anti-synuclein (1:100) antibodies. The secondary antibodies (1:50) were Texas red-labeled donkey anti-rabbit and fluorescein-labeled

donkey anti-goat (Jackson laboratories, Inc.). Slides were mounted with vectashield medium containing DAPI (Vector). Cell staining was always visualized with an Optiphot-2 fluorescence microscope (Nikon).

3.4. RESULTS

(only those performed by Lisette T. ARNAUD)

3.4.1 THE CHYMOTRYPSIN-LIKE ACTIVITY OF PROTEASOMES IS SIGNIFICANTLY REDUCED IN MUTA β 5 STABLE TRANSFECTANTS TREATED WITH CADMIUM - To

verify whether the hypersensitivity of the Muta β 5 stable transfectants correlated with a decrease in proteasome chymotrypsin-like activity, cells were incubated for 24h with a concentration of Cd²⁺ (9 μ M) at which only Muta β 5 stable transfectants show an increase in the levels of ubiquitinated proteins and a loss in cell viability. Equal amounts of cell lysates (1mg protein/sample) from WT β 5 and Muta β 5 stable transfectants were then fractionated by glycerol density gradient centrifugation. Each fraction was assayed for chymotrypsin-like activity with two proteasome substrates (Z-GGL-pNA and Suc-LLVY-AMC) and for caspase-like activity with Z-LLE-NA. Values were normalized to maximal activity observed in WT β 5 and Muta β 5 stable transfectants. As shown in Fig. 11 (*upper panels*), in wild type and mutant stable transfectants incubated without Cd²⁺ (*open squares, solid lines*), active enzyme with chymotrypsin-like activity sedimented with two

sedimentation coefficients corresponding to 26S and 20S proteasomes, respectively. No differences in chymotrypsin-like activity were detected in WT β 5 and Muta β 5 stable transfectants in the absence of cadmium. These results are in agreement with those obtained with crude cell extracts, since they also correspond to the combined chymotrypsin-like activity of proteasome particles containing endogenous as well as c-myc-tagged β 5 subunits.

Only slight alterations in the peptidase activity profiles were observed in WT β 5 stable transfectants incubated for 24h with 9 μ M Cd²⁺ (*closed squares, broken lines*). However, the chymotrypsin-like activity of the Cd²⁺-treated Muta β 5 stable transfectants was noticeably reduced (*closed squares, broken lines*), particularly in the fractions corresponding to the 26S proteasome elution position (Fig. 11, *hatched area*). These findings correlate well with the accumulation of ubiquitinated proteins observed only in the Cd²⁺-treated Muta β 5 stable transfectants. The caspase-like activity of the proteasome was also perturbed in the Cd²⁺-treated Muta β 5 stable transfectants (Fig. 11). These results indicate that Cd²⁺ may increase the structural instability of 26S proteasome

particles harboring the Muta β 5/c-myc subunit when they are subjected to glycerol gradient centrifugation.

Western blot analyses of the glycerol gradient centrifugation fractions (Fig. 11, *lower panels*) demonstrate that the mature forms of WT β 5/c-myc (24kDa, *arrows #1*) and Muta β 5/c-myc (26kDa, *arrows #3*) are present in the fractions in which proteasome activity can be measured, attesting to their incorporation into the proteasome. Notably, the levels of mature Muta β 5/c-myc detected in Fig. 11 are significantly lower than those of mature WT β 5/c-myc.

3.4.2 MUTA β 5 STABLE TRANSFECTANTS TREATED WITH CADMIUM DEVELOP LARGE UBIQUITIN CONJUGATE AGGREGATES CONTAINING SYNUCLEIN - Ub-conjugates

accumulate in cytosolic inclusions, such as Lewy bodies present in the substantia nigra of brains from Parkinson's disease patients. These inclusions also contain synuclein, which was shown to be degraded by the proteasome ²⁰⁸. For these reasons, we investigated the distribution of Ub-conjugates and synuclein in the mouse HT4 cells. The Cd²⁺-treated Muta β 5 stable transfectants exhibited large Ub-protein aggregates detected with the same anti-Ub antibody

employed in the Western blot analysis. Immunofluorescence studies revealed that synuclein was co-localized with most of the Ub-protein aggregates detected in the Cd²⁺-treated Mutaβ5 stable transfectants (Fig. 12a'-d'). Most of vector and WTβ5 stable transfectants treated with Cd²⁺ exhibited only minor Ub-protein aggregates, although large aggregates could also be detected in a few of these cells. Furthermore, in Cd²⁺-treated WTβ5 stable transfectants, synuclein appeared to be evenly distributed throughout the cell excluding the nucleus (Fig. 12a-d). No Ub-protein aggregates could be found in untreated cells. These results strongly support a role for proteasome impairment in inclusion formation.

3.5. DISCUSSION

Impairment of the ubiquitin/proteasome pathway is implicated in a variety of neurodegenerative disorders, such as Parkinson's disease [reviewed in ²⁰⁹]. Proteasome dysfunction triggered by harmful conditions such as oxidative stress [reviewed in ²¹⁰] or aging [reviewed in ²¹¹] is likely to decrease the degradation rate of ubiquitinated proteins causing their aggregation and accumulation in inclusions. Whether proteasome impairment or the accumulation of ubiquitinated proteins are a cause or consequence of the neurodegenerative process has not been clearly demonstrated.

Most studies addressing the outcome of proteasome inhibition on neuronal cells, such as those in ²¹², draw on a pharmacological approach with synthetic proteasome inhibitors. We decided to take advantage of a genetic manipulation of proteasome function because, although it is not a replacement for pharmacology, it offers a greater degree of specificity. We generated stable transfected mouse neuronal HT4 cells expressing a mutant $\beta 5$ subunit of the 20S proteasome that differs from the wild type $\beta 5$ subunit by only one amino acid in the active site.

This strategy enabled the generation of stable transfectants in which the chymotrypsin-like activity was impaired but not completely abolished. While there was no difference in the survival rate of WT β 5 and Muta β 5 stable transfectants under homeostatic conditions, the latter were hypersensitive to cadmium.

A reduction in the chymotrypsin-like activity of 20S proteasomes harboring Muta β 5/c-myc subunits was detected. Furthermore, aggregates of ubiquitinated proteins forming large inclusions were only detected in the Muta β 5 stable transfectants. Remarkably, the only disparity between Muta β 5 and WT β 5 stable transfectants is the single T1A mutation in the β 5 subunit of the 20S proteasome. The increase in Cd²⁺-sensitivity conferred by a single amino acid substitution in the β 5 subunit reinforces the notion that a proteasome deficiency unequivocally potentiates the harmful effects of oxidative stress as suggested by other investigators^{213, 214}. These studies underscore a synergy that may exist between proteasome deficiency and cellular stress in triggering neurodegeneration. Cells must constantly rely on the proteasome to remove mutant proteins or abnormal proteins produced under stress conditions. Impairment of proteasome function would thus

exacerbate the deleterious effects of stress conditions, most likely jeopardizing the recovery process. In conclusion, disruption of proteasome activity could thus be central to the development of familial as well as sporadic cases of neurodegeneration.

CHAPTER IV**AGING PERTURBS 26S PROTEASOME ASSEMBLY
IN *DROSOPHILA MELANOGASTER***

The FASEB Journal 2007 Sep;21(11):2672-82

by

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4.1. ABSTRACT

Aging is associated with loss of quality control in protein turnover. The ubiquitin-proteasome pathway is critical to this quality control as it degrades mutated and damaged proteins. We identified a unique aging-dependent mechanism that contributes to proteasome dysfunction in *Drosophila melanogaster*. Our studies are the first to show that the major proteasome form in old (43-47 days old) flies is the weakly active 20S core particle while in younger (1-32 days old) flies highly active 26S proteasomes are preponderant. In conclusion, our data reveal a previously unknown mechanism that perturbs proteasome activity in "old-age" female and male *Drosophila* most likely depriving them of the ability to effectively cope with proteotoxic damages caused by environmental and/or genetic factors.

4.2. INTRODUCTION

A fundamental characteristic of aging²¹⁵ and age-related neurodegenerative disorders^{216;217} is the accumulation and aggregation of ubiquitinated proteins in abnormal neuronal inclusions, such as neurofibrillary tangles in Alzheimer's disease and Lewy bodies in Parkinson's disease. The mechanisms causing the aggregation of ubiquitinated proteins and its role in aging and age-related neurodegeneration remain elusive. A decline in proteasome activity with the aging process has been shown in a variety of mammalian organs and tissues [reviewed in²¹⁸]. The loss of proteasome activity with aging has been associated with decreased subunit expression, alterations and/or replacement of proteasome subunits and formation of inhibitory cross-linked proteins [reviewed in^{219;220}]. Food restriction, which is currently the only experimental paradigm 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 in²²¹]. Proteasome dysfunction thus provides a link between environmental and genetic factors associated with aging and aging-related neurodegeneration.

Herein, we demonstrate a significant decline in

proteasome activity in female and male old (43-47 days of age) flies compared to younger (1 to 32 days of age) flies. Notably, we found that the major proteasome form in old flies is the 20S core particle, while in younger flies the 26S holoenzyme is the preponderant proteasome form. These findings establish that autoinhibited 20S proteasomes prevail in old flies whereas the fully-assembled 26S proteasome is highly active in young flies. Our results support the view that an aging-dependent disassembly of the 26S proteasome is an important risk factor in aging.

4.3. MATERIALS AND METHODS

(only those performed by Lisette T. ARNAUD)

4.3.1 IN-GEL PROTEASOME ACTIVITY AND DETECTION

Mixed populations of females and males for each age group were analyzed. Flies were harvested with buffer A [50mM Tris-HCl, pH 7.4, 5mM MgCl₂, 5mM ATP (grade 1; Sigma, St. Louis, MO), 1mM DTT and 10% glycerol], which preserves 26S proteasome assembly ²²². Following homogenization on ice with a teflon pestle for microcentrifuge tubes (100 up and down strokes), sonication on ice (2X10s with a 5s interval) and centrifugation (19,000xg, 15-min at 4°C) the protein content of the cleared supernatants was determined with the Bradford assay (BIO RAD). The cleared supernatants were resolved by non-denaturing PAGE using a modification of the method described in ²²³.

We used a three step gradient gel with approximately similar amounts of 5%, 4% and 3% polyacrylamide containing RhinohideTM polyacrylamide strengthener (Invitrogen-Molecular Probes, Carlsbad, CA). Bromophenol blue was added to the samples prior to loading. Non-denaturing minigels were run at 125Volts for 3 hours.

