"cAMP AND CHAPERONES: POTENTIAL THERAPEUTIC STRATEGIES TO PREVENT INFLAMMATION-LINKED TAU PATHOLOGY IN ALZHEIMER'S DISEASE"

by

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ABSTRACT

Senile plaques and neurofibrillary tangles are hallmarks of Alzheimer's disease (AD). The main component of neurofibrillary tangles (NFTs) is Tau, a highly soluble microtubule-associated protein whose major function is to stabilize microtubules, specifically in axons, in a phosphorylation-dependent manner. Neurodegenerative diseases collectively designated "Tauopathies" are linked to Tau mutations and/or Tau post-translational modifications. Accordingly, Tau hyperphosphorylation and cleavage are important events leading to Tau intracellular accumulation, aggregation and neuronal cell death. Caspase-cleaved Tau is detected in NFTs supporting the view that the apoptosis cascade is involved in the formation of NFTs. It is thought that Tau cleavage at its C-terminus by caspases renders Tau prone to aggregation and formation of NFTs. At the sites of

damage, AD brains also exhibit signs of chronic inflammation manifested by reactive astrocytes and microglia, which produce cytotoxic agents among them prostaglandins. There is a profound gap in our understanding of how cyclooxygenases and their prostaglandin products redirect cellular events to promote neurodegeneration.

In our studies we treated E18 cortical neurons with prostaglandin J2 (PGJ2), because it is potently neurotoxic. We show that PGJ2, a neurotoxic product of inflammation, induces the formation of the aggregate-prone form of Tau (Tau cleaved at Asp421, ΔTau) in a time- and dose-dependent manner. Furthermore, PGJ2 activates caspase 8 (extrinsic apoptotic pathway) and the effector caspase 3, thus inducing apoptosis in the cortical neuronal cultures.

In addition, we addressed the potential of increasing cAMP levels to prevent the toxic effects of neuroinflammation. Our studies focused on increasing cAMP because PGJ2 signals through a Gi protein-coupled receptor that reduces cAMP levels, promoting neuronal loss. Notably, increasing intracellular cAMP levels with dibutyryl-cAMP (db-cAMP) or PACAP27 prior to PGJ2 treatment decreased the levels of ΔTau and caspase activation, mitigating the loss of cell viability. These protective results of cAMP were only observed at early time points (4h and 8h) upon

treatment with PGJ2, indicating that they are only effective when applied before the neurons reach a point of no return.

We confirmed that PGJ2 treatment for 24h inhibits the proteasome and induces the accumulation and aggregation of ubiquitinated proteins. Elevating cAMP moderately increased the activity of the 26S proteasome. Surprisingly, db-cAMP or PACAP27 pre-treatment failed to prevent the accumulation/aggregation of ubiquitinated proteins induced by PGJ2.

We also addressed the potential of targeting molecular chaperones, such as Hsp90 and Hsp105 to prevent the toxic effects of neuroinflammation. Our studies focused on Hsp105 because it is a newly characterized chaperone that is highly abundant in the brain, and it is active under low ATP conditions. Nevertheless its role in neurodegeneration has yet to be determined. Promoting Hsp105 overexpression by its transient transfection in human neuroblastoma SK-N-SH cells improved cell survival upon treatment with PGJ2 for 24h. Furthermore, we investigated the protective effect of EC102, a small molecule Hsp90 inhibitor, in PGJ2-induced cell toxicity. EC102 blocks the Hsp90 ATPase activity, inhibiting its foldase capacity, instead targeting substrates for degradation by the proteasome. Rat E18 primary cortical neurons showed an increase in cell viability when cells were pre-treated with EC102 prior to PGJ2 treatment. These beneficial effects of molecular

chaperones support targeting them as a potential therapeutic target for AD.

Based on our studies, 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 toxic products released by glia, including PGJ2, act on the neighboring neurons causing among other effects, intracellular protein misfolding. If these proteins fail to be cleared by the ubiquitin/proteasome pathway (UPP) or fail to be refolded by chaperones, apoptosis is triggered launching caspase-mediated proteolysis. Caspase activation is responsible for generating protein fragments, including truncated Tau, which serve as seeds for cytotoxic protein aggregation. This sequence of events could explain many pathological features of the AD neurodegenerative process.

In conclusion, these studies showed that products of inflammation affect proteasome activity and alter protein turnover, which leads to protein aggregation, and neuronal injury. All of these processes are relevant to the pathology in AD. Moreover, our data indicate that the accumulation/aggregation of ubiquitinated proteins is a very stable phenomenon, and that once formed, the cell has difficulty in removing these aggregates. Overall, our studies suggest a new potential therapeutic approach for AD that involves maintenance

of intracellular levels of cAMP and, in a separate approach, enhancing the activity of heat shock proteins.

Elucidation of neurotoxic mechanisms linked to products of inflammation is highly significant, as it will offer new targets for anti-inflammatory drugs that more effectively prevent AD neurodegeneration linked to chronic inflammation and protein aggregation.

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Mariajose Metcalfe

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17-DMAG, 17-NN-dimethyl ethylene diamine-geldanamycin

Δ Tau, Tau cleaved at Asp421

Aβ, Amyloid Beta fragment

AD, Alzheimer's disease

ATP, adenosine triphosphate

cAMP, cyclic adenosine monophosphate

CHIP, Chaperone dependant E3 ligase

CNS, Central nervous system

COX, Cycloxygenase

CSF, Cerebrospinal fluid

DP1/DP2, PGD2 receptor 1 and 2

db-cAMP, dibutyryl-cAMP

DMSO, dimethyl sulfoxide

DTT, dithiothreitol

E18, embryonic day number 18

ECL, enhanced chemiluminescence

FTDP-17, Hereditary Frontotemporal dementia with parkinsonism-17

Hsp90 Hsp105, heat shock protein 90-105

MAPK, Mitogen activated protein kinase

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide;

NFT, Neurofibrillary tangle

NSAID, Non steroidal anti inflammatory drug

PACAP, pituitary adenylate cyclase, activating polypeptide

PAGE, polyacrylamide gel electrophoresis

PGJ2 PGD2 PGE2, Prostaglandin J2-D2-E2

15d-PGJ2, 15-deoxy-Δ12

PHF, Paired helical filament

PKA, cAMP-dependent protein kinase

PP1 PP2A PP2B, Protein phosphatase 1-2A-2B

PPARγ, Peroxisome proliferator activated receptor gamma

Rp-cAMPS, Adenosine 3',5'-cyclic monophosphorothioate, Rp-Isomer, Triethylammonium salt

s.d., standard deviation

SDS, sodium dodecyl sulfate

s.e., standard error

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

Ub-conjugates, ubiquitin conjugates

UPP, Ubiquitin proteasome pathway

CHAPTER I

INTRODUCTION

"RELATIONSHIP BETWEEN TAU PATHOLOGY AND NEUROINFLAMMATION IN ALZHEIMER'S DISEASE"

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1.1. ABSTRACT

Alzheimer's disease (AD) is a chronic, age-related neurodegenerative disorder. Neurofibrillary tangles (NFTs) are one of the pathologic hallmarks of AD. NFTs consist of abnormal protein fibers known as paired helical filaments (PHFs). Accumulation of PHFs is one of the most characteristic cellular changes in AD. Tau protein, a microtubule-associated protein, is the major component of PHF. Tau in PHFs is hyperphosphorylated, truncated and aggregated. What triggers PHF 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 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 review we focus on the pathological properties of Tau thought to play a role in NFT formation and summarize how CNS inflammation might be a critical contributor to AD pathology. A better understanding of the mechanisms that cause NFT formation is of clinical importance for developing therapeutic strategies to prevent and treat AD. One of the major challenges that we are faced with is to single out neuroinflammation as a therapeutic target for

prevention of AD neurodegeneration. The challenge rests on developing therapeutic strategies that prevent neurotoxicity linked to inflammation without compromising its neuroprotective role.

1.2. TAU, A MICROTUBULE BINDING PROTEIN

Alzheimer's disease (AD) is histophathologically characterized by extracellular β-amyloid-containing plaques and intracellular Tau-containing neurofibrillary tangles (NFT) (Johnson, Gotz et al., 2004). The main component of NFTs is Tau, a highly soluble microtubule-associated protein whose major function is to stabilize microtubules, specifically in axons, in a phosphorylation-dependent manner (Espinoza et al., 2008;Mandelkow et al., 2007). Tau promotes the assembly of microtubules into evenly spaced bundles in the axons and regulates the growing and shortening dynamics of individual microtubules (Rosenberg et al., 2008). Tau is a highly soluble protein that is heat and acid stable, and is rich in polar and charged amino acids with a basic character (Mandelkow et al., 2007). Five residues (Gly, Lys, Pro, Ser, and Thr) make up half of the Tau sequence, justifying its high solubility and the unfolded nature of the protein.

The Tau protein has four different regions that include from the C-terminus to the N-terminus: *(a)* the C-terminal domain with acidic and basic subregions that indirectly control Tau binding to microtubules via regulated phosphorylation; *(b)* the "microtubule-binding" domain with three or four imperfect repeats; *(c)* a positively charged Pro-rich domain that indirectly regulates the association between Tau and microtubules via regulated phosphorylation; *(d)* the N-terminal domain containing none, one or two negatively charged inserts (Mandelkow et al., 2007). The Pro-rich domain and the N-terminus comprise the "projection" domain that extends outward from the microtubule surface (Rosenberg et al., 2008).

There are six Tau isoforms in the human brain, which are product of alternative RNA splicing at exons 2, 3 and 10 (Goedert et al., 1989). The six different Tau isoforms can be designated as 3R0N, 3R1N, 3R2N, 4R0N, 4R1N, 4R2N, depending on the number of N-terminal inserts and C-terminal repeats. The microtubule-binding region contains three (3R) or four (4R) imperfect 18 amino acid long repeats with a characteristic Pro-Gly-Gly-Gly motif separated from each other by a 13 to 14 amino acid long interrepeat (Mandelkow et al., 2007;Crowther et al., 1989). Fetal human brain only expresses 3R Tau, whereas adult brain expresses approximately equal amounts of 3R and 4R Tau. Mutations in the *Tau* gene lead to extensive neuronal cell death

and dementia manifested in diseases such as hereditary Pick's disease and frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) (Rosenberg et al., 2008). Exonic mutations involve amino acid substitutions in the microtubule-binding repeat region or close to it, suggesting that they impair Tau binding to microtubules. Intronic mutations located near the splice-donor site of the intron following exon 10, are regulatory and affect Tau RNA splicing without affecting the amino acid composition of the protein (Spillantini and Goedert, 1998).

Neurodegenerative diseases collectively designated "Tauopathies" are linked to Tau mutations and/or Tau post-translational modifications. Accordingly, Tau hyperphosphorylation and cleavage are important events leading to Tau intracellular accumulation, aggregation and neuronal cell death (Corsetti et al., 2008).

1.3. TAU PHOSPHORYLATION

An important characteristic of Tau is that it possesses a large number of potential phosphorylation sites. Kinases that phosphorylate Tau are distributed into two groups (Gomez-Ramos et al., 2004):

(a) proline-directed serine-threonine protein kinases, which phosphorylate Ser-Pro or Thr-Pro Tau motifs; these include glycogen synthase kinase-3β (GSK3β), cyclin-dependent kinase (cdk) 5 and stress kinases such as JNK and p38.

(b) non-proline directed protein kinases that phosphorylate Ser or Thr residues not followed by Pro; among them are protein kinase A and C, calcium-calmodulin-dependent kinase II, serum and glucocorticoid-dependent kinase (SGK), protein kinase B, microtubule-affinity regulating kinase (MARK) and synapses of amphids defective (SAD) kinases, also known as brain specific serine/threonine kinases (Brsk).

Phosphorylation of Tau to physiological levels is a means by which Tau function is regulated (Johnson and Stoothoff, 2004). The physiological role of Tau phosphorylation includes regulation of microtubule dynamics, neurite outgrowth and axonal transport (Johnson and Stoothoff, 2004). Under normal physiological conditions, there is a balance between Tau phosphorylation and dephosphorylation. Phosphatases such as PP1, PP2A, PP2B and PP2C (protein phosphatase 1-2A-2B-2C, respectively) are known to reverse Tau phosphorylation, with PP2A postulated to be the phosphatase that dephosphorylates most Tau phosphorylation sites (Tian and Wang, 2002). Abnormal hyperphosphorylation of Tau within a consensus sequence for proline-directed kinases is an important factor in the

conversion of normal Tau into PHFs (Rosenberg et al., 2008). For example, combinations of the kinases cdk5/GSK-3 and CaMKII/GSK3β are involved in the rapid phosphorylation of Tau at Thr231 and Ser235, which is required for PHFs formation in AD (Sengupta et al., 1998;Wang et al., 2007).

Dephosphorylation of Tau by PP2A inhibits its aggregation into PHFs and restores its ability to bind to microtubules. However, re-phosphorylation of Tau by different combinations of PKA, calcium calmodulin kinase II (CaMKII), GSK3β and cdk5 promotes its self-assembly into PHFs leading to NFT formation (Wang et al., 2007).

When Tau is abnormally hyperphosphorylated it loses its biological activity, it becomes resistant to degradation (Rosenberg et al., 2008) and goes through conformational changes that render it insoluble and aggregation prone. Abnormal hyperphosphorylation of Tau leads to its aggregation into PHFs, which are the main components of NFTs. Understanding PHF assembly and how to prevent it, is relevant to AD and other neurodegenerative disease, such as FTDP-17, which are diseases in which Tau plays a critical role.

1.4. PIN-1 RESTORES TAU FUNCTION

The large family of proline-directed serine-threonine protein kinases, which include GSK3β, cdk5 and stress kinases such as JNK, play a critical role in cellular growth regulation, stress responses and neuronal survival (Lu and Zhou, 2007). These kinases specifically recognize Ser and Thr residues that precede Pro. Due to its unique stereochemistry, Pro can adopt two different conformational states, i.e. *cis* and *trans* isomeric states (Lu and Zhou, 2007). While the uncatalyzed isomerization of Pro is slow, it can be accelerated by peptidyl-prolyl *cis*/*trans* isomerases, which are enzymes able to change protein conformation. One of these enzymes, the peptidyl-prolyl isomerase PIN-1 [protein interacting with NIMA (never in mitosis A)-1] regulates phosphorylation signaling by proline-directed serine-threonine protein kinases (Lu and Zhou, 2007).