For detection of proteasome activity, the gels were incubated on a rocker for 10 to 30-min (depending on

protein amount loaded) at 37°C with 15ml of 0.4mM Suc-LLVY-AMC in buffer B (buffer A modified to contain 1mM ATP). Proteasome bands were visualized upon exposure to UV light (360nm) and were photographed with a NIKON Cool Pix 8700 camera with a 3-4219 fluorescent green filter (Peca Products, Inc.). Semi-quantitative analysis of the bands corresponding to proteasome activity was performed by image analysis with the ImageJ program (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997-2006).

Proteins on the native gels were then transferred (110mA) for 2.5h onto PVDF membranes. Western blot analysis was carried-out for detection of the 26S and 20S proteasomes with our anti-d β 5 affinity purified antibody (1:4000, Bio-Synthesis, TX). The peptide NH₂-(GC)DSGYHWDLEDKEAQE-COOH was used to produce the anti-d β 5-specific antibody and corresponds to amino acids 213-227 of the *Drosophila* d β 5 subunit. The anti-d β 5 antibody reacts with a core particle subunit (d β 5) thus detecting both the 26S and 20S proteasomes. Upon incubation with the secondary antibody, antigens were visualized by a chemiluminescent horseradish peroxidase standard method with the ECL reagent.

To determine the total protein pattern of young and old flies, parallel native gels were stained with Coomassie blue after assessment of proteasome activity with Suc-LLVY-AMC.

4.4. RESULTS

(only those performed by Lisette T. ARNAUD)

4.4.1 26S PROTEASOME ASSEMBLY AND ACTIVITY ARE IMPAIRED IN OLD (43-47 DAYS) FLIES - We postulated that the decline in proteasome activity observed in old flies could be due to impaired proteasome assembly. To test this premise we compared proteasome activity in flies of different age groups. Proteasome activity in cleared supernatants was assessed by an in-gel assay as described under "methods". No proteasome activity could be detected in the residual pellet fraction (not shown). As a marker, an aliquot of partially purified 20S proteasomes from *Srabbitt* reticulocyte lysates (20S) was run in a parallel lane.

Proteasome levels and activity (Fig. 13) from mixed populations of females and males were compared between two age groups: young (1-2 days) and old (43-47 days). In young flies, most of the proteasome activity assessed with the short substrate Suc-LLVY-AMC coincided with the 26S holoenzyme (not the 20S) form of the proteasome (Fig. 13, *left panel*). The in-gel chymotrypsin-like activity assay revealed that the proteasome activity in young flies (Y) coincided almost exclusively with the 26S holoenzyme, in

its symmetrical (two capped) and asymmetrical (one capped) forms. Only extremely low levels of chymotrypsin-like activity were associated with the 20S proteasome, demonstrating that the 26S holoenzyme is the most active *in vivo* form of the proteasome in young flies. In contrast, the chymotrypsin-like activity of both the 26S and the 20S proteasomes was low in old flies (0).

Immunoblot analysis of the native gels with our anti-d β 5 antibody revealed that, in young flies, proteasomes were detected as the 26S holoenzyme in its symmetric (two caps) and asymmetric (one cap) forms as well as the 20S proteasome (Fig. 13, *middle panel*). In contrast, in old flies, proteasomes were found almost exclusively as 20S particles (Fig. 13, *middle panel*). These findings establish that low-activity 20S proteasomes prevail in old flies while the assembled 26S holoenzyme is highly active in young flies.

Following assessment of proteasome activity with Suc-LLVY-AMC, native gels were stained with Coomassie blue (Fig. 13, *right panel*). The total protein pattern of young and old flies was slightly but consistently different. This difference was not caused by post-harvesting protein degradation as similar patterns were observed when flies

were harvested with or without a protease inhibitor cocktail (not shown).

4.5. DISCUSSION

In this paper we show for the first time that the aging process is associated with the disassembly of the 26S proteasome with a clear loss of its activity. The decline in 26S holoenzyme levels in old flies (43-47 days of age) coincides with increased levels of the 20S proteasome. Clearly, the activity of the 20S proteasome failed to decline. However, our studies demonstrate that the free 20S core particle is nearly inactive in all flies independently of their age group. Accordingly, the majority of the proteasome chymotrypsin-like activity coincides with the 26S proteasome, the levels of which decline sharply in 43-47 days old female and male flies. That the 20S proteasome is almost inactive is not particular to flies, as a similar phenomenon was observed in yeast ²²⁴. It is well established that in the outer rings of the 20S core particle, the conformation of the α subunits is such that it seals the entrance into the catalytic chamber ²²⁵. Activation of the 20S proteasome thus requires disruption of the inflexible and passive barrier provided by the outer α rings. The opening of these gates to the catalytic chamber is triggered by regulatory complexes, such as the 19S and the 11S

regulatory particles [reviewed in ²²⁶]. *In vitro* studies with purified 20S proteasomes demonstrated that the closed-gate conformation is also destabilized by the binding of some hydrophobic peptides to non-catalytic sites on the core particle ²²⁷ or by certain proteins, such as p21 and α -synuclein ²²⁸. The latter mechanism for activation of the 20S core particle has not yet been demonstrated to occur *in vivo*. However, it was proposed that because proteasomes diffuse rapidly in the cytoplasm and nucleus of mammalian cells, they may continuously collide with some of their substrates ²²⁹.

Our finding that the disassembly of the 26S proteasome is an "old-age" event could explain the presence of protein deposits containing ubiquitinated proteins and oxidatively modified proteins in non-pathologic aging ²³⁰ as well as in a variety of aging-related neurodegenerative disorders including Alzheimer's and Parkinson's diseases and amyotrophic lateral sclerosis, to name a few ²³¹. The reduction in assembled 26S proteasomes not only will affect normal protein turnover but also most likely deprives old flies of the ability to effectively cope with proteotoxic damages caused by life long environmental and/or genetic factors.

CHAPTER V

MODEL AND CONCLUSIONS

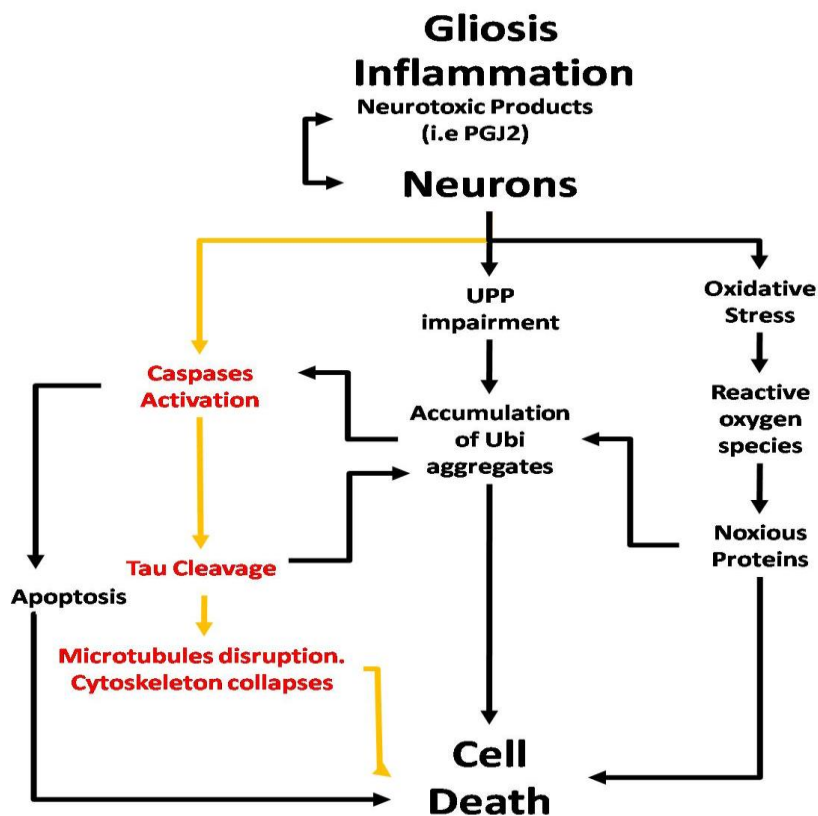
Neurodegenerative diseases such as AD and PD are age related diseases, and determining the sequence of events that might span over an entire life is today's challenge. In the case of AD, even though studies, models and hypotheses have flourished over the last two decades little is known about the beginning of the pathology and when symptoms are detected the neurodegeneration is so advanced that little can be done. There are however ubiquitous components to these neurodegenerative disorders: early neuroinflammation and ubiquitinated protein aggregates.

Our hypothesis is that inflammation more than just being a side effect of the neurodegenerative pathology is an important contributor to the progression of the disease. Indeed, we investigated the role of one neurotoxic product of inflammation (PGJ2) and demonstrated for the first time, that this CNS prostaglandin was able to modulate tau cleavage at Asp421 through caspase-mediated proteolysis in neuronal cells. This discovery is very important since for tau to seed PHF formation it must be cleaved first in order for its conformation to change. PHFs lead to NFT formation the levels of which correlate with cognitive impairment in AD. We linked two known

hallmarks of AD, neuroinflammation and tau cleavage, suggesting how a physiological event i.e. inflammation, can lead to pathological features through caspase-mediated proteolysis and cell death through apoptosis. Furthermore, we demonstrate that upon PGJ2-treatment tau accumulates in perinuclear aggregates in SK-N-SH cells or in bulb-like structures along dystrophic-like neuritis in rat primary cortical neurons. These observations confirm cytoskeleton collapse and tau aggregation.

In AD, tau is a component of NFTs which also contain ubiquitinated proteins. The latter are normally target for degradation by the proteasome. Other neurodegenerative diseases besides AD are also characterized by ubiquitin-protein aggregates. The formation of these aggregates suggests that the activity of the proteasome is perturbed. In this study we demonstrate that proteasome impairment due to a mutation in the subunit that carries-out the chymotrypsin-like, increases the vulnerability of cells to stress conditions and causes the formation of ubiquitin-protein aggregates. These findings support the notion that proteasome impairment is indeed associated with the formation of ubiquitin-protein aggregates. The sequence of events suggested by our results (*see scheme below*) starts with the triggering of inflammatory events by a variety of

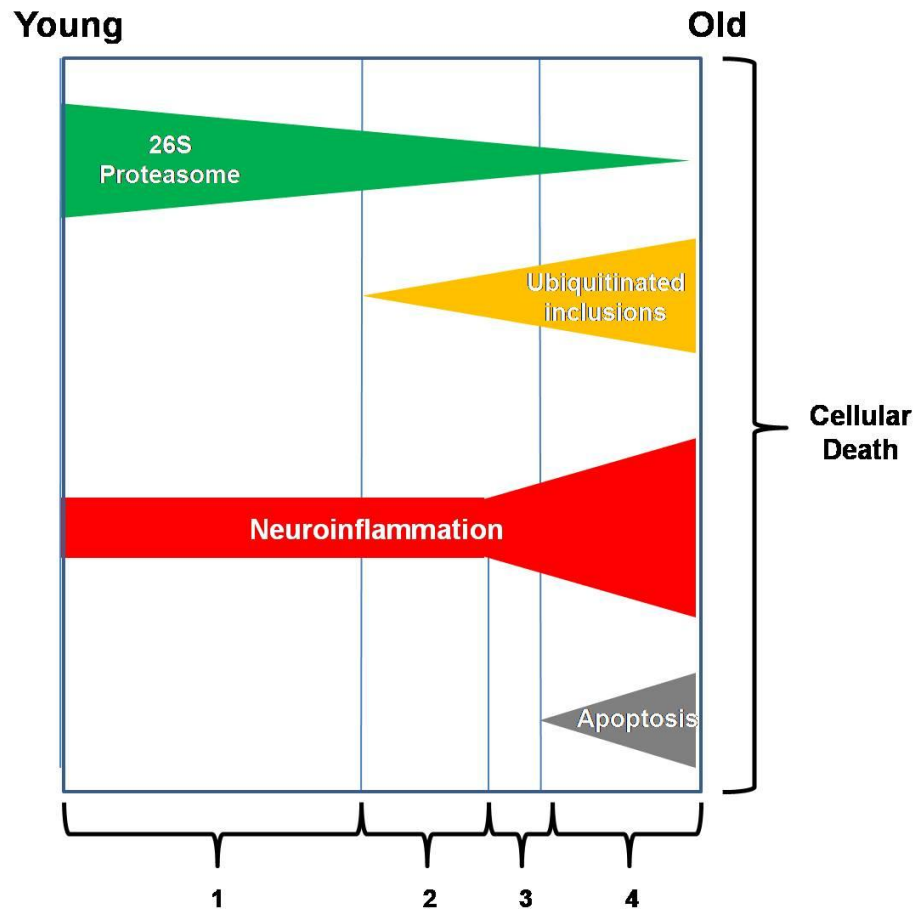
external stimuli. The resulting gliosis and pro-inflammatory factors (such as PGJ2) released in the proximity of the neurons affect a diversity of cellular functions with dramatic consequences. Indeed the impairment of the UPP pathway prevents removal of misfolded proteins generated by inflammation-induced oxidative stress, leading to accumulation of ubiquitinated proteins. As the ubiquitinated proteins accumulate they overwhelm the proteasome the activity of which is affected by the inflammatory factors. Proteasome impairment in concert with the accumulation of ubiquitinated proteins in the cell trigger apoptosis and caspase-mediated proteolysis. Caspases will then cleave the microtubule binding protein Tau generating an insoluble fragment prone to aggregation thus promoting formation of protein aggregates. In addition, caspases cleave other cytoskeletal proteins, such as tubulin and actin, resulting in the complete collapse of the cell scaffold. All of these events accelerate the deadly fate of the cell leading to neurodegeneration.



It is not clear why AD and other neurodegenerative disorders associated with the build-up of ubiquitinated proteins, such as in PD and ALS, are associated with aging. We demonstrate in *Drosophila* that aging is associated with a decrease in proteasome activity, supporting a role for the aging-dependent proteasome impairment in protein aggregation observed in these diseases. We established that in *Drosophila* the 26S fully assembled proteasome was preponderant in “young” flies while the 20S core particle, which is catalytically inactive, prevailed in “old” flies. These results provide

a basis for understanding how proteasome function is affected by aging.

Overall our hypothesis (scheme next page) is that, during the lifespan of an individual, the CNS is frequently subjected to proteotoxic damages caused by environmental and/or genetic factors, some of which may induce neuroinflammation. Toxic products of inflammation can further exacerbate the generation of abnormal and misfolded proteins that would normally get ubiquitinated and processed by the proteasome (1). However as the individual ages, the well oiled machinery gets gripped. The proteasome disintegrates and so it fails to remove the ubiquitinated proteins (2). These ubiquitinated proteins tend to aggregate within the cells disrupting its metabolism and launching a death pathway, such as apoptosis, that could further fuel neuroinflammation (3). Activation of apoptosis also initiates the caspase cascade leading to cleavage (among other proteins) of tau (4) and culminating in massive neurodegeneration.



CHAPTER VI

FUTURE DIRECTIONS

The studies described in this thesis revealed that:

1) Products of inflammation, such as PGJ2, induce tau pathology manifested by tau cleavage which coincides with apoptosis in human neuroblastoma SK-N-SH cells. These

results suggest that chronic inflammation could be critical to tau pathology in the brain. To confirm this hypothesis it would be interesting to test conditioned media from LPS-activated microglia on our SK-N-SH cell model. We would establish if neuroinflammation factors released by microglia, which are usually detected surrounding neurons in AD, can induce the same tau cleavage as PGJ2. We would also like to study if neuronal cells subjected to stress conditions that lead to tau cleavage, would attempt to compensate for the loss of tau by increasing its expression.

2) PGJ2 induces the formation of a large perinuclear tau aggregate next to a nuclear indentation. Upon PGJ2-treatment of SK-N-SH cells we detected a large perinuclear aggregate detected with a pan tau antibody. However, we did not define if the aggregates contain cleaved and/or full length tau. It would be of interest to further investigate the components of the aggregates to determine which tau forms (full length or cleaved) are present as

well as if tau is **ubiquitinated** and/or **hyperphosphorylated** in the same manner as in NFTs.

We would also like to address the potential toxic role of the aggregates, which remains a controversial issue. It is thought that aggregated proteins are "neutralized toxic proteins". When cells fail to proteolyse proteins that could become toxic if remaining soluble in the cytoplasm, it was hypothesized that the aggregation of those "stable" proteins is a survival mechanism to segregate them from the rest of the cell. We could attempt to prevent tau aggregation with, for example, N744 tosylate, known to prevent tau aggregation *in vitro*²³². By treating cells with PGJ2 alone or in combination with N744, we would establish if attenuating protein aggregation would prevent cell death.

The studies proposed in the "future directions" should provide valuable information on the involvement of inflammation in tau pathology and on the mechanisms that drive neurodegenerative disorders, such as AD, that are associated with inflammation and protein aggregation.

CHAPTER VII

FIGURES

Figure 1 (next page) - PGJ2 induces accumulation of ubiquitinated proteins, tau cleavage and apoptosis in a dose-dependent manner.

(A) and (E) - Western blot analyses to detect tau in total extracts of human SK-N-SH neuroblastoma cells (30µg of protein/lane) treated with PGJ2 for 16h. In (A) the blot was probed with the TauC3 antibody (tau cleaved at Asp421, epitope a.a. 412-421). After stripping (A) the blot was reprobed with the pan tau (clone 13, epitope a.a. 2-18) antibody (E) which reacts with all tau isoforms. Equal protein loading was demonstrated by probing the immunoblots with the anti-actin antibody (D). (B) Blots were stripped and reprobed with the anti-PARP antibody, which detects cleaved PARP, an apoptosis marker. (C) Blots were stripped and reprobed again with the anti-ubiquitinated (Ub) proteins antibody. (F) Cell viability was assessed with the MTT assay. Data represent the mean \pm SEM from at least three determinations. The viability for each condition was compared to the viability of cells treated with vehicle only (control, 100%). The asterisk (*) identifies the values that are significantly different ($p < 0.001$) from the control. (G) The levels of Ub-proteins (*crosses*), Δ tau (*solid squares*) and cleaved PARP (*open squares*) were semi-quantified by densitometry.

Data represent the number of pixels (arbitrary units) for each PGJ2-concentration as fold-increase over control (vehicle only). Molecular mass markers in kDa are shown in the middle. Boxes highlight the peaks in Δ tau levels (A) and Ub-proteins (C). Similar results were obtained in duplicate experiments. Δ TAU, tau cleaved at Asp421; TAU FL - full length TAU.