PIN-1 has been linked to AD as it is detected in NFTs from AD brains (Lu et al., 1999), and is thought to be neuroprotective against AD neurodegeneration (Lu et al., 2003). PIN-1 possesses an N-terminal WW domain responsible for mediating the binding of PIN-1 to Tau, specifically at the pT231 residue. PIN-1 promotes Tau dephosphorylation at the pThr231-Pro motif by the *trans*-specific phosphatase PP2A. Notably, PIN-1 only binds to Tau after its mitosis-specific phosphorylation (Lu

et al., 1999). Microtubule polymerization assays in conjunction with PIN-1 localization studies in normal and AD brains suggest that one of the functions of PIN-1 is to bind directly to pThr231 on Tau, promote its dephosphorylation, and restore the ability of Tau to bind to microtubules (Lu et al., 1999).

Neurons are post-mitotic cells but some studies support the view that aberrant neuronal cell cycle re-entry plays an important role in AD neurodegeneration (Illenberger et al., 1998;Billingsley and Kincaid, 1997;Kosik et al., 1996). Aberrant neuronal reactivation of mitosis is associated with Tau hyperphosphorylation. A reduction of PIN-1 protein levels as observed in AD will result in higher levels of hyperphosphorylated Tau, thus compromising its ability to promote microtubule polymerization and increasing Tau aggregation and NFT formation. PIN-1 and the associated prolyl isomerization process are thus potential therapeutic targets to prevent AD neurodegeneration (Maudsley and Mattson, 2006).

1.5. TAU CLEAVAGE

Tau cleavage independently by caspases and calpain is an important post-translational modification that together with hyperphosphorylation plays a critical role in the formation of PHFs (Wang et al., 2007). Caspase-cleaved Tau is detected in

NFTs supporting the view that the apoptosis cascade is involved in the formation of NFTs (Gamblin et al., 2003). It is thought that Tau cleavage at its C-terminus by caspases renders Tau prone to hyperphosphorylation and formation of NFTs (Mandelkow et al., 2007). The N-terminal of Tau is cleaved by calpain, which induces a change in Tau conformation from an unfolded state to a β-sheet structure rendering its C-terminus susceptible to cleavage by caspases (Wang et al., 2007).

The role of apoptosis in AD neurodegeneration is still unclear although caspases seem to be activated early in the progression of AD (Eckert et al., 2003). The two main apoptotic pathways are *(a)* the death-receptor pathway involving the initiator caspase 8 and *(b)* the mitochondrial pathway involving the initiator caspase 9 (Rissman et al., 2004). Both pathways converge on pro-caspase 3 cleavage leading to a cascade that triggers caspase 3 activation, Tau cleavage and Tau pathological aggregation. In AD brains a co-localization of caspase-8 (Guillozet-Bongaarts et al., 2006) and caspase-9 (Rohn et al., 2002) with NFTs was observed, suggesting that both apoptotic pathways occur within the same neuronal populations in AD brains. However, it is not clear whether caspase 8 or 9 activation precedes or coincides with NFT formation (Rohn et al., 2002).

Tau is cleaved by multiple caspases at a highly conserved

Asp residue (Asp421) in its C-terminus producing the N-terminal product Asp421Tau (Gamblin et al., 2003;Berry et al., 2003). Cleavage assays of full-length Tau in the presence of various caspases show that Tau is more susceptible to cleavage by executioner caspases (-3, -7) than by initiator caspases (-1, -4, -5, -8, -10) (Dickson, 2004). Tau aggregation assays indicate that Asp421Tau generated by caspase-3 or caspase-9 aggregates faster and has a stronger seeding effect for aggregation than full-length Tau (Rohn et al., 2002).

In AD brains Asp421Tau is widespread in the CA1 region of the hippocampus. This distribution co-localizes with intra-neuronal and extra-neuronal Aβ deposits and correlates with cognitive impairment (Rissman et al., 2004). Asp421Tau is also detected in other Tauopathies, such as Pick's disease (Mondragon-Rodriguez et al., 2008), supranuclear palsy, cortical degeneration and dementia with Lewy bodies (Wray et al., 2008).

There seems to be a correlation between Aβ, the major component of senile plaques in AD, and NFT formation. Aβ may lead to activation of apoptosis through the death-receptor as well as the mitochondrial pathways. Studies with E18 rat primary cortical neurons show that upon treatment with Aβ peptides Tau is cleaved to Asp421Tau (Rohn et al., 2002;Rissman et al., 2004). Other studies demonstrated that Tau cleavage induced by Aβ

treatment is prevented when the cultures are pre-incubated with caspase inhibitors (Gamblin et al., 2003). Furthermore, treatment of hippocampal neurons with Aβ induces neurite degeneration and microtubule collapse only when Tau is present. Tau depleted neurons show no signs of degeneration in the presence of Aβ, supporting a role for Tau in Aβ-induced neurodegeneration (Rapoport et al., 2002).

1.6. CORRELATION BETWEEN TAU HYPERPHOSPHORYLATION AND CASPASE CLEAVAGE

The relationship between Tau hyperphosphorylation and its cleavage by caspases remains poorly defined. Some studies suggest that phosphorylation precedes cleavage in tangle evolution (Guillozet-Bongaarts et al., 2006). *In vitro* phosphorylation of Tau at Ser422 renders Tau more resistant to caspase 3 proteolysis supporting the notion that phosphorylation at Ser422 prevents caspase cleavage some time during the progression of AD (Guillozet-Bongaarts et al., 2006).

The family of c-Jun N-terminal kinases (JNKs) are involved in processes such as cell differentiation, proliferation, apoptosis and neurodegeneration (Borsello and Forloni, 2007). JNKs are activated under stress conditions, such as those induced by reactive oxygen species and UV (Song and Lee, 2007).

Studies with cell culture models (Sahara et al., 2008) established that JNKs induce Tau hyperphosphorylation leading to caspase activation, thus promoting Tau cleavage. The JNK signaling pathway can be activated by a number of stress factors including oxidative stress and pro-inflammatory cytokines (Cho and Choi, 2002). JNK pathways are altered in AD causing abnormal phosphorylation of proteins that, under normal homeostatic conditions, would not be JNK targets (Borsello and Forloni, 2007). There are many potential substrates for JNK, but there is a great interest in determining whether JNK activation is involved in Tau phosphorylation and if this process occurs before or after caspase cleavage and Tau aggregation. Tau phosphorylation by JNK primes Tau for phosphorylation by GSK3β resulting in Tau hyperphosporylation. Only then will Tau form toxic aggregates that will in turn activate caspases and induce neuronal death. This sequence of events is further supported by the co-localization of phospho-JNK with Tau inclusions (Pei et al., 2001).

1.7. TAU UBIQUITINATION

Whether Tau is ubiquitinated and degraded *in vivo* by the ubiquitin proteasome pathway (UPP) remains controversial. In addition, it is not clear if Tau present in NFTs is

ubiquitinated. However, it is well established that proteasome activity is impaired in AD brains (Keller et al., 2000).

There are least two mechanisms associated with AD that could be responsible for proteasome impairment. The first mechanism is associated with frameshift mutants of ubiquitin B (UbB+1) found to co-localize with NFTs and senile plaques in the cerebral cortex of patients with sporadic AD (van Leeuwen et al., 1998). The UbB+1 mutant lacks the C-terminal glycine, which is a critical amino acid for ubiquitination and formation of polyubiquitin chains. UbB+1 impairs the degradation of ubiquitinated proteins by competing with wild-type ubiquitin for binding to the 26S proteasome (Lam et al., 2000). The second mechanism linked to AD is related to the direct binding of PHFs to the proteasome, an event that impairs proteasome activity (Keck et al., 2003).

A direct link between Tau aggregation and proteasome impairment is missing, although 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 (Dorval and Fraser, 2006). Some *in vitro* and cell culture studies suggest that Tau is degraded by the proteasome (David et al., 2002). Tau degradation by the proteasome seems to be dependent on Tau ubiquitination with K63-polyubiquitin chains. These chains are required for Tau interaction with the

sequestosome 1/p62, a "shuttling" partner for some proteasome substrates (Babu et al., 2005). Other studies with rat primary hippocampal neurons (Brown et al., 2005) and human neuroblastoma SH-SY5Y cells (Shimura et al., 2004) report that Tau degradation is independent of proteasome activity. This controversy remains to be solved.

Similar to ubiquitination, sumolation is a post-translational modification consisting of the addition of small ubiquitin-like modifiers (SUMO) to lysine residues on target proteins. Protein sumolation is known to prevent proteasomal degradation and/or to change protein function (Johnson, 2004). Tau sumolation was found to be up-regulated in cells treated with phosphatase inhibitors suggesting a new regulatory modification of Tau that may have an implication on its pathology (Dorval and Fraser, 2006).

1.8. ROLE OF THE CHIP/HSP90 COMPLEX IN TAU DEGRADATION

Tau undergoes hyperphosphorylation prior to its aggregation. One of the hypotheses for the neuronal accumulation of fibrillary Tau in neurodegenerative disorders exhibiting NFTs, is that chaperones fail to prevent the aggregation of hyperphosphorylated Tau. Hyperphosphorylated Tau is recognized and ubiquitinated by the U-box protein CHIP (carboxyl terminus

of Hsp70-interacting protein) (Sahara et al., 2005). CHIP is a co-chaperone with intrinsic ubiquitin ligase activity, which allows its chaperone function to switch from protein folding to protein degradation (Johnson, 2004). CHIP interacts with Hsp70 and inhibits its ATPase activity suggesting that it has another function besides protein refolding. CHIP also binds to HSP90 and forms a complex that selectively ubiquitinates hyperphosphorylated Tau along with UbcH5B (Sahara et al., 2005). The levels of CHIP are inversely proportional to insoluble Tau accumulation in AD brains (Goryunov and Liem, 2007). In addition, mice lacking CHIP exhibit high levels of insoluble Tau in their brains (McClellan et al., 2005). These results suggest that CHIP complexed with Hsp90 delays the formation of Tau aggregates and may thus play a protective role.

The serine/threonine kinase Akt is another protein that is ubiquitinated by the CHIP/Hsp90 complex and then targeted for proteasomal degradation (Dickey et al., 2008). Akt kinase is one of the kinases that hyperphosphorylates Tau promoting Tau accumulation/aggregation (Takashima, 2006). Studies with a variety of cell models indicate that under stress conditions the CHIP/Hsp90 complex ubiquitinates both Akt and Tau for proteasomal degradation (Dickey et al., 2008). Low Akt levels cause down-regulation of CHIP expression either by direct transcriptional regulation or via a feedback mechanism (Dickey

et al., 2008). However, due to aging or under disease conditions, the levels of Akt increase and because the CHIP/Hsp90 complex has a higher affinity for Akt than for hyperphosphorylated Tau, Akt is preferentially ubiquitinated and degraded. As a result, hyperphosphorylated Tau fails to be degraded and its levels increase promoting Tau accumulation/aggregation (Iqbal et al., 2008;Dickey et al., 2008). Higher levels of Tau increase its chances of being hyperphosphorylated by kinases such as Akt and MARK/Par-1 (Mandelkow et al., 2004), the latter being a microtubule affinity regulating kinase that plays a role in phosphorylating Tau during AD neurodegeneration. Notably, Par-1 phosphorylation of Tau at S262/S356 sharply decreases Tau affinity for the CHIP/Hsp90 complex, therefore enhancing Tau accumulation (Dickey et al., 2007). In conclusion, the role played by Akt in Tau accumulation supersedes its role in Tau phosphorylation.

1.9. INFLAMMATION AND TAU PATHOLOGY

Inflammation is implicated in AD (McGeer and McGeer, 2007). The association between inflammation and AD manifests itself through *(a)* the presence of activated microglia and astrocytes surrounding senile plaques and *(b)* higher levels of inflammatory mediators in brains of AD patients than in age matched controls

(Sastre et al., 2008). Current animal models of AD fail to address the mechanisms by which products of inflammation produced by activated microglia and astrocytes induce AD neurodegeneration. This is a very important issue since some products of inflammation are neuroprotective and others neurotoxic.

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 Tauopathies (Yoshiyama et al., 2007). Prominent microglial activation preceded tangle formation and immunosuppression of young P301S transgenic mice diminished Tau pathology and increased their lifespan. It was concluded that neuroinflammation is linked to early progression of Tauopathies (Yoshiyama et al., 2007).

What triggers the formation of NFTs is not known, but neuroinflammation could play a critical 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. We

recently reviewed the link between inflammation and Tau pathology (Arnaud et al., 2006).

We propose a model in which any stimulus (physical, chemical or infectious) capable of inducing inflammation in brain areas affected in AD, directly activates microglia and astrocytes. These stimuli could also directly induce neuronal injury, which through yet unknown factors trigger the reactive response of glial cells. Either way, the activated glia surround and drench neurons with their toxic products, such as prostaglandins, nitric oxide, IL1β, IL6, TNFα and reactive oxygen species (e.g. superoxide anion). All of these cytotoxic agents must work in concert to induce synergistic neurotoxicity leading to neurodegeneration. This sequence of events could explain many pathological features of the AD neurodegenerative process. Elucidation of the mechanisms mediated by neurotoxic products of inflammation and that lead to AD neurodegeneration will open up new and important possible targets for pharmacological treatment of AD (Klegeris et al., 2007).

1.10. LATE-BREAKING STUDIES

Two recent studies address the proteolytic events leading to Tau pathology. One study (Arnaud et al., 2009) proposes that impairment of the ubiquitin/proteasome pathway leading to

accumulation of ubiquitinated proteins is an early response to neuronal damage. If cells cannot clear the accumulated ubiquitinated proteins, apoptosis kicks in triggering caspase activation, which leads to Tau cleavage. Failure to remove these aggregation prone Tau fragments by cathepsins would promote Tau pathology. The other study (Wang et al., 2009) suggests that chaperone-mediated autophagy is involved in the delivery of cleaved Tau to lysosomes for additional cleavage. Impaired translocation of cleaved Tau across the lysosomal membrane seems to promote Tau oligomerization and Tau aggregation at the membrane, thus interfering with lysosomal functioning. Both studies agree that disruption of the lysosomal pathway plays a role in the final stages of Tau pathology. However, as suggested by the first study, impairment of the ubiquitin/proteasome pathway followed by caspase activation seems to be earlier events in the proteolytic cascade involved in Tau pathology.

1.11. OVERALL CONCLUSIONS

Alzheimer's disease is an age related neurodegenerative disorder that is associated with neuroinflammation. 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. Neurofibrillary tangles (NFT) are a pathologic hallmark of AD. The major component of NFTs is Tau, a microtubule associated protein that is abundant in neurons and is highly soluble, yet in AD it appears as abnormal aggregates. Tau is hyperphosphorylated, truncated at Asp421 and aggregates into insoluble PHFs. Elucidation of the sequence of these and other Tau post-translational modifications and their contribution to neuronal cell death is highly significant for identifying the upstream steps that can be therapeutically targeted to prevent neurons from reaching a point of no return.