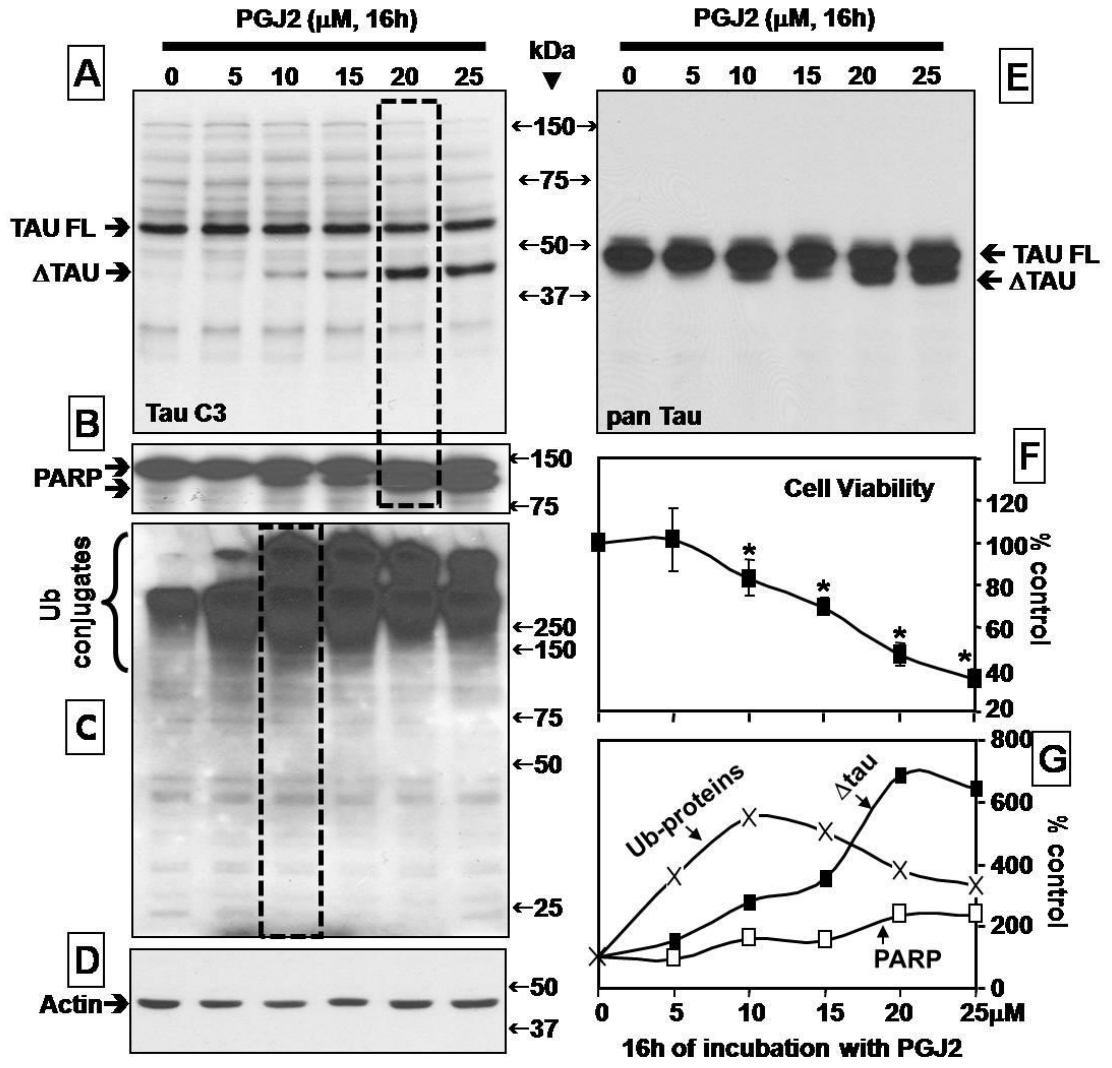


Figure 1

Figure 2 (next page)- PGJ2 induces accumulation of ubiquitinated proteins, tau cleavage and apoptosis in a time-dependent manner. (A) and (E) - Western blot analyses to detect tau in total extracts of human SK-N-SH neuroblastoma cells (30µg of protein/lane) treated with 20µM PGJ2 for different times (2h, 4h, 8h, 16h, and 20h). In (A) the blot was probed with the TauC3 antibody (tau cleaved at Asp421, epitope a.a. 412-421). After stripping the blot in (E) was reprobed with the pan tau (clone 13, epitope a.a. 2-18) antibody which reacts with all tau isoforms. Equal protein loading was demonstrated by probing the immunoblots with the anti-actin antibody (D). (B) Blots were stripped and reprobed with the anti-PARP antibody, which detects cleaved PARP, an apoptosis marker. (C) Blots were stripped and reprobed again with the anti-ubiquitinated (Ub) proteins antibody. (F) Cell viability was assessed with the MTT assay. Data represent the mean ± SEM from at least three determinations. The viability of cells treated with 20µM PGJ2 for each time point was compared to the viability of cells treated with vehicle only for 24h (control, 100%). The *asterisk* (*) identifies the values that are significantly different ($p < 0.05$) from the control. (G) The levels of Ub-proteins (*crosses*), Δ tau (*solid squares*) and cleaved PARP (*open*

squares) were semi-quantified by densitometry. Data represent the number of pixels (arbitrary units) for each time point in cells treated with 20 μ M PGJ2 as fold-increase over control (vehicle only). Molecular mass markers in kDa are shown in the middle. Boxes highlight the peaks in Δ tau levels (A) and Ub-proteins (C). Similar results were obtained in duplicate experiments. Δ TAU, tau cleaved at Asp421; TAU FL - full length TAU.

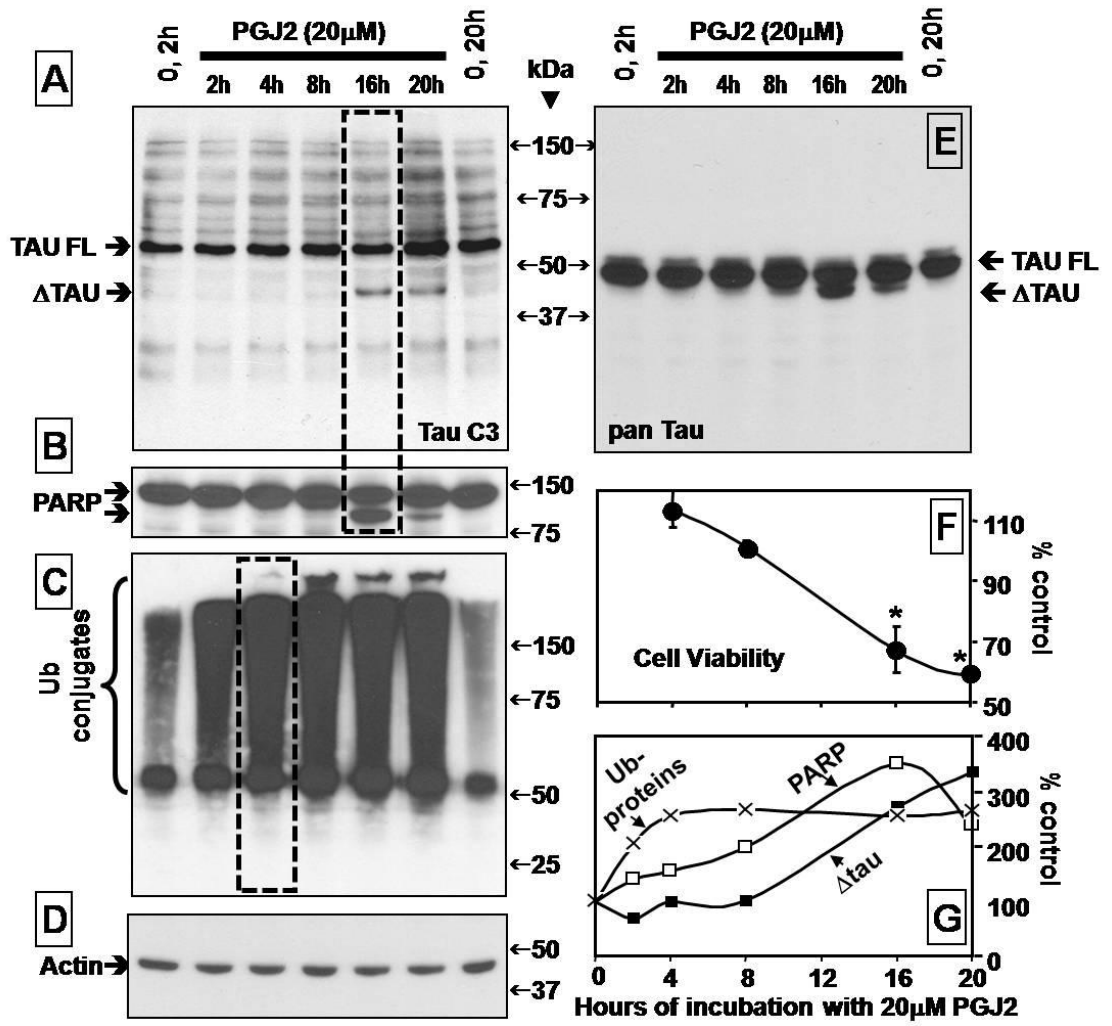


Figure 2

Figure 3 (next page) - PGJ2 increases caspase activity.

(A) The activities of caspases 2, 3, 8 and 9 were determined in SK-N-SH cells treated with DMSO (vehicle, *black bars*) or treated with 20 μ M PGJ2 (*white bars*) for 16h. Relative caspase activities (RFU = relative fluorescent units) normalized for protein (200 μ g/assay) are shown. Results from two determinations are presented (mean \pm SD). The *asterisk* (*) identifies the values that are significantly different ($p \leq 0.014$, T-test) from the controls. **(B)** Caspase 3 activity was determined in SK-N-SH cells treated with DMSO (vehicle, 2h and 20h, *black bars*) or treated with 20 μ M PGJ2 (*white bars*) for 2h, 4h, 8h, 16h and 20h. Relative caspase activities (RFU = relative fluorescent units) normalized for protein (200 μ g/assay) are shown. Results from three determinations are presented (mean \pm SEM). The *asterisk* (*) identifies the values that are significantly different ($p < 0.001$, ANOVA, Tukey-Kramer multiple comparison test) from the control.

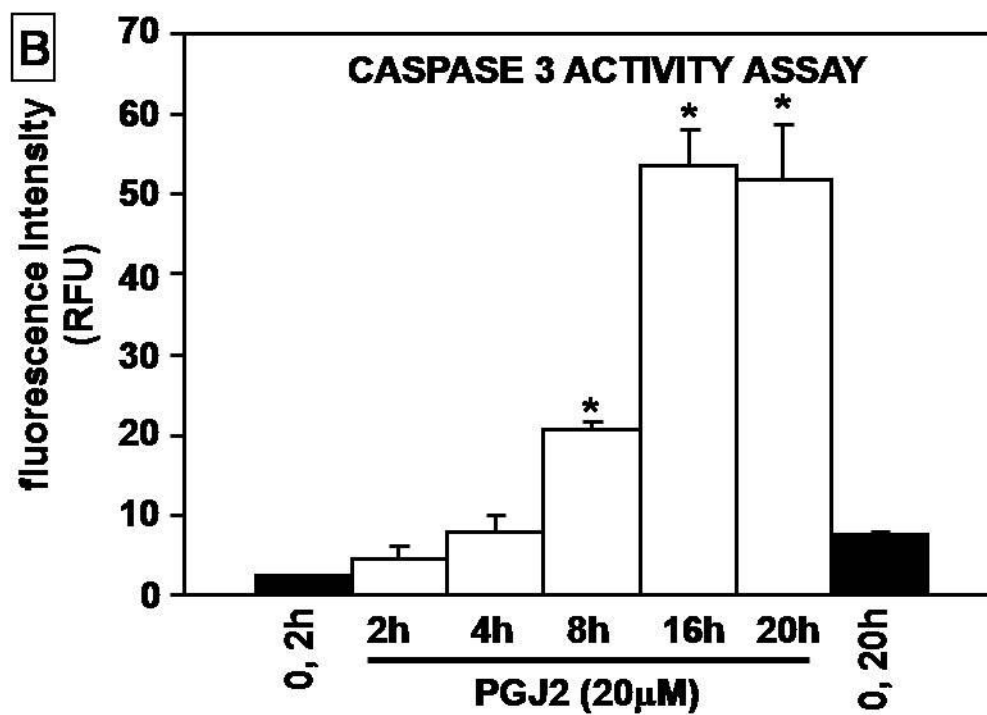
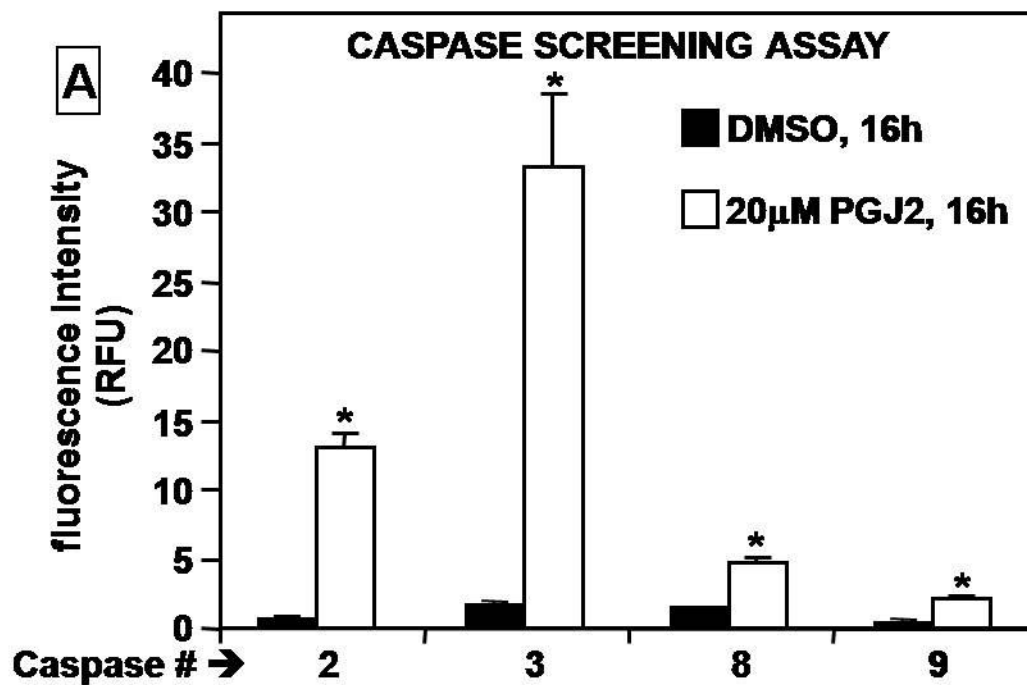


Figure 3

Figure 4 - Individual caspase inhibitors (2 μ M) attenuate the PGJ2-dependent increase in Δ tau levels. Δ Tau levels in total extracts of human SK-N-SH neuroblastoma cells (50 μ g of protein/lane) were assessed by Western blot analysis with the Tau C3 antibody (tau cleaved at Asp421, epitope a.a. 412-421). Cells were treated for 16h with DMSO (vehicle, control, lanes 1 and 9) or with 20 μ M PGJ2 alone (lanes 2 and 10, boxed) or with 20 μ M PGJ2 after a 40-min pre-incubation with 2 μ M of individual caspase inhibitors (1 through 6, lanes 3 - 8; 8 through 10, lanes 11-13, and 13, lane 14) or the pan caspase inhibitor (Z-VAD-FMK, lane 15). As an FMK negative control, cells were separately incubated with Z-FAD-FMK (lane 16). Similar results were obtained in duplicate experiments.

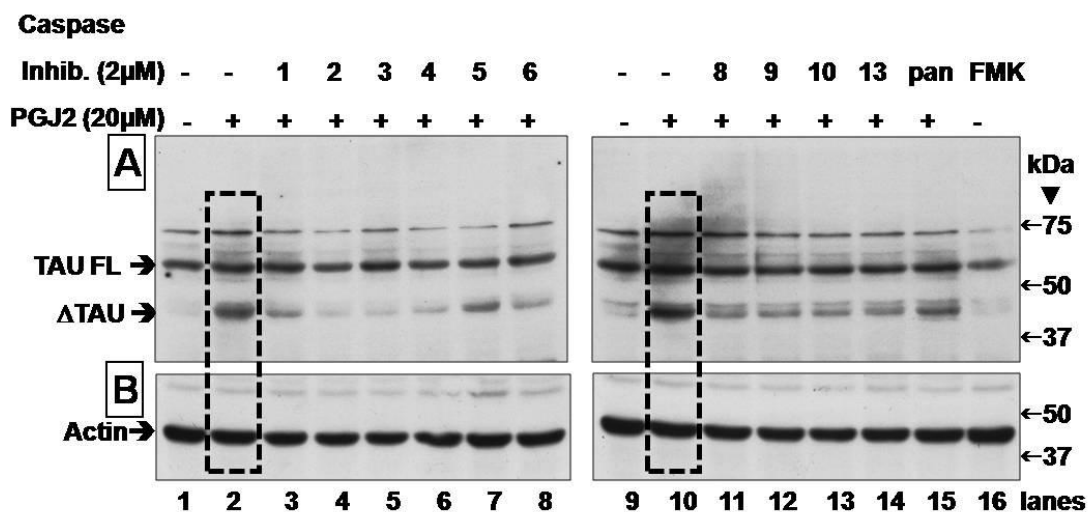


Figure 4

Figure 5 - Pharmacological manipulation of Δ tau with PPAR γ agonist/antagonist, proteasome or calpain inhibitors and the anti-oxidant NAC (next page). Δ Tau levels in total extracts of human SK-N-SH neuroblastoma cells (30 μ g of protein/lane) were assessed by Western blot analysis with the Tau C3 antibody (tau cleaved at Asp421, epitope a.a. 412-421). Cells were treated for 16h with DMSO (vehicle, control, lanes 1A and 1B) or with 20 μ M PGJ2 alone (lanes 2A and 2B, boxed) or with 20 μ M PGJ2 after a 40-min pre-incubation with BADGE (lane 3A, PPAR γ antagonist), pan caspase inhibitor (Z-VAD-FMK, lane 4A), or NAC (lane 6B, boxed, anti-oxidant). Cells were also separately incubated with BADGE (lane 5A), pan caspase inhibitor (lane 6A), ciglitazone (PPAR γ agonist, lane 7A), proteasome inhibitor (lanes 4B and 5B), calpain inhibitor (lane 3B), and NAC (lane 7B). Similar results were obtained in duplicate experiments.

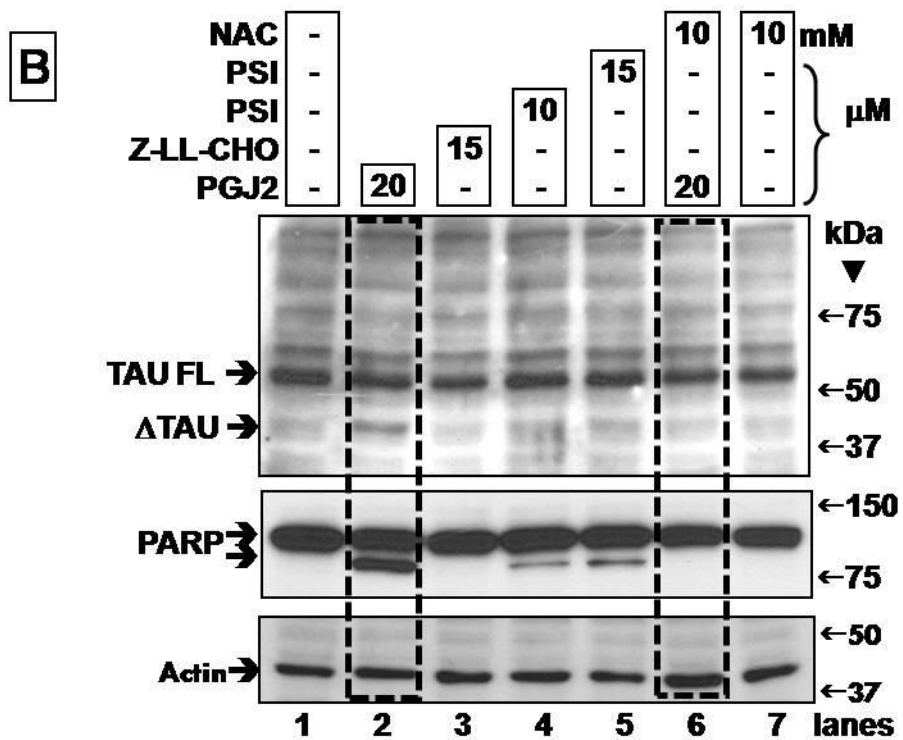
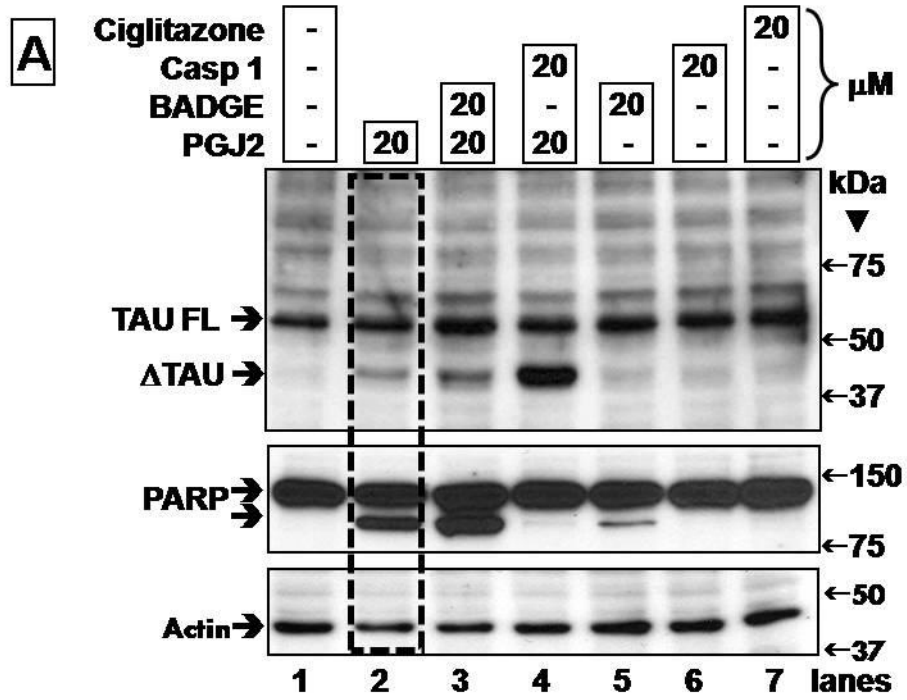


Figure 5

Figure 6 - Calpain inhibitors fail to attenuate the PGJ2-dependent increase in Δ tau levels while cathepsin inhibition stabilizes Δ tau (next page). Δ Tau levels in total extracts of human SK-N-SH neuroblastoma cells (40 μ g of protein/lane) were assessed by Western blot analysis with the Tau C3 antibody (tau cleaved at Asp421, epitope a.a. 412-421). Cells were treated for 16h with DMSO (vehicle, control, lanes 1A and 9A) or with 15 μ M PGJ2 alone (lanes 2 A and 10A) or with 15 μ M PGJ2 after a 40-min pre-incubation with calpain inhibitors (CP1, lane 4A, CP3, lane 6A, Z-LL-CHO, lane 8A, and calpeptin, lanes 12A and 14A). Cells were also treated for 16h with 20 μ M PGJ2 after a 40-min pre-incubation with a cathepsin inhibitor alone (pepstatin, lane 2B) or in combination with pan caspase inhibitor (lane 3B). Cells were also separately incubated with the calpain inhibitors or pepstatin (lanes 3A, 5A, 7A, 11A, 13A and 1B). Similar results were obtained in duplicate experiments.