The dynamics of Tau phosphorylation/dephosphorylation are a main focus of attention for scientists that try to find a means by which to regulate this process to avoid Tau hyperphosphorylation and aggregation, which impairs its clearance. However, since Tau is a target for so many kinases and phosphatases understanding this process has proven to be very difficult. The challenge is to dissect which pathways render Tau prone to hyperphosphorylation and aggregation, such as its cleavage by caspases or priming by a cascade of kinases. Resolving the pathways that lead to Tau hyperphosphorylation and aggregation will provide a basis for designing more effective therapeutic strategies for AD.

Neuroinflammation with the ensuing gliosis could be an early manifestation of AD neurodegeneration. A better

understanding of the mechanisms by which products of inflammation mediate neuronal injury may lead to more effective anti-inflammatory therapeutic strategies to prevent or treat AD neurodegeneration, which is associated with chronic inflammation.

CHAPTER II

"ELEVATING CAMP WITH PACAP27 PREVENTS CASPASE-MEDIATED TAU CLEAVAGE RESCUING CORTICAL NEURONS FROM THE NEUROTOXIC PRODUCT OF INFLAMMATION PROSTAGLANDIN J2: A NEW THERAPEUTIC APPROACH FOR ALZHEIMER'S DISEASE?"

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2.1. ABSTRACT

Senile plaques and neurofibrillary tangles are hallmarks of Alzheimer's disease (AD). The microtubule-associated protein Tau is a major component of AD neurofibrillary tangles. At the sites of damage, AD brains also exhibit signs of chronic inflammation manifested by reactive astrocytes and microglia, which produce cytotoxic agents among them prostaglandins. To address the relation between inflammation and neurodegeneration in AD, we treated rat E18 cortical neurons with prostaglandin J2 (PGJ2), a potently neurotoxic endogenous mediator of inflammation. In addition, we investigated the potential of increasing cAMP levels to prevent the toxic effects of neuroinflammation. Our studies focused on increasing cAMP because PGJ2 signals through a Gi protein-coupled receptor that reduces cAMP levels, promoting neuronal loss. We show that PGJ2 induces the formation of the aggregate-prone form of Tau (Tau cleaved at Asp421, ΔTau) in a time- and dose-dependent manner in the primary neuronal cultures. Furthermore, PGJ2-treatment induces apoptosis via the extrinsic pathway, as it activates the initiator caspase 8 and the effector caspase 3. PGJ2, previously demonstrated to inhibit the proteasome, also induced the accumulation/aggregation of ubiquitinated proteins. Notably, increasing intracellular cAMP levels with dibutyryl-cAMP (db-cAMP) or PACAP27 prior to PGJ2 treatment decreased the levels of ΔTau and caspase activation,

mitigating the loss of cell viability. These protective results of cAMP were only observed at early time points (4h and 8h) upon treatment with PGJ2, indicating that they are only effective when applied before the neurons reach a point of no return. Elevating cAMP moderately increased the activity of the 26S proteasome. Surprisingly, db-cAMP or PACAP27 pre-treatment failed to prevent the accumulation/aggregation of ubiquitinated proteins induced by PGJ2. These results indicate that the accumulation/aggregation of ubiquitinated proteins is a very stable phenomenon, and that once formed, the cell has difficulty in removing these aggregates. Overall, our studies suggest a new potential therapeutic approach for AD that involves maintenance of intracellular levels of cAMP. Future studies will address more efficient regimens to prevent accumulation of ubiquitinated proteins and thus more effectively avert neurodegeneration in AD.

2.2. INTRODUCTION

Chronic neuroinflammation is a critical factor in the pathogenesis of Alzheimer's disease (Herrup, 2010). Cyclooxygenases are major players in inflammation and they produce a variety of prostaglandins, some with pro-survival others with pro-death effects in the CNS (Lucin and Wyss-Coray, 2009). Cyclooxygenases and their prostaglandin products have

been implicated in AD neurodegeneration (Yoshiyama et al., 2007;Zagol-Ikapitte et al., 2005;Liang et al., 2007). Inhibiting cyclooxygenases with NSAIDs is being explored as a therapeutic strategy to mitigate chronic inflammation and to prevent the onset or progression of AD pathology (McGeer and McGeer, 2007;Vlad et al., 2008). The effectiveness of NSAIDs may be compromised because they block the production of neuroprotective and neurotoxic prostaglandins. A better understanding of downstream neurotoxic prostaglandin pathways is critical to the development of more effective anti-inflammatory approaches to slow the progression or delay the onset of AD associated with neuroinflammation.

Here we focus on prostaglandin J2 (PGJ2) because it is an endogenous product of inflammation that causes pleiotropic changes that mimic many of the pathological processes that occur in AD. PGJ2 induces neuronal cell death (Kondo et al., 2002;Li et al., 2004b), inhibits 26S proteasome activity (Ishii et al., 2005;Wang et al., 2006;Ogburn and Figueiredo-Pereira, 2006), triggers accumulation/aggregation of ubiquitinated proteins (Li et al., 2004b), perturbs the cytoskeleton (Ogburn and Figueiredo-Pereira, 2006) and up-regulates cyclooxygenase-2 (Li et al., 2004a). Furthermore, we recently showed that in cortical neurons PGJ2 induces Tau cleavage at Asp421, Tau aggregation, neuritic dystrophy, and apoptosis assessed by PARP and caspase 3

cleavage (Arnaud et al., 2009). Truncation of Tau at Asp421 is an early event in AD tangle pathology (Gamblin et al., 2003;Rissman et al., 2004). Together these data suggest that PGJ2 could act as a novel inflammatory mediator in the AD brain and targeting it could influence the course of AD.

PGJ2 is a bioactive prostaglandin produced *in vivo* during inflammation (Rajakariar et al., 2007) by non-enzymatic dehydration of prostaglandin D2 (PGD2), the most abundant prostaglandin in the CNS (Abdel-Halim et al., 1977;Pentreath et al., 1990). PGJ2 is metabolized into Δ12-PGJ2 and 15-deoxy-Δ12,14-PGJ2 (15d-PGJ2) (Uchida and Shibata, 2008). 15d-PGJ2 is produced in large amounts by LPS-activated microglia (Bernardo et al., 2003). Prostaglandins of the J2 series are unique among prostaglandins because they are highly reactive and form covalent Michael adducts with free thiol groups in glutathione and cysteines in cellular proteins (Straus and Glass, 2001). Electrophile binding to key protein cysteine(s) by endogenous compounds such as PGJ2 is regarded as playing an important role in determining whether neurons will live or die (Satoh and Lipton, 2007). In addition, prostaglandins of the J2 series signal via DP2, a *Gi* coupled PGD2 receptor that induces inhibition of basal cAMP production. Thus, we set out to determine whether agents that restore and/or elevate intracellular cAMP can overcome the toxic effects of PGJ2 on

cortical neurons. These agents could be used as anti-inflammatory drugs to protect neurons from the neurotoxic effects of chronic inflammation without halting its benefits in AD patients.

2.3. MATERIALS AND METHODS

2.3.1. Materials: Prostaglandin J2 was from Cayman Chemical (Ann Arbor, MI, USA). Adenosine 3′, 5′-cyclic monophosphate dibutyryl sodium salt (db-cAMP), adenosine 3′,5′-cyclic monophosphorothioate, Rp-Isomer, triethylammonium salt (Rp-cAMPS) and the cAMP colorimetric direct immunoassay Kit, were from Calbiochem/EMD Bioscience (Gibbstown, NJ). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was from Sigma-Aldrich (St. Louis, MO). The substrate Suc-LLVY-AMC and pituitary adenylate cyclase activating polypeptide-27 (PACAP-27) were from BACHEM Bioscience Inc. (King of Prussia, PA). Antibodies: rabbit polyclonal anti-ubiquitinated proteins (1:1,500, cat# Z0458, Dako North America, Carpinteria, CA); rabbit polyclonal anti-β5 (1:1,000, cat# PW8895, BIOMOL, Plymouth Meeting, PA); mouse monoclonal anti-β-actin (1:10,000, cat# A2228, Sigma, Saint Louis, MO); rabbit polyclonal anti-caspase 8 (1:500, cat# 3020) and anti-caspase 9 (1:1,000, cat# 3016) from BioVision, Mountain View, CA); mouse monoclonal Tau C3 (Tau cleaved at Asp421, 1:5,000) and mouse monoclonal Tau 5

(1:50,000) were courtesy of Dr. L. Binder (Northwestern University, Chicago, IL, USA); rabbit polyclonal anti-LC3 (1:1,000, cat# NB100-2331, Novus Biologicals, Littleton, CO); rabbit polyclonal anti-caspase 3 (1:1,1000, cat# 9662), anti-CREB (1:1,000, cat# 9197), and anti-phosho-CREB (1:1,000, cat# 9198) from Cell Signaling Technology (Danvers, MA) . The respective secondary antibodies with HRP conjugate (1:10,000) were from Bio-Rad Laboratories (Hercules, CA).

2.3.2. Cell cultures: Dissociated cultures from Sprague Dawley rat embryonic (E18) cerebral *cortical neurons* were prepared as described in (Biederer and Scheiffele, 2007) with some modifications. The isolated cortices free of meninges were digested with papain (0.5 mg/ml from Worthington Biochemical Corp., Lakewood, NJ) in Hibernate E without calcium (BrainBits LLC., Springfield, IL) at 37°C for 30min in a humidified atmosphere containing 5% CO_2. After removal of the enzymatic solution the tissues were gently dissociated in Neurobasal media (Invitrogen, Carlsbad, CA). The dissociated tissues were centrifuged at 300Xg for 2min. The pellet was resuspended in Neurobasal media without antibiotics and plated on 10-cm dishes pre-coated with 50 µg/mL poly-D-lysine (Sigma, Saint Louis, MO). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. The cells were maintained at 37 °C in 5% CO2 in Neurobasal media supplemented with 2% B27 and 0.5 mM

glutamine (all from Invitrogen) and half of the medium was changed every 4 days.

2.3.3 Culture treatments: E18 primary neuronal cortical cultures were treated for 4h, 8h, 16h or 24h with DMSO or ultra pure filtered water (controls) or with different drugs: PGJ2 in DMSO, db-cAMP and PACAP27 in ultra pure filtered water added directly to DMEM without serum supplemented with 0.5 mM glutamine and 1 mM sodium piruvate (all from Invitrogen). 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.

2.3.4 Cell viability assay: Cells were plated at a density of $2.5X10^5$ cells per well on 24-well plates under various conditions for 4h, 8h, 16h or 24h. Cell viability was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described in (Mosmann, 1983). This assay assesses mitochondrial viability.

2.3.5. Western blotting: Western blot analysis was carried out following SDS-PAGE. Cells were plated at a density of $1X10^6$ cells per 10cm dish. After treatment, cells were rinsed twice with PBS and harvested by gently scraping into ice-cold lysis buffer [20mM Tris-HCl, pH 7.5, 137mM NaCl, 1mM EGTA, 2.5mM $Na_4P_2O_7$, 1mM β-glycerophosphate, 50mM NaF, 1mM

phenylmethylsulfonyl fluoride, 1% NP40, 1mM Na_3VO_4, 1% Glycerol and protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO)]. Following lysis (15min) and centrifugation (14,000xg for 10min) at 4°C the protein concentration of the cleared supernatants was determined (BCA kit, Pierce, Rockf., IL). Normalized samples were boiled for 5min in Laemmli buffer and loaded onto gels (40µg of protein/lane). Following electrophoresis, proteins were transferred onto an Immobilon-P membrane (Millipore, Bedford, MA, USA). The membrane was probed with the respective antibodies and antigens were visualized by a standard chemiluminescent horseradish peroxidase method with the ECL reagent. Semi-quantitative analysis of protein detection was done by image analysis with the ImageJ program (Rasband, W.S., ImageJ,U.S. NIH, Maryland, http://rsb.info.nih.gov/ij/, 1997-2006). Relative intensity (no units) is the ratio between the value for each protein and the value for β-actin (loading control).

2.3.6. Filter trap: Cells were plated at a density of $1X10^6$ cells per 10cm dish. After treatment media was removed and cells were lysed as for western blotting. Samples were normalized to 0.5 µg/µl using a buffer containing 2% SDS and 10mM Tris-EDTA pH 7.5. 50 µg of proteins from the different extracts were filtered through a 0.2 µm nitrocellulose membrane (BIO-RAD, Hercules, CA) using a 96-well dot blot apparatus (Schleicher & Scheull Inc.).

Each well was washed twice with washing buffer containing 0.1% SDS and 10mM Tris-EDTA, pH 7.5. Captured aggregates were detected by immunoblotting using the anti-ubiquitin and the Tau-C3 antibody.

2.3.7. In-gel proteasome activity and levels: Cells were plated at a density of $1X10^6$ cells per 10cm dish. Upon treatment with vehicle (control, DMSO or water) or the respective drugs, cells were washed twice with PBS and harvested for the in-gel assay as described in (Ogburn and Figueiredo-Pereira, 2006) with 30µg protein/lane loaded for proteasome activity and 40µg protein/lane loaded for western blotting. The native gels were run at 150V for 120min. The in-gel proteasome activity was detected by incubating the native gel on a rocker for 10min at 37°C with 15ml of 400µM Suc-LLVY-AMC followed by exposure to UV light (360nm) and photographed with a NIKON Cool Pix 8700 camera with a 3-4219 fluorescent green filter (Peca Products, Inc). Proteins on the native gels were transferred (110mA) for 2h onto PVDF membranes. Immunoblotting was then carried-out sequentially for detection of the 20S and 26S proteasomes with anti-β5 subunit antibody. The anti-β5 antibody reacts with a core particle subunit, therefore detects both the 26S and 20S proteasomes. Antigens were visualized by a chemiluminescent horseradish peroxidase method with the ECL reagent. For loading control, aliquots of the samples were also boiled for 5min in

Laemmli buffer and loaded onto 10% gels (40µg of protein/lane) for immunoblotting with anti-β-actin.

2.3.8. Intracellular cAMP: cAMP levels were determined with a nonradioactive assay kit. Cells were plated at a density of $1X10^6$ per 10cm dish. After treatment, media was removed and 500µl of 0.1 N HCl were added to each dish followed by an incubation for 5 minutes. Cell lysates were harvested and centrifuged at 600xg for 10-min at room temperature. The supernatant was used directly in the non-acetylated version of the assay. cAMP levels were determined following manufacturer's specifications. Absorbance was measured at 405nm and a 570nm correction, with a PowerWave HT Spectrophotometer. cAMP concentration (pmol/µg protein) for each sample was determined according to the kit's instructions.