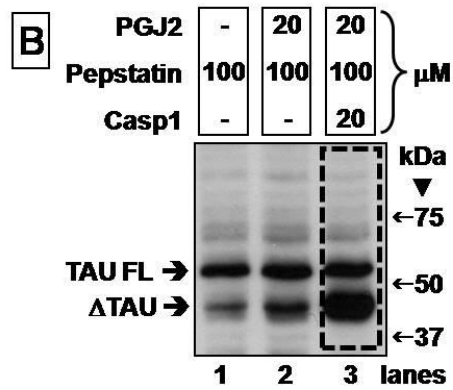
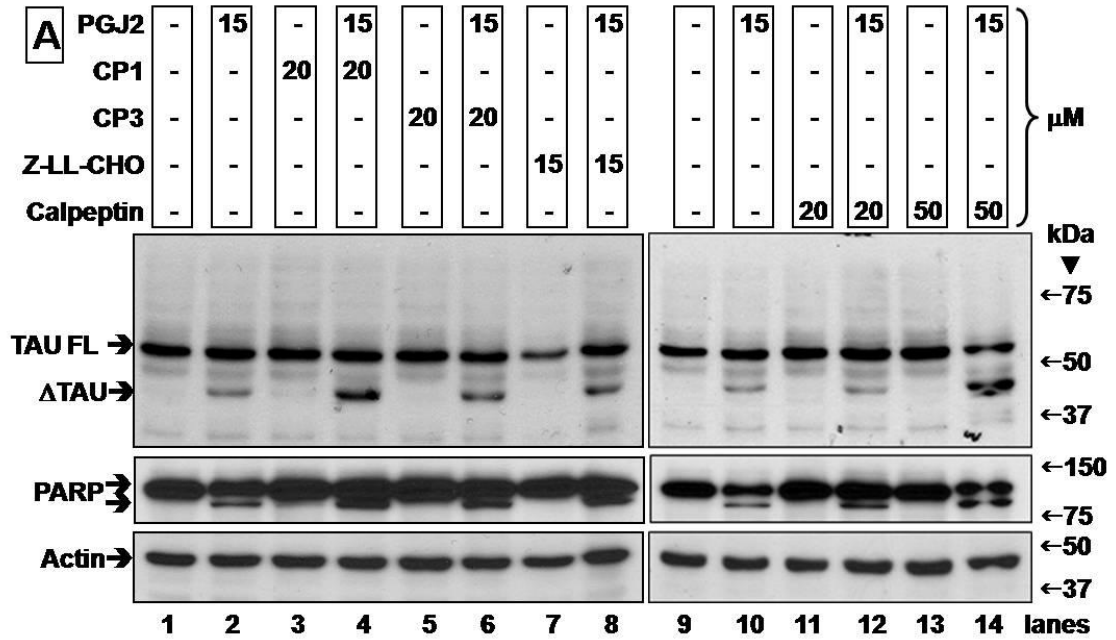


Figure 6

Figure 7 - Subcellular localization of Δ tau. Total lysates from SK-N-SH cells treated for 16h with vehicle (C, control) or 20 μ M PGJ2 (T, treated) were fractionated into cytoplasmic, enriched nuclear and pellet fractions. Western blot analysis was performed to detect levels of Δ tau [(A) TauC3 antibody and (B) pan tau antibody], cleaved PARP (C), HSP90 (D) and γ -tubulin (E). Equal amounts of protein (40 μ g) were loaded per lane. Molecular mass markers in kDa are shown on the right. Similar results were obtained in duplicate experiments.

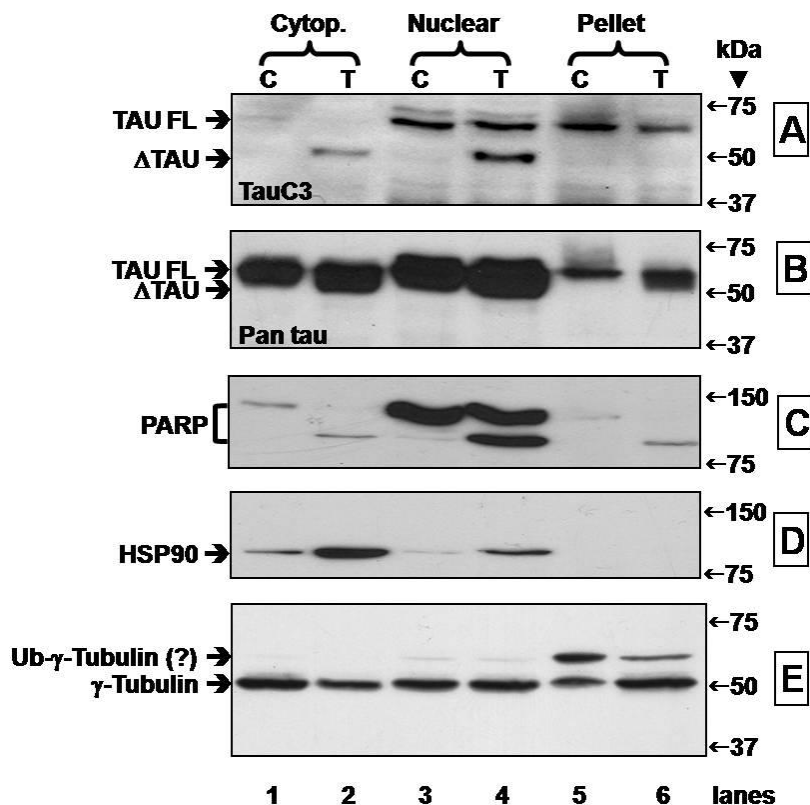


Figure 7

Figure 8 - Detection of PGJ2-induced Δ tau by four different antibodies. Δ tau was detected in the enriched nuclear fraction (as in fig. 7) of SK-N-SH cells treated with DMSO (vehicle, control, C) or PGJ2 (20 μ M, 16h, T) with monoclonal and polyclonal antibodies that react with different epitopes: Tau C3 (mono) from Covance, tau clone 5 (mono) courtesy Dr. Binder, Tau Y9 (poly rabbit) from BioMol and pan tau clone 13 (mono) from Santa Cruz. Δ tau, tau cleaved at Asp421; TAU FL - full length tau. Molecular mass markers in kDa are shown on the right.

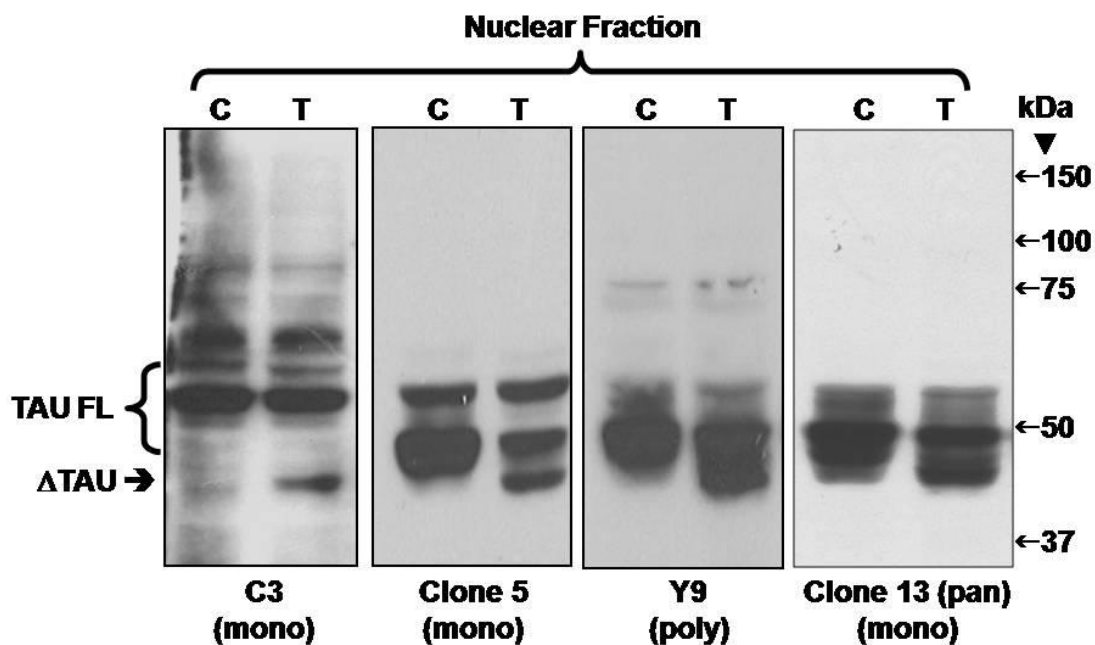


Figure 8

Figure 9 - PGJ2 triggers the formation of tau aggregates

(next page). Immunofluorescence staining of SK-N-SH cells treated with vehicle only (DMSO, *A and D*) or 20 μ M PGJ2 (*B and C*) for 16h. Tau (*green*) was visualized by immunostaining with the pan tau antibody (Santa Cruz). Nuclei (*red*) were detected with TO-PRO-3. Merged images are shown for tau/To-Pro. Arrows point to large tau aggregates. The scale bar is = 7.3 μ m (*A*), 11.9 μ m (*B*), 5.5 μ m (*C*) and 3.6 μ m (*D*).

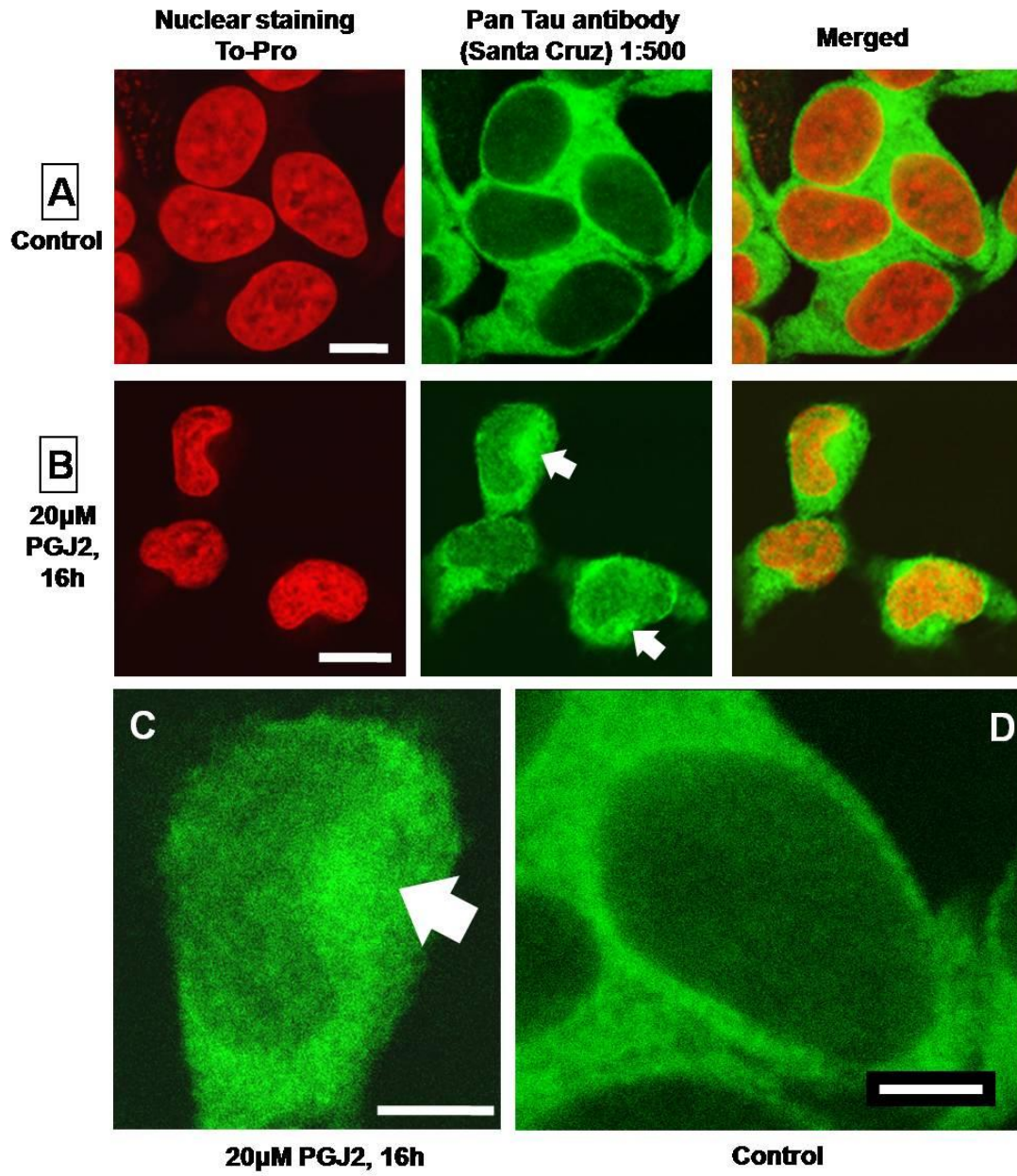


Figure 9

Figure 10 - PGJ2 induces tau cleavage in rat (E18) primary cortical neuronal enriched cultures (next page). Western blot analyses to detect tau in total extracts of rat primary cortical cultures (35µg of protein/lane) treated with DMSO (vehicle, control, C) or with 20µM PGJ2 (T) for 16h. The blot was probed with the TauC3 antibody (A) then stripped and reprobed with the pan tau clone 5 antibody (B). Equal protein loading (6µg and 8µg of protein per lane for cortical neurons and SK cells, respectively) was demonstrated by probing parallel immunoblots with the anti-actin antibody (C). As a positive control, TAU FL and ΔTAU detected in PGJ2-treated SK-N-SH cells are shown in lanes 1A and 2A. ΔTAU, tau cleaved at Asp421; TAU FL - full length tau.

(D and E), ΔTAU immunofluorescent staining (Tau C3 antibody) of rat cortical neurons treated with 20µM PGJ2 for 16h. The *arrows* are pointing to dystrophic neuritis and the *arrowheads* to bulb-like accumulations of tau. The scale bar = 30µm.

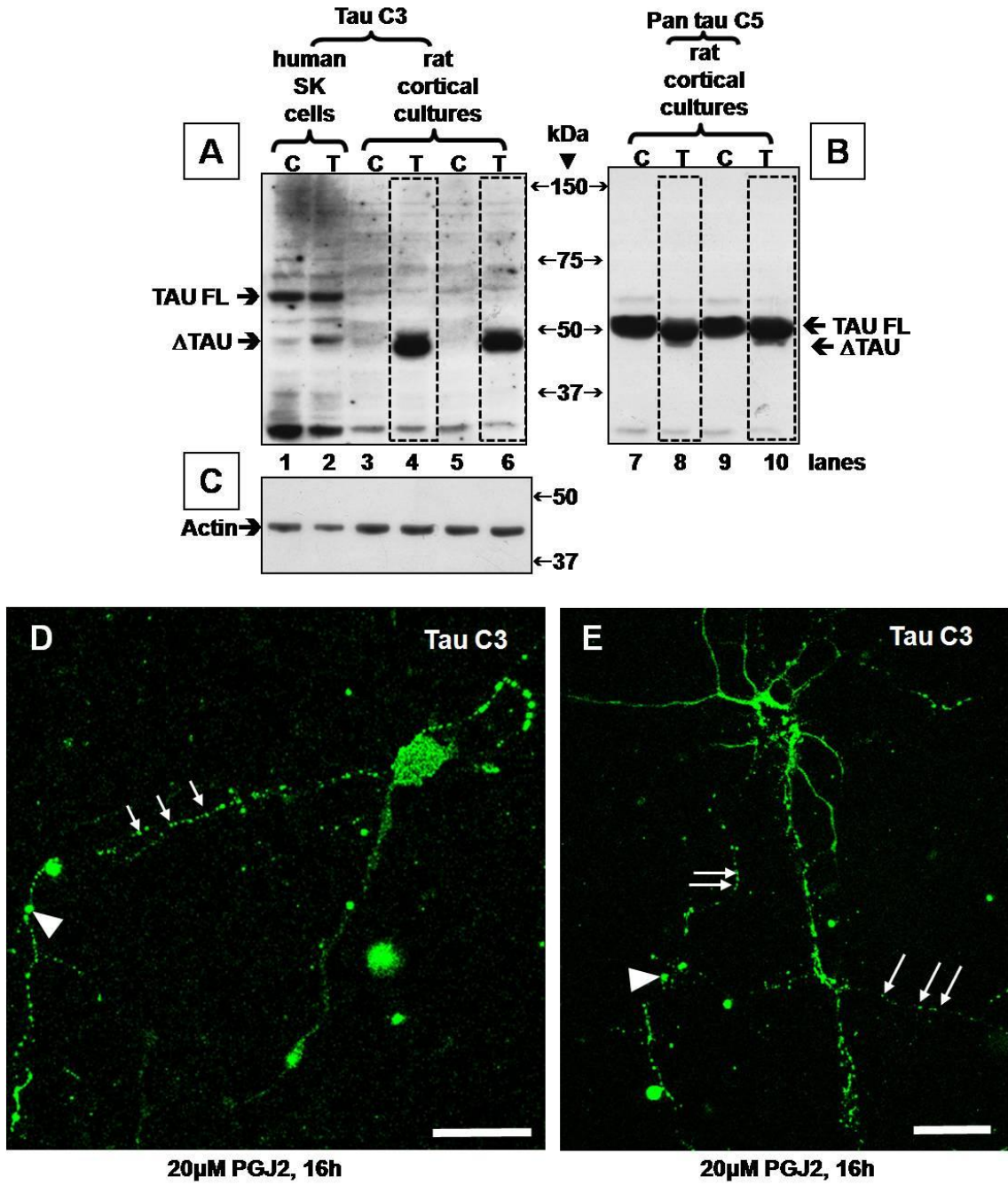


Figure 10

Figure 11 - Sedimentation velocity analysis of cell lysates obtained from WT β 5 and Muta β 5 stable transfectants incubated for 24h without and with 9 μ M Cd²⁺ (next page)-

The cleared supernatants (1mg of protein/sample) were fractionated by glycerol density gradient centrifugation (10-40% glycerol from fractions 14 to 1). Elution positions of the 26S and 20S proteasomes are indicated.

(a) Aliquots (50 μ l) of each fraction obtained from cells incubated without (*open squares, solid lines*) and with 9 μ M Cd²⁺ (*closed squares, broken lines*) were assayed for proteasome peptidase activities with three different substrates. Values were normalized to maximal activity observed in WT β 5 and MUTA β 5 stable transfectants for each peptidase activity, respectively. The hatched area highlights the difference in chymotrypsin-like activity between untreated and Cd²⁺-treated Muta β 5 transfectants.

(b) Anti-c-myc immunoblot analysis of the same fractions used in **(a)**. Proteins were precipitated with acetone from 300 μ l of each fraction obtained from cells incubated without (*top panels*) and with 9 μ M Cd²⁺ (*bottom panels*). The arrows point to mature (#1, 24kDa) and precursor (#2, 31kDa) forms of WT β 5/c-myc and mature (#3, 26kDa) and precursor (#4, 31kDa) forms of Muta β 5/c-myc.