2.3.9. Caspase activity assays: Caspase activity assays (for caspases 3, 8 and 9) were carried out with caspase colorimetric assay kits from Biovision (Mountain View, CA). Cells were plated at a density of $1X10^6$ per 10cm dish. After treatment, media was removed and cells were washed once with PBS and harvested with 75µl of cell lysis buffer. After harvesting, cells were incubated on ice for 15-min and centrifuged at 10,000xg for 10-min at 4°C. The protein concentration of the cleared supernatants was determined (BCA kit, Pierce, Rockf., IL) and samples were

normalized to 3 μg/μl. The normalized supernatants were used directly in the caspase activity assay following manufacturer's specifications. Absorbance was measured at 400nm, with a PowerWave HT Spectrophotometer in a kinetic assay for 2 hours and caspase activity was determined as absorbance at 400 nm per 150 μg of protein.

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

2.4. RESULTS

2.4.1. The accumulation of soluble ubiquitinated proteins induced by PGJ2 precedes ub-protein aggregation, activation of pro-caspases 8 and 3 as well as caspase-mediated cleavage of TAU in rat cerebral cortical neuronal cultures – Aggregates of ubiquitinated proteins and the microtubule associated protein Tau co-localize in intraneuronal neurofibrillary tangles in AD. Moreover, caspase-mediated cleavage of Tau at Asp421 (ΔTau) seems to be an early event in AD tangle pathology. To determine the temporal relation between caspase-mediated cleavage of Tau (ΔTau) and the accumulation/aggregation of ubiquitinated proteins we treated rat E18 cortical neurons with the neurotoxic product of inflammation PGJ2, which induces both ubiquitin-protein aggregation and ΔTau. Initially, we treated cells for 24h with different concentrations of PGJ2 to determine the optimal conditions to establish a time course of the two events, i.e. ubiquitin-protein accumulation/aggregation and ΔTau. As shown in Figure 1A, a significant increase in ubiquitinated proteins (Ub-proteins) and ΔTau were detected with PGJ2 concentrations as low as 5μM. We then performed a time-course experiment wherein the cortical neurons were treated with 20μM PGJ2 and harvested after 4h, 8h, 16h and 24h. Data in Figure 1B show that 4h after treatment with PGJ2, the levels of Ub-proteins were already significantly ($p<0.001$) higher than

control, while the levels of ΔTau were only slightly increased above control reaching a peak by 16h. Notably, when compared to 8h of PGJ2-treatment, the levels of ub-proteins after 16h and 24h were lower, indicating a decline in NP40 "soluble" ub-proteins with a corresponding increase in NP40 "insoluble", SDS-soluble ub-proteins, as shown in Fig. 1B. These data demonstrate that the accumulation of "soluble" ub-proteins precedes the formation of ΔTau and ub-aggregates.

PGJ2 was previously shown to induce neuronal apoptosis in SH-SY5Y (Kondo et al., 2002) and SK-N-SH (Arnaud et al., 2009) cells. Since the production of ΔTau is linked to caspase cleavage (Rissman et al., 2004;Guillozet-Bongaarts et al., 2005) we investigated the temporal relation between caspase activation, ΔTau detection and ub-protein accumulation. PGJ2 was shown to induce neuronal apoptosis via the extrinsic (caspase 8) pathway (Kondo et al., 2002). We validated that indeed PGJ2 activates initiator caspase 8 as well as the effector caspase 3 in a concentration- (Fig. 2A) and time-dependent (Fig. 2B) manner in rat primary cortical cultures. Caspase activation was assessed by conversion of pro-caspases 8 and 3 into their active forms caspase 8 and 3, respectively. From the concentration curve (Fig. 2A), it is clear that activation of the two caspases peaked upon 24h treatment with 10μM PGJ2, which coincided with the maximum production of ΔTau. A similar trend was observed for

the time course (Fig. 2B) as the highest level of caspase activities and ΔTau coincided. These data support a tight correlation between caspase activation and formation of ΔTau. We also established that PGJ2 did not inhibit autophagy as the levels of the autophagic marker LC3-II were not altered (Fig. 2A and B).

Overall, the data in these two figures (1 and 2) suggest that the accumulation of soluble ubiquitinated proteins is one of the events that initiates a toxic cascade culminating in apoptosis and caspase-mediated cleavage of TAU (ΔTau).

2.4.2. Reversing the PGJ2-induced decline in basal cAMP levels in rat cerebral cortical neuronal cultures - PGJ2 signals via one of the PGD2 receptors, i.e. the DP2 receptor, with a nanomolar affinity similar to PGD2 (Monneret et al., 2002;Hata et al., 2003). DP2 is coupled to inhibitory G-proteins and decreases cAMP concentrations (Hata et al., 2003). Based on its receptor-mediated action we reasoned that raising cAMP levels could prevent some of the PGJ2 toxic effects. We tested two strategies to elevate intracellular cAMP levels: (1) dibutyryl-cAMP (db-cAMP), a cell permeable analog of cAMP that is significantly less susceptible to hydrolysis by phosphodiesterases (Miller et al., 1973), and (2) PACAP27 (pituitary adenylate cyclase activating polypeptide) which is a

peptide that binds to PAC1R (pituitary adenylate cyclase 1 receptor), a seven transmembrane domain, G-coupled receptor. At nanomolar levels, PACAP-binding to PAC1R activates adenylate cyclase and elevates intracellular cAMP (Moody et al., 2011). We confirmed that indeed PGJ2 (10μM, 4h) decreased cAMP levels by almost three-fold compared to control conditions in the rat E18 neuronal cultures (Fig. 3). In addition, pre-treating cells with dibutyryl-cAMP (1mM, db-cAMP) or PACAP27 (100nM) prior to PGJ2, significantly raised cAMP (Fig. 3). It is notable that PACAP27 elevated intracellular cAMP at drastically lower (nanomolar) concentrations than db-cAMP (millimolar).

2.4.3. At early time points (4h and 8h), db-cAMP and PACAP27 prevent PGJ2-induced caspase-mediated cleavage of Tau and activation of pro-caspases 8 and 3 in rat cerebral cortical neuronal cultures – Since both db-cAMP and PACAP27 prevented the decreased in intracellular cAMP induced by PGJ2 (Fig. 3) we tested if these agents that elevate cAMP counteract some of the effects of PGJ2. It is clear that pre-treatment with db-cAMP (1mM) or PACAP27 (100nM) prevent TAU cleavage at Asp421 (ΔTau) when the rat cortical neurons are treated with PGJ2 (10μM) for 4h or 8h (Fig. 4). However, at the later time points (16h and 24h) the effect of the two cAMP elevating drugs on Tau was

abolished, as they were incapable of mitigating the PGJ2-induced Tau cleavage (Fig. 4).

Since Tau cleavage at Asp421 (ΔTau) is dependent on caspase activation, we analyzed the effect of pre-treating the cortical neuronal cultures with db-cAMP or PACAP27, on caspases. We focused on caspase 8, which mediates the extrinsic apoptotic pathway, and caspase 3, the effector caspase, because these two caspases seem to be preferentially activated by PGJ2 (Fig. 2). Just like what we observed for the production of ΔTau, conversion of pro-caspases 8 and 3 to the respective cleaved caspases, was prevented by db-cAMP (1mM, Fig. 5A) or PACAP27 (100nM, Fig. 5B) at least up to 8h of treatment with PGJ2. PACAP27 seemed to be less efficient than db-cAMP. At the latest time points, i.e. 24h, both db-cAMP and PACAP27 failed to prevent the switch from pro-caspases to their cleaved forms (Fig 5A and 5B).

Besides preventing the conversion of pro-caspase 8 and 3 into their cleaved forms, pre-treatment with db-cAMP or PACAP27 abolishes activation of these caspases by PGJ2, as assessed with a colorimetric assay kit (Fig. 6). Both caspase 8 and 3 are significantly ($p<0.001$) activated by PGJ2 (8h, 10μM) but pre-treatment with either db-cAMP or PACAP27 mitigates the PGJ2 activation of both caspases. For caspase 3, db-cAMP was more

effective than PACAP27. As seen in Fig. 6, caspase 9 is not significantly activated by PGJ2.

Overall these data support the notion that after 16h of treatment with PGJ2 the cells reach a point of no return, whereby the damage is such that the cells can no longer recuperate from PGJ2 toxicity.

2.4.4 Db-cAMP and PACAP27 do not abolish the accumulation/aggregation of ubiquitinated proteins induced by PGJ2, even though db-cAMP *per se* elevates proteasomal activity -

We previously demonstrated that PGJ2 induces the accumulation and aggregation of ubiquitinated proteins in neuronal cells (Li et al., 2004b). In an effort to determine if elevating intracellular cAMP prior to PGJ2-treatment would prevent the accumulation/aggregation of ubiquinated proteins we pre-treated primary neuronal cultures with db-cAMP or PACAP27 before adding PGJ2 to the media. Surprisingly, none of the pre-treatments decreased the levels of ubiquitinated proteins induced by PGJ2 (Fig. 7), even at the early time points (4h and 8h), when increasing cAMP levels overcame caspase-mediated TAU cleavage (Fig. 4). It seems that in terms of accumulation/aggregation of ubiquitinated proteins, PGJ2 prevails over any protective effect that increasing cAMP may have.

Two factors could account for the failure of cAMP to prevent the accumulation of ubiquitinated proteins induced by PGJ2. One is proteasome inhibition and the other protein aggregation. We first addressed proteasome inhibition. We and others established that PGJ2 inhibits proteasome activity (Shibata et al., 2003;Ishii and Uchida, 2004;Wang et al., 2006;Ishii et al., 2005). We thus assessed how elevating cAMP influences this PGJ2 effect. Proteasome activity was measured with the native in gel assay that provides a means to differentiate the three forms of the proteasome, i.e. 26S proteasomes with either two regulatory caps [26S (2)] or just one [26S(1)] and the 20S core particle by itself (20S). Proteasome activity was determined with the substrate Suc-LLVY-AMC, which evaluates the chymotrypsin-like activity. In addition, proteasome levels were established by immunobloting with an anti-β5 antibody. As the β5 subunit is a component of the 20S core, the antibody provides for the detection of both forms of the 26S as well as the 20S proteasome. As seen in Fig. 8, db-cAMP by itself increases the activity [*top panel, left* (*arrows on graphs*)] and level [*lower panel, left (arrows on graphs*)] of both forms of the 26S proteasome; the activity of the 20S particle was also elevated without much change in its levels. It is clear that PGJ2 decreased both the activity and levels of the 26S proteasome without altering the 20S core [Fig.

8, *right panels* (*black bars on graphs*)]. Pre-treatment with db-cAMP slightly improved the PGJ2-negative effect on 26S proteasomes, without complete recovery of activity and levels [Fig. 8, *right panels* (*arrows on graphs*)]. PACAP27 did not alter proteasomes (Fig. 8, *left and right panels*), most likely because of the hydrolyzable nature of the cAMP generated by PACAP27 action. Overall these studies demonstrate that while db-cAMP *per se* improves proteasome activity, PGJ2 negative effects prevail over cAMP in the cortical neuronal cultures.

The db-cAMP positive effects on the proteasome were mediated by protein kinase A (PKA), as they were abolished (Fig. 8, *left and right panels*) by pre-treating cells with Rp-cAMPS, a PKA inhibitor that acts as a competitive antagonist of the cyclic-nucleotide-binding domains on PKA (de Wit et al., 1984). Furthermore, the cAMP effect could be mediated, among others, via the CREB signaling pathway, as we show that cAMP alone or in combination with PGJ2 increases phospho-CREB (Fig. 9).

We next focused on protein aggregation as a possible cause for the failure of cAMP to prevent the PGJ2-dependent accumulation of ubiquitinated proteins. Once aggregated, ubiquitinated proteins are not degraded and even inhibit the proteasome (Snyder et al., 2003;Bennett et al., 2005). We first evaluated the effect of PGJ2 in a concentration- and time-dependent manner on the aggregation of ubiquitinated proteins

and ΔTau, by using the filter trap assay. Upon treatment with PGJ2 aggregates with a size > 0.2μm (the pore size of the filter paper) were detected with antibodies for ubiquitinated proteins (Ub-conjugates) and ΔTau (Fig. 10). The PGJ2-concentration curve exhibited a similar trend for ubiquitin-protein and ΔTau aggregation, as both types of aggregates were detected at ≥ 10μM PGJ2. The time course differed, as aggregates of ubiquitinated proteins were detected by 8h while those of ΔTau appeared later by 16h. These date confirm what was observed in Fig. 1, i.e. that the accumulation/aggregation of ubiquitinated proteins are an early event in the PGJ2 toxic cascade. In addition, the low levels of ΔTau aggregates detected after 8h of PGJ2 (10μM) treatment were abolished by pre-treatment with db-cAMP (1mM) or PACAP27 (100nM), while the Ub-aggregates were diminished by the pre-treatment but not eliminated. These data indicate that by 8h of PGJ2-treatment the Ub-aggregates are more abundant and stable than the ΔTau aggregates, thus harder to prevent.

2.4.5. Shorter incubations (8h) but not longer (24h) with db-cAMP and PACAP27 prevent the loss in cell viability induced by PGJ2 - We previously showed that prostaglandins of the J2 series were the most neurotoxic of the prostanoids that we tested which also included PGA1, D2 and E2, in neuroblastoma cells and mesencephalic cultures (Li et al., 2004b). As shown in Figure 11A, rat cerebral cortical cultures are also sensitive to PGJ2 toxicity in a time- and concentration-dependent manner. Notably, pre-treatment with db-cAMP (1mM, Fig. 11B) or PACAP27 (25nM to 100nM, Fig. 11C) significantly ($p < 0.001$) diminished the loss of viability of the primary neuronal cultures upon 8h treatment with 10μM PGJ2. However, elevating cAMP with each of the drugs failed to stop the loss in cell viability induced by 24h of incubation with 10μM PGJ2. It seems that after 24h with PGJ2 the cells reach a point of no return in which their demise is no longer avoidable.

2.5. DISCUSION

In these studies we demonstrate that elevating intracellular cAMP in rat primary cortical neuronal cultures can prevent some of the events in the toxic cascade induced by PGJ2, such as caspase activation, caspase-mediated Tau cleavage, and loss of cell viability. However, this protection by cAMP is only observed with shorter (approximately up to 8h) and not longer

(24h) incubations with PGJ2, which seems to correlate with the formation of stable ubiquitin protein aggregates. Once these aggregates are formed and attain a certain (undefined) level, apoptosis is activated, via the extrinsic pathway for PGJ2, and the cells reach a point of no return.

We tested two approaches to elevate cAMP: treating cells with db-cAMP or with PACAP27. Db-cAMP is a derivative of cAMP that is more lipophilic and membrane permeable, and is a very poor substrate for a variety of cAMP phosphodiesterases, exhibiting an ~ 14-fold lower rate of hydrolysis than cAMP (Miller et al., 1973). PACAP27 is a peptide (sequence HSDGIFTDSYSRYRKQMAVKKYLAAVL-NH_2) that binds to PAC1R (pituitary adenylate cyclase 1 receptor), a seven transmembrane domain G-coupled receptor expressed in the cerebral cortex and hippocampus as well as in other brain areas (Joo et al., 2004). Nanomolar concentrations of the two forms of PACAP, PACAP38 and the truncated form PACAP27 (the latter used in our experiments), activate adenylate cyclase and elevate intracellular cAMP (Moody et al., 2011). In our studies, db-cAMP was more effective than PACAP27 in counteracting the PGJ2 toxic effects. This is not surprising as db-cAMP is significantly less susceptible to hydrolysis by the intracellular cAMP phosphodiesterases than endogenous cAMP, which is generated by PACAP27 when it binds to PAC1R. Nevertheless, it is notable that PACAP27 mimics some of

the protective effects of db-cAMP at drastically lower (nanomolar) levels than db-cAMP (millimolar). PACAP27 has a relative low molecular weight, is lipophilic, and crosses the blood-brain barrier reaching the brain by a nonsaturable mechanism after systemic injection (Dogrukol-Ak et al., 2004). Stimulation of PAC1R by PACAP enhances α-secretase activity (Kojro et al., 2006) and improves memory in rats (Sacchetti et al., 2001). PACAP27 could be an effective protective agent against other stressors besides PGJ2. Its overall properties make PACAP27 a very interesting agent that could be useful in the treatment of AD [reviewed in (Reglodi et al., 2011)]. Because its action depends on the production of intracellular hydrolyzable cAMP, it is conceivable that instead of one dose of PACAP27, a repetitive dosing regimen may be required to improve its efficacy against neurodegeneration in AD.

Elevating intracellular cAMP in neurons is considered to be mostly protective [reviewed in (Silveira and Linden, 2006)] although there are some reports suggesting that, for example, the $A\beta_{31-35}$ peptide toxicity in cortical neurons is mediated by activation of the cAMP/PKA pathway (Zhao et al., 2008). In our studies, elevating cAMP via db-cAMP or PACAP27 was neuroprotective against PGJ2 toxicity in short, up to 8h treatments, but not upon longer 24h incubations with the prostanoid. We demonstrate that neuroprotection by increasing

cAMP levels is mediated by PKA activation and downstream phosphorylation of CREB, the cyclic AMP response element-binding transcription factor. CREB is implicated in AD and is being considered as a target for novel therapeutic strategies to ameliorate cognitive decline in aging and cognitive disorders [reviewed in (Saura and Valero, 2011)].

The raise in intracellular cAMP can affect many cellular pathways, but we were particularly interested on its effects on the ubiquitin/proteasome pathway as our studies focused on the accumulation/aggregation of ubiquitinated proteins, which occurs in AD. We demonstrate that db-cAMP *per se* enhances the activity and levels of 26S proteasomes in cortical neuronal cultures. As far as we know, this is the first time that db-cAMP is shown to raise proteasomal activity in primary neurons, but others established a similar phenomenon in myocardium upon PKA stimulation (Drews et al., 2010;Asai et al., 2009). In these latter studies, PKA activation enhanced the levels and/or phosphorylation of particular proteasomal subunits. The transcription factor(s) mediating the PKA-dependent up-regulation of proteasome subunits in mammalian cells are currently unidentified. A recent study suggested that CREB could regulate the expression of some of the components of the UPP (Seo and Chung, 2008). Accordingly, transcriptional activation of CREB increased the rate of degradation of the protein

regulator of calcinurin 1(RCNA1), mostly likely by raising the levels of unidentified components of the UPP as proteasome inhibitors blocked this increase. RCNA1 is a protein that is highly expressed in the brains of Down Syndrome and Alzheimer's disease patients (Fuentes et al., 1995). It is tempting to speculate that CREB may function to directly or indirectly regulate proteasome subunit levels. In addition to transcription regulation, both *in vitro* and *in vivo* studies demonstrated that PKA controls proteasome activity through the phosphorylation of proteasome regulatory and core subunits (Zhang et al., 2007;Tsukamoto et al., 2010;Lu et al., 2008). A search for a more effective strategy to increase proteasome activity in the cortical neurons is necessary, since in our studies the inhibitory effect of PGJ2 on the proteasome prevailed over stimulation by db-cAMP.

Pre-treatment with db-cAMP or PACAP27 failed to prevent the accumulation of ubiquitinated proteins induced by short (up to 8h) and long (24h) incubations with PGJ2. One of the factors contributing to this failure could be that the elevation of cAMP did not fully counteract the negative effect of PGJ2 on the proteasome. We postulate that even low levels of ubiquitinated proteins that begin to accumulate rapidly due to mild proteasome inhibition associated with the initial exposure to PGJ2, could exacerbate proteasome dysfunction. Several studies demonstrate

that increasing the levels of polyubiquitin chains (Piotrowski et al., 1997) and/or aggregation prone proteins, such as α-synuclein (Snyder et al., 2003) or GFP fused to truncated huntingtin with 150 glutamines (Bennett et al., 2007), decreases proteasome activity. A similar phenomenon was observed in *Drosophila*, in which expression of mutant androgen receptor with expanded polyglutamine repeats markedly inhibited the proteasome (Pandey et al., 2007). When these proteins accumulate in the cell, they can bind to proteasomes and block access of other substrates to the proteasomal degradation machinery, thus further aggravating the proteotoxic situation. Our data clearly demonstrate that the accumulation of ubiquitinated proteins is an early event that cannot be prevented by pre-treating cells with db-cAMP or PACAP27. Initially, the low levels of ubiquitinated proteins that escape degradation seem to be quite stable; these proteins can act in an additive or synergistic manner with PGJ2 to further exacerbate proteasome malfunction. With time, proteasome activity continues to deteriorate to a point that the cells can no longer cope with the proteotoxic stress and reach a point of no return. This feed-forward incremental accumulation of ubiquitinated proteins could be a major factor in neurodegeneration. Autophagy seems to clear the ubiquitin-protein aggregates [reviewed in (Yao, 2010)], but it may not be able to clear diffuse ubiquitinated proteins. Our

most recent studies indicate that autophagy does not play a large role in the breakdown of soluble ubiquitinated proteins (Myeku and Figueiredo-Pereira, 2011). It remains to be tested if activation of autophagy at early stages of the neurotoxic cascade induced by PGJ2 or other stressors prevents the accumulation/aggregation of ubiquitinated proteins and averts neurodegeneration. Perhaps a more effective strategy would be to devise ways to more efficiently elevate proteasome activity to prevent the accumulation of soluble ubiquitinated proteins early in the neurodegenerative process, to avoid protein aggregation and prevent cells from reaching a point of no return.

We demonstrated that PGJ2-treatment induces caspase activation and caspase-mediated cleavage of Tau at Asp421 (ΔTau), and that these events are detected later than the accumulation of ubiquitinated proteins in rat cerebral cortical cultures (Arnaud et al., 2009). Earlier studies by other groups showed that caspase-mediated cleavage of Tau is an early event in AD tangle pathology (Rissman et al., 2004;Guillozet-Bongaarts et al., 2005). Caspase-mediated cleavage of Tau was observed in rat cortical (Gamblin et al., 2003) and hippocampal (Park et al., 2007) neurons, and hippocampal organotypic slice cultures (Chong et al., 2006) treated with Aβ42. Tau was also shown to be cleaved by multiple caspases *in vitro* (Gamblin et al., 2003). All of these data strongly support that formation of ΔTau is

critical to Tau pathology in AD. We now show that agents that elevate cAMP, such as db-cAMP and PACAP27, prevent activation of caspases 8 and 3, formation of ΔTau, and toxicity induced by short (up to 8h) but not long (24h) incubations of cortical neurons with PGJ2. These data further support a close coordination between caspase activation and ΔTau formation. Since caspase activation and associated cleavage of Tau are preventable by increasing cAMP levels, but the accumulation of ubiquitinated proteins is not, we postulate that proteasome impairment and the ensuing ubiquitin-protein accumulation are among the main events that hinder cAMP neuroprotection against PGJ2.

In conclusion, our studies support the notion that enhancing proteasome activity to effectively reduce aberrant protein levels by exploring the PKA pathway could offer a very effective therapeutic strategy for AD and other chronic neurodegenerative disorders associated with proteinaceous aggregates and inflammation.

CHAPTER III

"PROTECTIVE ROLE OF CHAPERONES AGAINST PROTEIN AGGREGATION"

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3.1 ABSTRACT

Molecular chaperones are implicated in neurodegenerative disorders such as AD (Dou et al., 2003). These diseases are characterized by protein misfolding and subsequent aggregation. In some AD models overexpression of molecular chaperones (Hsp) such as Hsp70, reduce phenotypes associated with Aβ aggregation (Evans et al., 2006). Nevertheless, the chaperone activity of Hsp70 relies on the presence of physiological ATP levels and the assistance of co-chaperones (Mayer and Bukau, 2005). Under certain conditions of cellular stress, ATP levels decrease markedly. In consequence, most chaperones are no longer active and cannot refold misfolded proteins. We thus investigated if there are any Hsps that are functional under low ATP levels.

Hsp105, a subgroup of the Hsp70 family is constitutively expressed with high levels in the brain (Satoh et al., 1998). Previous studies by others (Yamagishi et al., 2003) showed that Hsp105 suppresses the aggregation of heat-denatured proteins in the presence of ADP. Studies carried-out with a specific Hsp90 inhibitor, which is an improved derivate of geldanamycin, 17-DMAG, that induces the expression of Hsp40-Hsp70-Hsp105 showed that at nanomolar concentrations it inhibits the formation of mutant huntingtin aggregates (Herbst and Wanker, 2007). These data suggest that heat shock proteins play an important role in

preventing the aggregation of proteins that are aggregation prone.

We postulate that during inflammation there is a decrease in ATP levels that will impair the ability of Hsp to prevent protein misfolding. Enhancing the expression of chaperones that are active under low ATP levels, such as Hsp105, or enhancing the heat shock protein response with Hsp90 inhibitors, should reduce the aggregation of aberrant proteins under pro-inflammatory conditions and thus promote cell survival. The results from our preliminary studies support this premise.

3.2. INTRODUCTION

Chaperones are essential for cellular homeostasis since they help in the folding and assembly of a nascent polypeptide, the transport of proteins across membranes and the selection of misfolded proteins for degradation (Cyr, 2008). Molecular chaperones have been implicated in neurodegenerative diseases, such as AD, characterized by the accumulation of protein aggregates. Previous results showed that under neurodegenerative conditions in the hippocampus, there is an induced overexpression of the heat shock protein 70 (Hsp70) as a protective reaction (Ayala and Tapia, 2008). Hsp105 is a subgroup of the Hsp70 family. Hsp105 is constitutively expressed and exhibits high levels of expression in the brain (Nakamura et

al., 2008). An important difference between Hsp105 and Hsp70 is that Hsp105 has a higher affinity for denatured polypeptides and does not bind to ATP *in vitro* (Easton et al., 2000).

The functions of Hsp105 are similar to those of other members of the Hsp70 family. So what is the distinctive role of these proteins? It is thought that Hsp105 cooperates with other chaperones and co-chaperones. Hsp105 exists as a complex with Hsp70 and Hsc70 (constitutive form of Hsp70) and suppresses the chaperone activity of Hsc70, probably acting as a negative regulator of Hsp70 (Saito et al., 2009). Hsp105 functions as a substitute for Hsp70 to avoid aggregation of denatured proteins in cells undergoing severe stress that causes ATP depletion (Yamagishi et al., 2003).

Enhancing chaperone activity is a potential therapeutic strategy for preventing/treating neurodegenerative diseases characterized by protein aggregates such as AD. The endoplasmic reticulum (ER) is an organelle that provides the perfect environment for protein folding and neurological stress and diseases can perturb its normal activity. When the ER stress is profound, the unfolded protein response (UPR) is triggered by the accumulation of misfolded proteins, causing cells to undergo controlled cell death by apoptosis through caspase-3 (Meares et al., 2008). Hsp105 plays an important role in the ER stress response since when Hsp105 is knocked down in COS-7 cells, cells

died independently from caspase-3, suggesting that Hsp105 is necessary for apoptotic signaling following the UPR activation (Meares et al., 2008)

Hsp90 is highly abundant in most tissues and is a key component of a multichaperone complex composed by Hsp90, Hsp70 and Hsp40 and other co-factors (Zhao and Houry, 2005). It serves as a chaperone in the folding and quality control for several client proteins that are involved in human diseases such as cancer, neurodegenerative diseases and viral infections (Solit and Chiosis, 2008). When Hsp90 is faced with a damaged protein it can send it down two pathways: a) refolding the protein or b) targeting it for degradation by the proteasome. In cancer it has been shown that there is an increase in the levels of Hsp90 and it selectively stabilize mutated or aberrantly modified oncoproteins (Trepel et al., 2010).

Hsp90 inhibitors derived from the antibiotic geldanamycin are under clinical trials for cancer therapy (Waza et al., 2006). Since Hsp90 plays an important role in maintaining the functional stability of cells under stress signals, it has been explored as a target in neurodegenerative disease, especially in Tauopathies, such as AD and frontotemporal dementia (FTD). These diseases are characterized by abnormal accumulation and aggregation of Tau (Luo et al., 2007).

Dickey et al(Dickey et al., 2007), proposed an important role for the Hsp90 complex in the pathogenesis of Tauopathies. They showed that EC102, a purine-scaffold Hsp90 inhibitor, selectively reduced phospho-Tau species, redirecting aberrant Tau towards the proteasome in a mouse model of Tauopathy. To explore the protection by EC102 against PGJ2 toxicity, we treated rat E18 cortical neurons with EC102 prior to PGJ2 treatment. Our preliminary results show that 1μM of EC102 increases cell viability when neurons are exposed to EC102 and PGJ2. In consequence, EC102 could be an alternative therapeutic approach to reduce the levels of aberrant Tau.