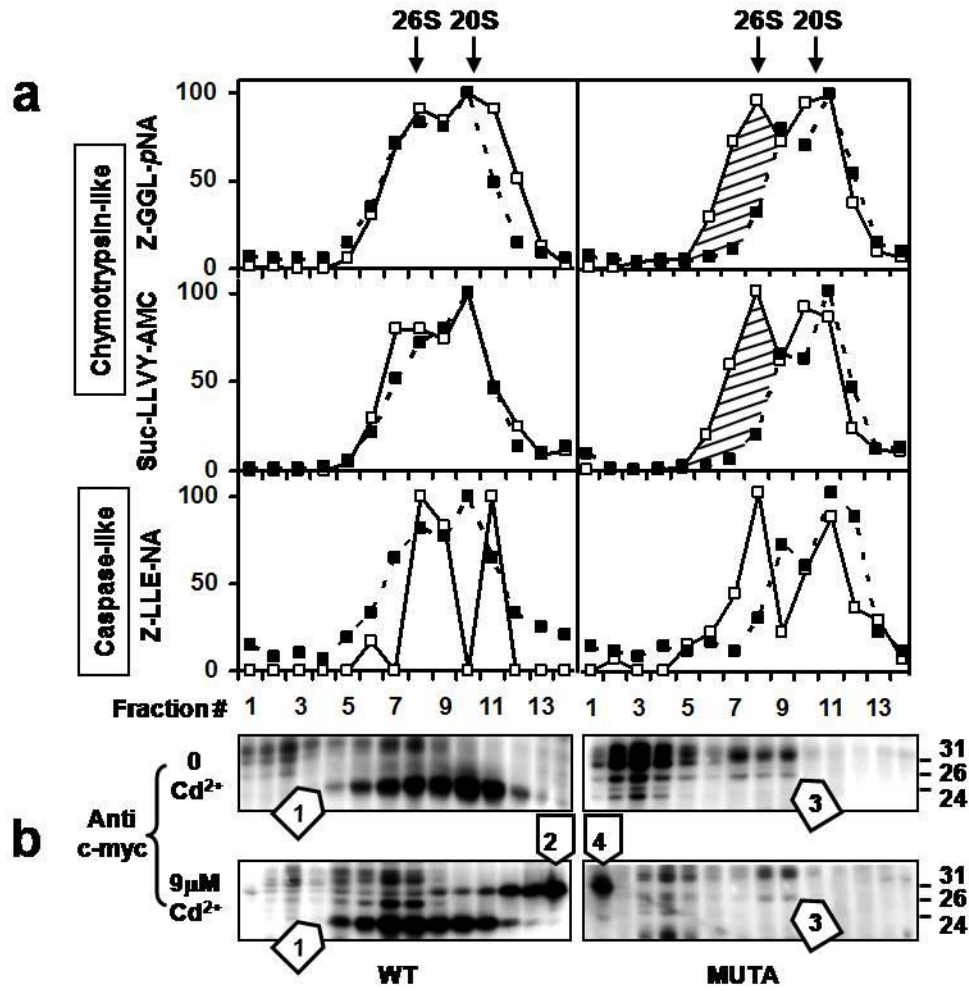


Figure 11

Figure 12 - Ub-conjugates and synuclein co-localize in aggregates in Muta β 5 stable transfectants treated with Cd²⁺

(next page)- Double immunofluorescent staining of WT β 5 (**a-d**) and Muta β 5 (**a'-d'**) stable transfectants incubated for 24h with 9 μ M Cd²⁺. Ub-aggregates were visualized with the anti-Ub conjugates (*a* and *a'*, red), synuclein with anti-synuclein (*b* and *b'*, green) and nuclei (*n*) with DAPI (arrows in *d* and *d'*, blue). Overlapped images are shown in (*c* and *c'*) and in (*d* and *d'*). The scale bar = 10 μ m.

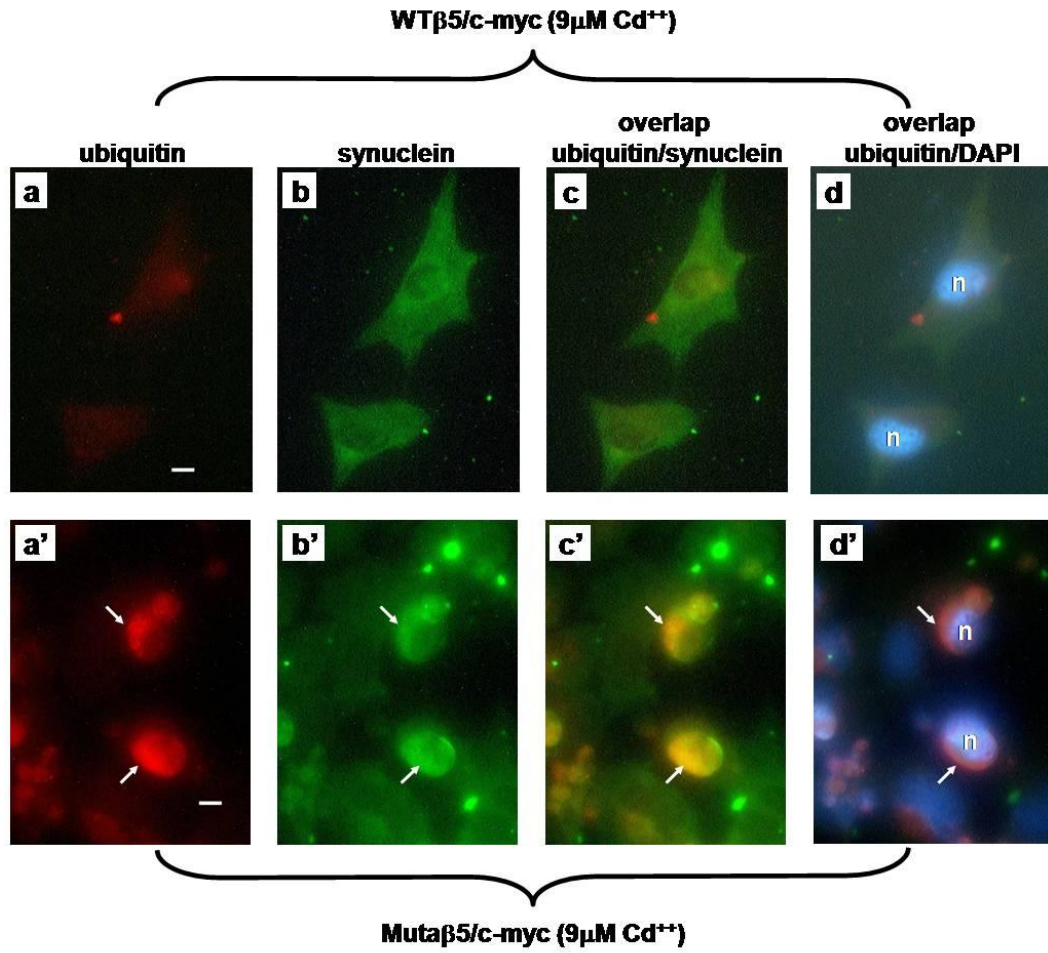


Figure 12

Figure 13 - Proteasome activity and levels in young (Y, 1-2 days) and old (O, 43-47 days) flies (next page)

Cleared fly lysates and 20S proteasomes partially purified from rabbit reticulocyte lysates (20S, as a marker) were subjected to non-denaturing gel electrophoresis as described under "Methods". Lysates from mixed populations of females and males of each age group were analyzed. The chymotrypsin-like activity was assessed with Suc-LLVY-AMC by the in-gel assay (*left panel*). 26S and 20S proteasomes were detected by immunoblotting with our antibody that reacts with $d\beta 5$, a subunit of the core proteasome particle (*middle panel*). As indicated on the left, this antibody recognizes the symmetric [26S(2), two caps] and asymmetric [26S(1), one cap] 26S holoenzymes as well the 20S core particle. Total protein pattern was established by Coomassie blue staining of native gels (*right panel*) following assessment of proteasome activity with Suc-LLVY-AMC. Similar results were obtained in at least quadruplicate experiments.

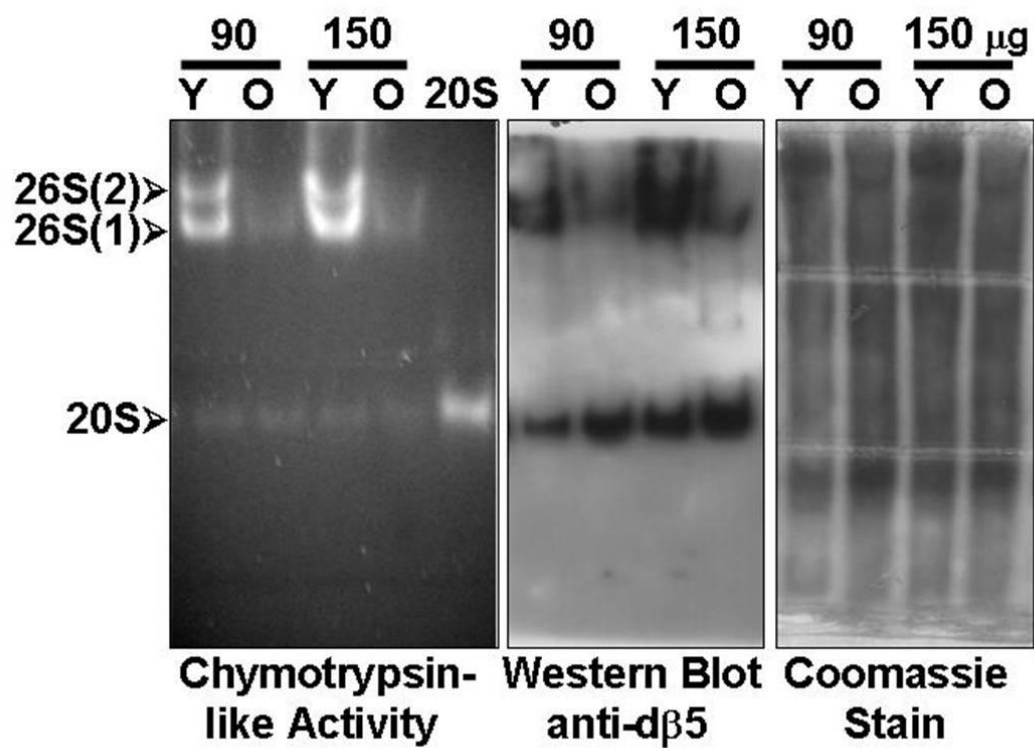


Figure 13

CHAPTER VIII

REFERENCE LIST

REFERENCE LIST

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