Overall, Hsps may have a unique role in the brain as neuroprotective agents under pro-inflammatory conditions associated with an increase in the levels of ubiquitinated proteins.

3.3. MATERIALS AND METHODS

3.3.1. Materials: Prostaglandin J2 was from Cayman Chemical (Ann Arbor, MI, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was from Sigma-Aldrich (St. Louis, MO). EC102 was courtesy of BiogenIdec (San Diego, CA). Antibodies: Hsp105 (rabbit polyclonal) 1:500 from Santa Cruz (Santa Cruz, CA), V5 (mouse monoclonal) 1:5000 from Invitrogen (Carlsbad, CA), actin (mouse monoclonal) 1:10000 from Sigma (St.

Louis, MO). The respective secondary antibodies with HRP conjugate (1:10,000) were from Bio-Rad Laboratories (Hercules, CA).

3.3.2 Cell cultures: SK-N-SH cells are a human neuroblastoma cell line derived from peripheral tissue (Biedler et al., 1978). The cells were maintained at 37°C in MEM with Earle's salts containing 5% normal fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.4% MEM vitamins, 0.4% MEM nonessential amino acids, and 100 units/ml penicillin, 100 µg/ml streptomycin in 5% CO_2. Rat E18 cortical neuronal cultures were obtained from rat E18 embryos. The cells were maintained at 37°C in 5% CO_2 in Neurobasal media (Invitrogen) and half of the medium was changed every 3 days.

3.3.3 Cell transfection: Lipofectamine 2000 (Invitrogen, CA) was used to perform all cell transfections following manufacturer's recommendations.

3.3.4 Cell viability: Cell survival was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described in (Mosmann, 1983). In short, cells were grown in 24-well plates and treated with different PGJ2 concentration for different times. At the end of the incubation period, cells were subjected to the MTT assay. Water soluble yellow MTT in media is metabolized by the metabolically active cells to the water insoluble purple formazan. After one hour

incubation with MTT, the media was removed and the formazan dissolved in 0.04 N HCl in isopropanol. The resulting product was quantified by spectrophotometry using a plate reader at OD 550 nm.

3.3.5. Western Blotting: Western blot analysis was carried out by SDS-PAGE on 10% polyacrylamide gels. After treatment cells were rinsed twice with PBS and harvested by gently scraping into SDS buffer [0.01% SDS, 0.09 M Tris-EDTA pH 7.5]. Samples were boiled for 5 minutes before loading onto gels. Following electrophoresis, proteins were transferred to an Immobilon-P membrane (Millipore, Bedford, MA). Membranes were probed with the respective antibodies and antigens were visualized by a standard 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 (1:20000) 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). Relative intensity (no units) is the ratio between the value for each protein and the value for actin.

3.3.6 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 (CA).

3.4. RESULTS

3.4.1 Hsp105 overexpression mitigates PGJ2-cytotoxicity in SK-N-SH cells - Upon PGJ2-treatment we observed an increase in Hsp105 levels (Fig. 12). These results indicate that the cells induce the heat shock response including up-regulation of Hsp105, to overcome the toxic effects of PGJ2-treatment. It is possible that although Hsp105 is up regulated it could be entrapped in protein aggregates and therefore its function could be impeded. To further increase the intracellular levels of Hsp105 we attempted to overexpress it by transfecting the pLentiI/V5Hsp105 plasmid into SK-N-SH cells. Exogenously expressed Hsp105 fused to a V5 tag was detected with an anti-V5 antibody (mouse monoclonal, 1:5000, Invitrogen, CA). Upon transfection and treatment with PGJ2, we investigated if this strategy to raise Hsp105 levels would overcome the PGJ2-cytotoxicity. We measured cell viability upon treatment with different PGJ2 concentrations for 24h (Fig. 12)

According to the results shown in Fig. 12 *(middle panel)*, we chose to transfect the cells with 4μg of pDNA for 16h prior to treatment with increasing concentrations of PGJ2. Cells were incubated with PGJ2 for 24h and cell viability with the MTT assay was assessed (Fig. 12, *bottom panel*). These preliminary

results clearly establish that overexpression of Hsp105 renders the cells less susceptible to the cytotoxic effects of PGJ2. In future studies we plan to further explore the beneficial effect of Hsp105 overexpression.

3.4.2 EC102 pre-treatment mitigates PGJ2-cytotoxicity in rat E18 cortical neurons - Prior to 10μM PGJ2 treatment, we treated cells with 0.5, 1, 2, 5 and 10μM of EC102 for 1 hour. We observed that 0.5, 1, 2 and 5μM EC102 prevented PGJ2-induced cell toxicity. (Fig.13). These results indicate that the cells that activate the chaperone system can overcome the toxic effects of PGJ2-treatment, possibly by increasing clearance of protein aggregates. In future studies we plan to further explore the beneficial effect Hsp90 inhibitors.

3.5. DISCUSION

Regional and cellular localization of Hsp105 in the brain using northern blot, western blot, and immunohistochemical analyses demonstrated that Hsp105 is highly expressed in mouse and human brain regions with the exception of the cerebellum (Hylander et al., 2000). Recently, overexpression of Hsp105 in COS-7 cells was found to suppress cell toxicity caused by transiently expressing a polyglutamine tract-containing

truncated androgen receptor (Ishihara et al., 2003). Hsp105 is a nucleotide exchange factor for Hsp70 that acts as a chaperone under low ATP levels and in the presence of ADP. Raising Hsp105 levels through transient transfection moderately reduced cell toxicity induced by PGJ2-treatment. This effect may be explained due to our transfection protocol leads to transient transfection. To reduce transfection variability and improve its efficiency, in our future studies we plan to establish an SK-N-SH cell line stably expressing Hsp105.

Unfortunately, there is no compound that specifically increases Hsp105 levels. Geldanamycin analogs raise Hsp40, Hsp70 and Hsp105 levels. Although geldanamycin was a subject for preclinical studies, it was a poor candidate due to its *in vivo* toxicity and instability (Schulte and Neckers, 1998).17-DMAG (17-NN-dimethyl ethylene diamine-geldanamycin) a less toxic form of geldanamycin (Herbst and Wanker, 2007) induces expression of the molecular chaperones Hsp40, Hsp70, and Hsp105 in mammalian cells and inhibits the formation of mutant huntingtin aggregates (Herbst and Wanker, 2007). Nevertheless this inhibitor is quite large so it lacks the characteristics for a CNS therapeutic agent. EC102, on the other hand, is a small molecule Hsp90 inhibitor that crosses the brain blood barrier (Dickey et al., 2006). We showed that pre-treatment of rat E18 cortical neurons with EC-102 increased cell survival. For our future studies we

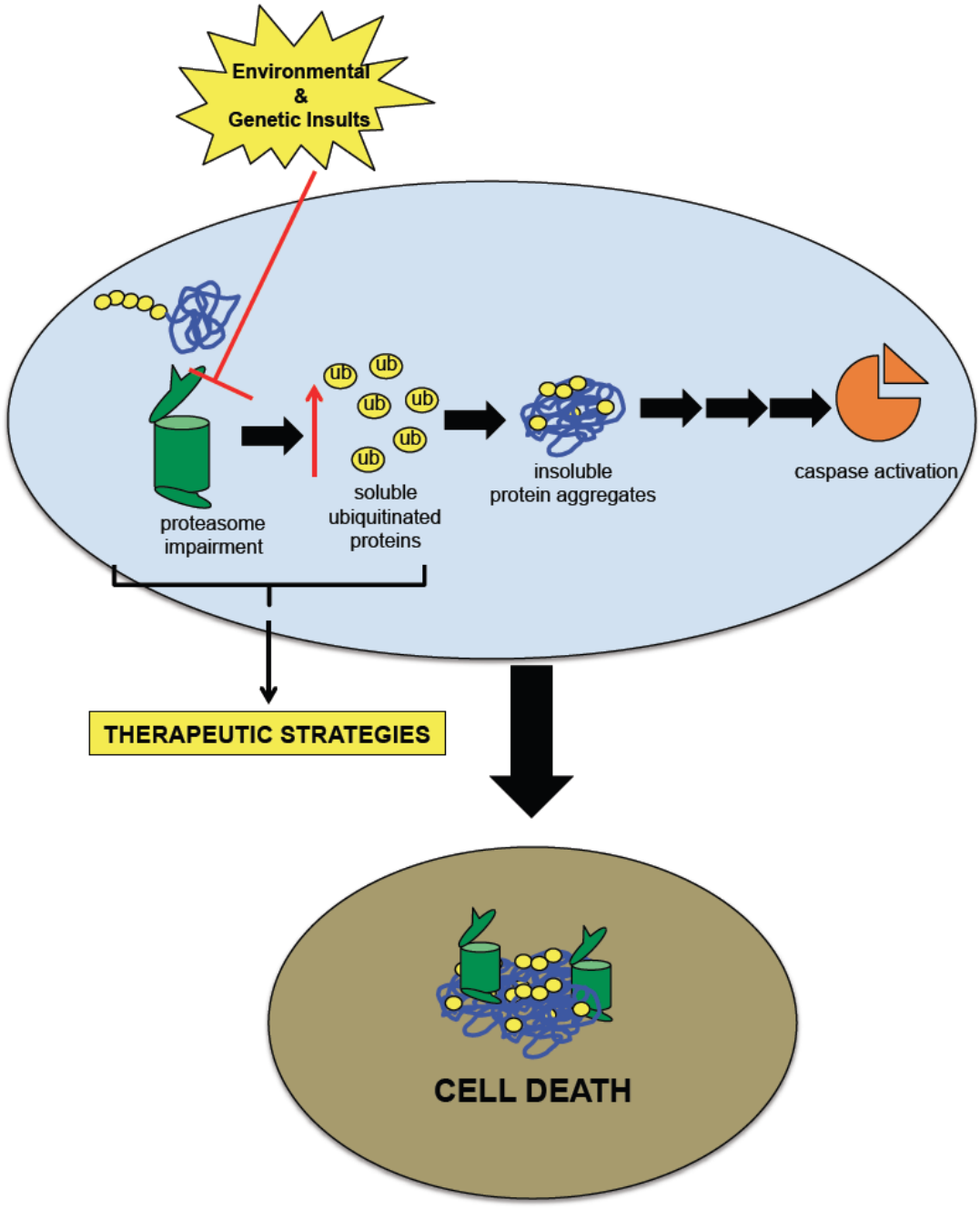
will determine if this concentration reduces de levels of ΔTau as well.

Using agents that can activate the chaperone system could potentially prevent protein aggregation under stress conditions, and thus be of therapeutic value to neurodegenerative disorders such as AD. Enhancing chaperone expression may prove be an effective strategy to prevent neurons from reaching a "point of no return" to avoid AD neurodegeneration.

CHAPTER IV

MODELS AND CONCLUSIONS

MODEL 1


Environmental
&
Genetic Insults
ub
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ub
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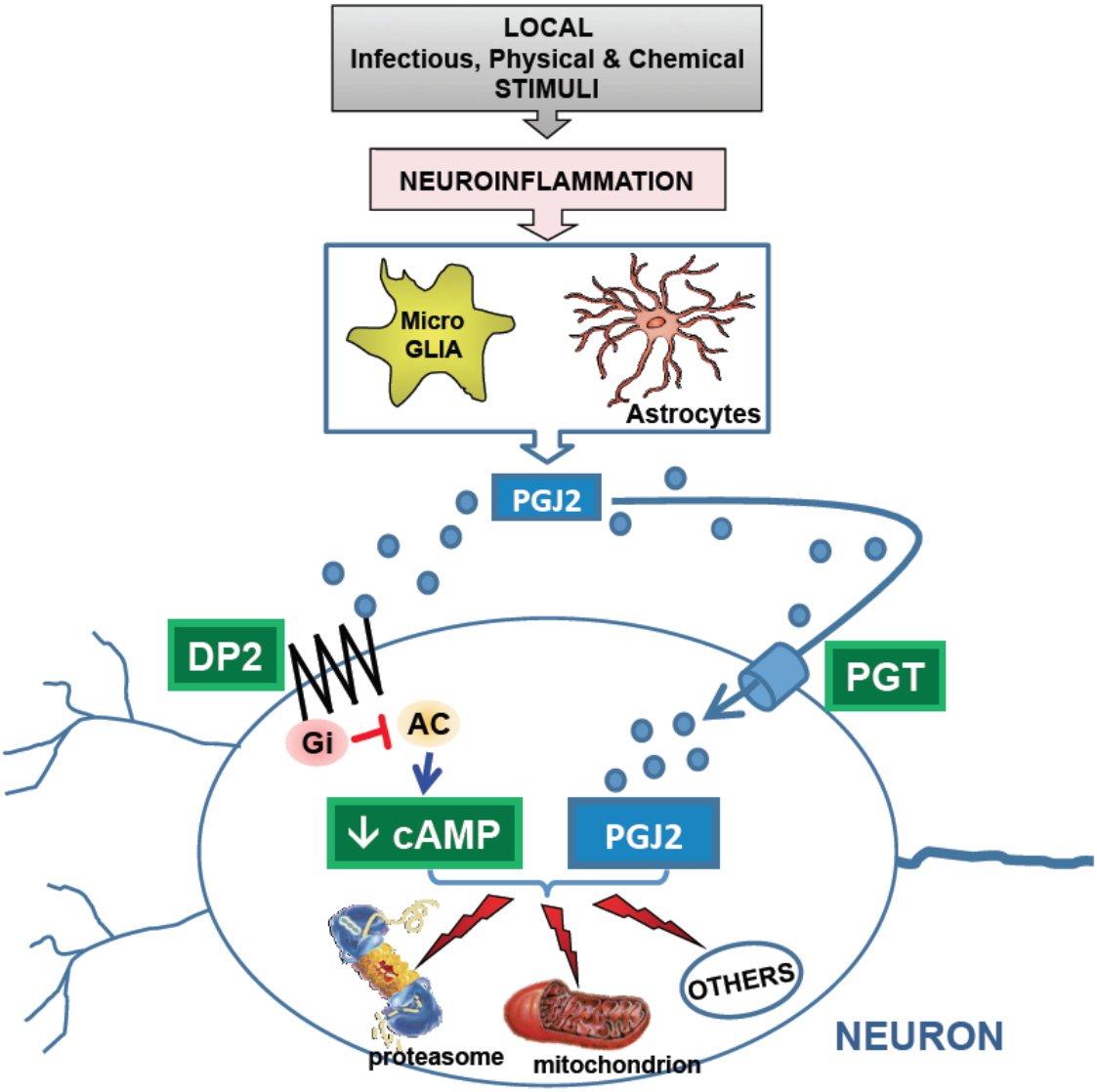
proteasome
impairment
soluble
ubiquitinated
proteins
insoluble
protein aggregates
caspase activation
THERAPEUTIC STRATEGIES
CELL DEATH

MODEL 2


LOCAL
Infectious, Physical & Chemical
STIMULI
NEUROINFLAMMATION
Micro
GLIA
Astrocytes
PGJ2
DP2
PGT
Gi
AC
↓ cAMP
PGJ2
OTHERS
proteasome
mitochondrion
NEURON

Neurodegenerative disorders are age related diseases. We propose that over an individual's life time, environmental and genetic insults down-regulate one of the major proteolytic pathways in mammalian cells, the ubiquitin-proteasome pathway, leading to gradual accumulation of misfolded and ubiquitinated proteins. In particular, AD is diagnosed at late stages of the disease when neuronal viability has already been compromised, a reason why is necessary to determine the principal components of the early stages of disease.

Sporadic AD is considered to be a multifactorial disease; events such as decreased blood circulation and oxygen in the brain, oxidative stress and inflammation have been described to compromise the survival mechanism of neurons (De, 2010). Our main hypothesis relates to this idea, where we postulate that inflammation is an important contributor to the progression of the disease rather than a consequence of the development of the pathology.

Indeed, we investigated the role of a neurotoxic product of inflammation (PGJ2) and demonstrated that accumulation of soluble ubiquitinated proteins precedes protein aggregation. One way to explain this phenomenon is that PGJ2 first induces proteasome impairment, which leads to an increase in the levels of soluble ubiquitin in the cytoplasm. These proteins are not being degraded by the proteasome, in consequence at a later time

point they form insoluble aggregates. If these aggregates cannot be degraded they will induce caspase activation (see model 1).

Caspase activation is a key step in apoptosis, and it has been reported to occur in AD (Cotman et al., 2005). Caspase activation leads to the proteolytic cleavage of several neuronal proteins. Among these is Tau, which gets cleaved by caspase 3 at the residue Asp421 in its C-terminal. Previous studies showed that PGJ2 induces caspase activation and Tau cleavage (Arnaud et al., 2009). We established the temporal relation between caspase-mediated cleavage of Tau and the accumulation and aggregation of ubiquitinated proteins. Upon PGJ2-treatment, first we observe an increase in soluble ubiquitinated proteins; nevertheless ΔTau reached its peak at 16h after PGJ2 treatment. We also validated that PGJ2 induces caspase activation through the extrinsic pathway. We show that the highest level of caspase activity coincided with the highest levels of ΔTau.

These data support our model were we propose that any stimulus that causes neuroinflammation and production of neurotoxic prostaglandins such as PGJ2, will impair the proteasome and lead to (1) the accumulation of soluble ubiquitinated proteins; (2) activation of the extrinsic apoptotic pathway; (3) Tau cleavage at Asp421; (4) protein aggregation and cell death. Overall, the accumulation of soluble

ubiquitinated proteins is one of the events that initiate a toxic cascade culminating in apoptosis (model 1).

The effects of prostaglandins of the J2 series are mediated through the DP2 receptor which is coupled to G_i proteins that inhibit adenylate cyclase causing inhibition of cAMP production [reviewed in (Pettipher et al., 2007;Hirai et al., 2001)], as well as through an active transport system [reviewed in (Harris et al., 2002)]. Once inside the cell, an additional mechanism allows transport of PGJ2 into the nucleus, where it affects gene transcription [reviewed in (Harris et al., 2002)].

In our studies described here, we demonstrate that PGJ2 down-regulates the basal cAMP levels in rat cerebral cortical neuronal cultures. Based on PGJ2 receptor-mediated action we reasoned that raising cAMP levels could prevent some of its toxic effects (Model 2). We used dibutyryl-cAMP (db-cAMP), which is cell permeable and less susceptible to hydrolysis by phosphodiesterases (Miller et al., 1973) and PACAP27, a potent neurotrophic and neuroprotective peptide shown to be protective in models of ischemic and traumatic brain injuries and neurodegenerative diseases (Reglodi et al., 2011). The reduction of intracellular cAMP levels by PGJ2-treatment in cortical neuronal cultures was diminished by pre-treatment with db-cAMP or PACAP27. This effect on intracellular cAMP levels raised the question whether increasing cAMP will potentially avoid or

reduce ΔTau levels that we observe in PGJ2-treated neurons. Pre-treatment of cortical neurons with db-cAMP or PACAP 27 prevented Tau cleavage at Asp421 at early time points (4h and 8h); however at later time points these agents were unsuccessful in diminishing PGJ2-induced Tau cleavage. Since in the case of PGJ2 the formation of ΔTau is carried out by activation of the extrinsic apoptotic pathway, we explored if pre-treatments with db-cAMP or PACAP27 reduce caspase 8 and caspase 3 activation. In our studies we show that increasing cAMP levels with db-cAMP or PACAP27 prevents the conversion of pro-caspase 8 and 3 into their cleaved forms, and abolishes the activation of these caspases induced by PGJ2. Nevertheless this protective effect was only observed at early time points (4h and 8h) that coincide with the formation of the ΔTau fragment.

It is interesting to mention that neither db-cAMP nor PACAP27 pre-treatments abolished the accumulation/aggregation of ubiquitinated proteins induced by PGJ2 at early time points, when we observed a reduction in the formation of ΔTau. These results support our model: when there is proteasome impairment, it leads to accumulation/aggregation of ubiquitinated proteins, which are very hard to get rid of by the cell. Failure to promptly remove ubiquitinated aggregates could trigger

apoptosis, leading the neurons to a point of no return and eventually cell death.

Our studies are based on the finding that Tau cleavage, through caspases, will generate the ΔTau fragment. This fragment was previously shown to be an aggregate-prone form of Tau *in vitro* (Gamblin et al., 2003). We show, in a cell culture model, that PGJ2 induces caspase activation and the formation of the aggregate-prone form of Tau. Following this event, truncated Tau will recruit normal Tau to form misfolded aggregates. Once aggregates are formed, they failed to be degraded probably because the major proteolytic pathways are overwhelmed by the excessive protein aggregation.

We also explored an alternative approach to avoid the formation of ΔTau, using agents that can activate the chaperone system. This alternative was investigated because one of the first defenses against protein aggregation involves the molecular chaperones system that helps in the normal folding and refolding of abnormal proteins back to the native state (McClellan et al., 2005). Our preliminary results show that either increasing the levels of Hsp105, an ATP independent chaperone, by transient transfection or using the small molecule Hsp90 inhibitor, EC102, increases cell viability when cells are exposed to PGJ2. In future studies we will explore if these

approaches also reduce the formation of ΔTau and ubiquitin aggregates.

Agents that increase the efficacy of the major proteolytic pathways, like increasing cAMP levels, could improve proteasome activity and clearance of soluble ubiquitinated proteins. Another method could be increasing the levels of molecular chaperones by using Hsp90 inhibitors. When Hsp90 is faced with a damaged protein it can either refold or target it for degradation by the proteasome. As a result, ubiquitinated protein build up is reduced accompanied by a decrease in cell toxicity.

Overall our hypothesis is that, during the lifespan of an individual, the CNS is frequently subjected to proteotoxic stimuli 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 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 V

FUTURE DIRECTIONS

Maintenance of protein homeostasis presents a critical task for post mitotic neuronal cells. Over their lifetime, these cells develop a loss of quality control and a gain of protein deposition indicating proteasome impairment. Therefore, developing therapeutic strategies that aim at up-regulating proteasome activity can be a very effective strategy to treat or delay chronic neurodegenerative diseases associated with protein aggregation.

We propose that future studies should focus on:

1) **Elucidate the mechanism by which elevating cAMP levels induce neuroprotection.**

   Our data indicate that enhancement of proteasome activity is mediated through the cAMP/PKA pathway. Nevertheless we do not know what the mechanism behind this effect is. We anticipate that elevating cAMP levels could increase the expression of some of the subunits of the proteasome. Alternatively, cAMP-dependent activation of PKA, could phosphorylate particular subunits. To elucidate these possibilities we will perform western blot and immunoprecipitation assays to assess proteasome subunit expression and/or phosphorylation.

2) **Investigate if UPP ligases, such as CHIP and Parkin, are downstream targets of cAMP/PKA to promote ubiquitination**

and proteasomal degradation.

Our data demonstrate that db-cAMP treatment of cortical neuronal cultures enhances proteasome activity. We hypothesize that other proteins that promote protein degradation by the proteasome such as ubiquitin ligases may be up-regulated under db-cAMP treatment. To test this hypothesis we will investigate the levels of CHIP (carboxyl terminus of Hsp70-interacting protein) and Parkin, which are ubiquitin ligases that induce ubiquitination and degradation of proteasomal substrates. The results will further substantiate our hypothesis that elevation of proteasome activity leads to a parallel increase in the levels/activity of proteins that promote substrate degradation by the proteasome, and act in a concerted fashion with proteasomes to degrade ubiquitinated substrates.

3) Increase the neuroprotective effect of PACAP27.

PACAP27 is a neuropeptide that signals through a Gs-protein coupled receptor, PACR1. We show that 100nM of PACAP27 increase intracellular cAMP levels, reduce the formation of ΔTau, and decrease caspase 8 and caspase 3 activation. This effect is not as potent as the one observed with 1mM db-cAMP. This could be explained by PACAP27 inducing an increase in intracellular cAMP, which is a target for phosphodiesterases that, through hydrolysis, control cAMP levels(Essayan, 2001).

We plan to bypass this shortfall of PACAP27 by changing the PACAP27 treatment to several doses, i.e. treating cells with 100nM of PACAP27 every 8 hours. We expect that maintaining intracellular cAMP levels constitutively high might increase PACAP27 protection against PGJ2, by significantly reducing caspase activation and the formation of the ΔTau fragment, thus averting protein aggregation and cell death.

4) Increase chaperone activity to reduce ΔTau levels.

Chaperones are essential for cellular homeostasis since they help in the folding and assembly of a nascent polypeptide, the transport of proteins across membranes and the selection of misfolded proteins for degradation (Cyr, 2008). In our studies we show that increasing the protein levels of Hsp105 or blocking Hsp90 can increase cell viability of cortical neurons exposed to PGJ2. We expect that under these conditions caspase activation and the levels of ΔTau are significantly less than when cells are exposed to PGJ2 alone. We will address these possibilities by performing western blot analyses for ΔTau and caspases using the TauC3 antibody as well as caspase 8 and 3 antibodies along with caspase activity assays.

Using agents that activate the chaperone system could potentially prevent protein aggregation under stress conditions

and thus be of therapeutic value to neurodegenerative disorders such as AD.

5) Target already formed aggregates by inducing autophagy.

Proteins destined for degradation are covalently tagged with an ubiquitin chain in which the terminal glycine residue of one ubiquitin is linked through an isopeptide bond to a Lys (K) residue of the substrate. If this Lys residue is in position 48 (K48) of ubiquitin, the polyubiquitin chain on the substrate targets it to degradation by the proteasome. However, polyubiquitin chains can also be formed at the K63 position of ubiquitin. Previous studies showed that substrates that carry K63 polyubiquitin chains are targeted to the autophagy-lysosomal system instead of the proteasome(Tan et al., 2008).

In our studies we showed that increasing proteasome activity by elevating cAMP levels is not sufficient to reduce the levels of ubiquitinated protein aggregates. We will explore the possible outcome of stimulating the autophagy-lysosomal system for degradation of the protein aggregates. Although autophagy is generally considered as a nonselective degradation machinery, growing evidence supports the existence of a selective mechanism that specifically targets protein aggregates to autophagy for clearance (Ross and Poirier, 2004). This pathway has the capacity to engulf protein aggregates, while the proteasome

lacks such ability due its narrow barrel that allows cleavage of single proteins but precludes the entry of aggregates.

In future studies we will induce autophagy in rat primary cortical neurons with rapamycin prior and/or post treatment with PGJ2. We expect that autophagy activation will be able to reduce the levels of ubiquitinated protein and ΔTau protein aggregates formed upon PGJ2 treatment.

6) OVERALL CONCLUSION

In our view, further characterization and optimization of the mechanisms by which the cAMP/PKA pathway and molecular chaperones affect proteasome activity, reduce caspase activation and the formation of ΔTau are critical to the development of a therapeutical approach to prevent and slow down the progression of neurodegenerative diseases that are associated with the abnormal accumulation and aggregation of ubiquitinated proteins.

It is equally important to characterize and optimize the mechanisms by which neurotoxic products of inflammation damage neuronal cells. Treatment with PGJ2, as we show in our present and previous studies, recapitulates many of the pathological processes relevant to AD neurodegeneration. Abolishing the neurotoxicity induced by inflammatory mediators such as PGJ2 is likely to be another effective therapeutic strategy to treat/prevent neurodegeneration in AD.

CHAPTER VI

FIGURES

Fig. 1 – PGJ2-treatment induces accumulation and aggregation of ubiquitinated proteins and caspase-mediated cleavage of TAU (ΔTau) in a dose- (A) and time-dependent (B) manner. Western blot analyses to detect NP40-soluble ubiquitinated proteins [Ub-conjugates (soluble)], aggregated (SDS-soluble) ubiquitinated proteins [Ub-conjugates (pellet)], Tau cleaved at Asp421 (ΔTau), full length TAU, and actin (loading control) in cell extracts of rat E18 cerebral cortical cultures (40 µg of protein/lane). The neuronal cultures were treated with increasing concentrations of PGJ2 for 24 hours **(A)** or with 20µM PGJ2 for different time points **(B)**. The blots were probed with anti-ubiquitinated (Ub) proteins antibody, the Tau C3 antibody (Tau cleaved at Asp421, epitope a.a. 412-421), Tau 5 antibody (clone 13, epitope a.a. 2-18, reacts with all Tau isoforms) and anti-actin antibody. The levels of soluble Ub-proteins (*open squares*) and ΔTau (*solid squares*) were semi-quantified by densitometry. Data represent the percentage of the pixel ratio for soluble ub-proteins or ΔTau over actin for each condition compared to control. Values are means and s.d. from at least two experiments. Molecular mass markers in kDa are shown in the middle. The *asterisk* (*) identifies the values that are significantly different (*$p<0.05$; **$p<0.01$; ***$p < 0.001$) from the control. Ub-conjugates,

ubiquitinated proteins; ΔTau, Tau cleaved at Asp421; Tau FL – full length TAU.

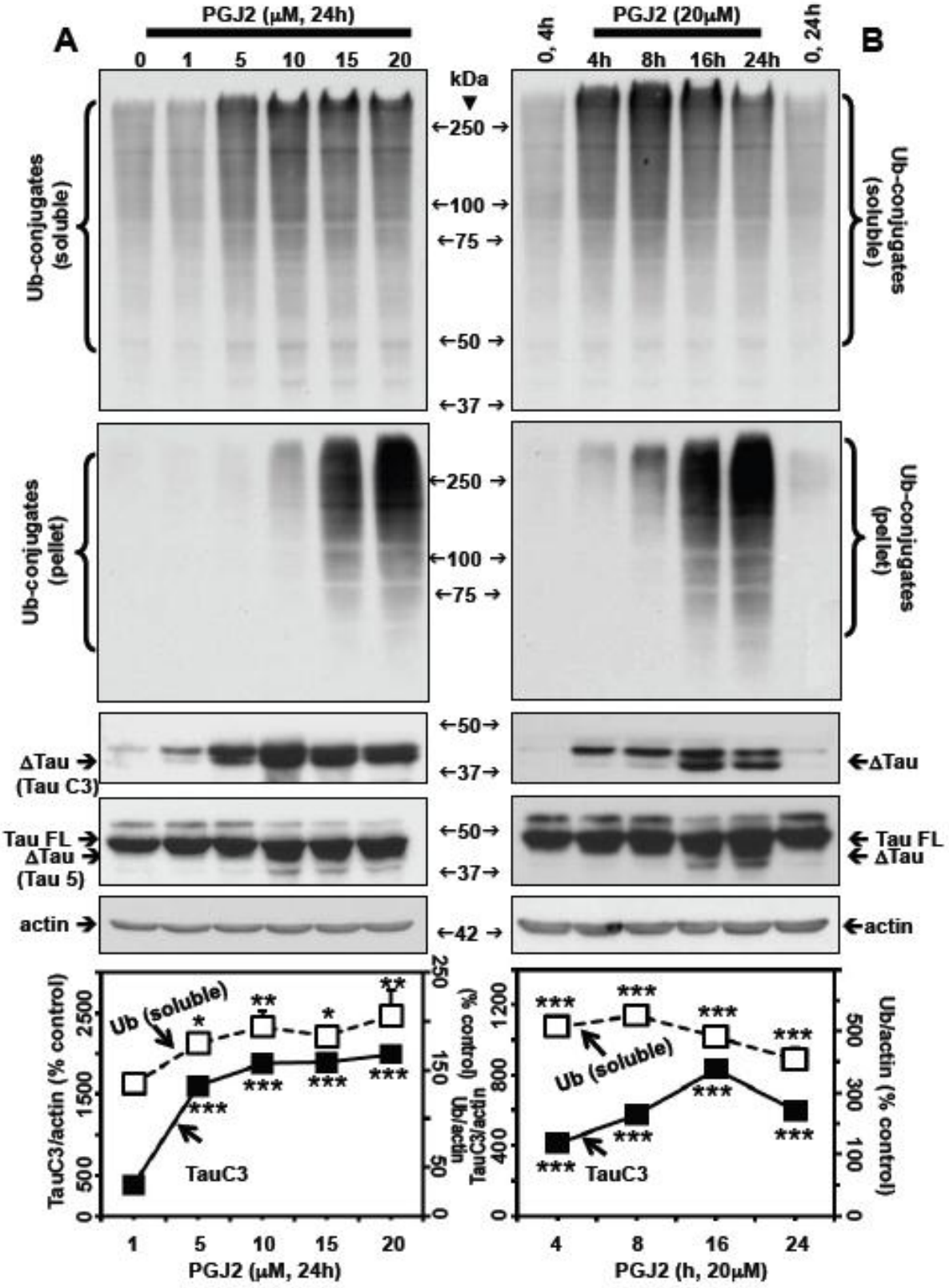

A
PGJ2 (μM, 24h)
0 1 5 10 15 20
B
PGJ2 (20μM)
0, 4h 4h 8h 16h 24h 0, 24h
kDa
Ub-conjugates (soluble)
Ub-conjugates (pellet)
←250 →
←100 →
←75 →
←50 →
←37 →
ΔTau → (Tau C3)
Tau FL → ΔTau → (Tau 5)
actin →
←42 →
←ΔTau
Tau FL ← ΔTau ←
←actin
Ub (soluble)
TauC3
TauC3/actin (% control)
Ub/actin (% control)
Ub/actin (% control)
PGJ2 (μM, 24h)
PGJ2 (h, 20μM)

Figure 1

Fig. 2 – PGJ2-treatment induces the conversion of pro-caspase 8 and pro-caspase 3 to their cleaved forms in a dose- (A) and time-dependent (B) manner; no changes in pro-caspase 9 or the autophagic marker LC3-II were observed upon these treatments. Western blot analyses to detect caspases 9, 8 and 3 in their zymogenic (Pro) and cleaved (Cl) forms, the autophagic proteins LC3-I and LC3-II, and actin (loading control) in cell extracts of rat E18 cerebral cortical cultures (40 µg of protein/lane). The *top panel* (ΔTau) is shown for comparison and was copied from figure 1. The neuronal cultures were treated with increasing concentrations of PGJ2 for 24 hours **(A)** or with 20µM PGJ2 for different time points **(B)**. The blots were probed with the respective antibodies. The levels of cleaved caspase 8 (*open squares*) and cleaved caspase 3 (*solid squares*) were semi-quantified by densitometry. Data represent the percentage of the pixel ratio for each of the cleaved caspases over actin for each condition compared to control. Values represent means and s.d. from at least two experiments. Molecular mass markers in kDa are shown in the middle. The *asterisk* (*) identifies the values that are significantly different (*$*p<0.05$; ***$p < 0.001$*) from the control. Pro, zymogenic, and Cl, cleaved forms of the caspases; ΔTau, Tau cleaved at Asp421.

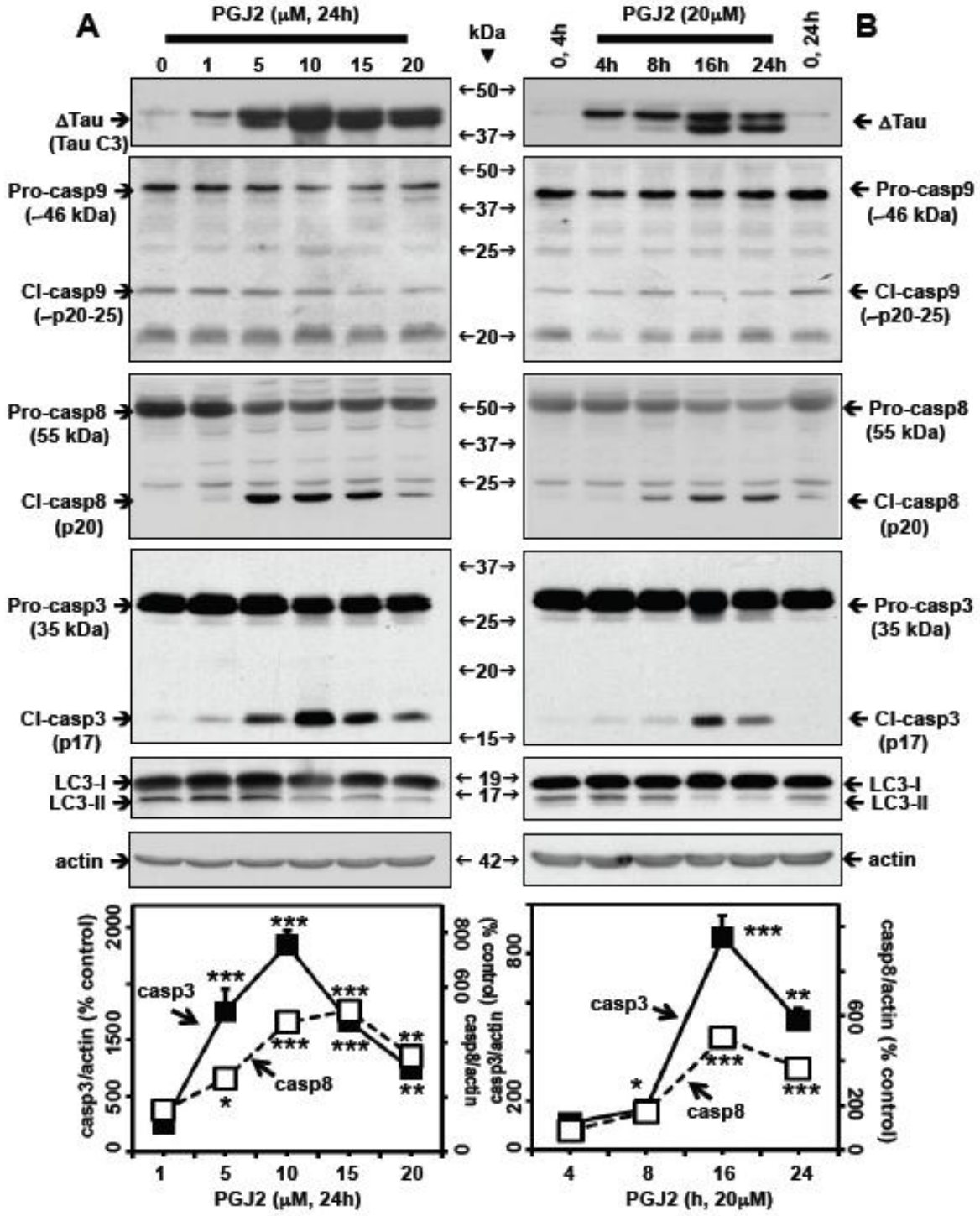

A
PGJ2 (μM, 24h)
0 1 5 10 15 20
kDa
B
PGJ2 (20μM)
0, 4h 4h 8h 16h 24h 0, 24h
ΔTau (Tau C3)
Pro-casp9 (–46 kDa)
Cl-casp9 (–p20-25)
Pro-casp8 (55 kDa)
Cl-casp8 (p20)
Pro-casp3 (35 kDa)
Cl-casp3 (p17)
LC3-I
LC3-II
actin
casp3
casp8
casp3/actin (% control)
casp8/actin (% control)
PGJ2 (μM, 24h)
PGJ2 (h, 20μM)

Figure 2

Fig. 3 - PGJ2 decreases cAMP levels in rat E18 neuronal cultures. Intracellular cAMP was measured with an EIA kit as described in "Materials and Methods". Rat E18 cerebral cortical cultures were pre-treated for 1h with water (vehicle, control), db-cAMP (1mM) or PACAP27 (100nM) prior to treatment for 4h with DMSO (vehicle, control, *white bars*) or with 10μM PGJ2 (*black bars*) alone. The levels of intracellular cAMP in the neuronal cultures are expressed as pmoles per ug of protein. Values represent means and s.e. from three experiments. The *asterisk* (*) identifies values that are significantly different in a comparison between water or each drug alone (*white bars*), with water or each drug in conjunction with PGJ2 (*black bars*); *$*p<0.05$ and $***p < 0.001$*.

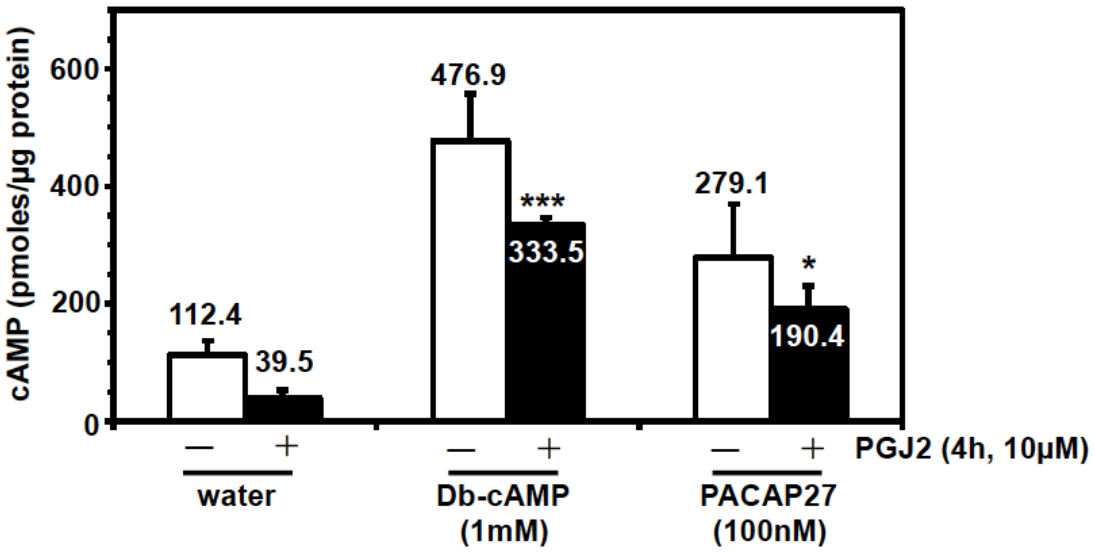

cAMP (pmoles/µg protein)
600
400
200
0
112.4
39.5
476.9
***
333.5
279.1
*
190.4
— +
— +
— +
PGJ2 (4h, 10µM)
water
Db-cAMP
(1mM)
PACAP27
(100nM)

Figure 3

Fig. 4 – Db-cAMP (1mM, *top panels*) and PACAP27 (100nM, *bottom panels*) diminish caspase-mediated cleavage of TAU (ΔTau) induced by PGJ2-treatment at early (up to 8h) but not late (16h and late) time points. Western blot analyses to detect Tau cleaved at Asp421 (ΔTau), full length TAU, and actin (loading control) in cell extracts of rat E18 cerebral cortical cultures (40 µg of protein/lane). The neuronal cultures were pre-treated for 1h with water (minus sign, vehicle, control), db-cAMP (plus sign, 1mM) or PACAP27 (plus sign, 100nM) prior to DMSO (vehicle, control) or 10µM PGJ2 for different time points (4h, 8h, 16h and 24h). The blots were probed with the Tau C3 antibody (Tau cleaved at Asp421, epitope a.a. 412-421), Tau 5 antibody (clone 13, epitope a.a. 2-18, reacts with all Tau isoforms) and anti-actin antibody. The levels of ΔTau were semi-quantified by densitometry. Data represent the percentage of the pixel ratio for ΔTau over actin for each condition compared to control. Values are means and s.e. from three experiments for db-cAMP or means and s.d. from two experiments for PACAP27. Molecular mass markers in kDa are shown on the right. The *asterisk* (*) identifies values that are significantly different in a comparison between PGJ2 alone (*white bars*) with PGJ2 in conjunction with db-cAMP or PACAP27 (*black bars*); ****p < 0.001*. ΔTau, Tau cleaved at Asp421; Tau FL – full length TAU.

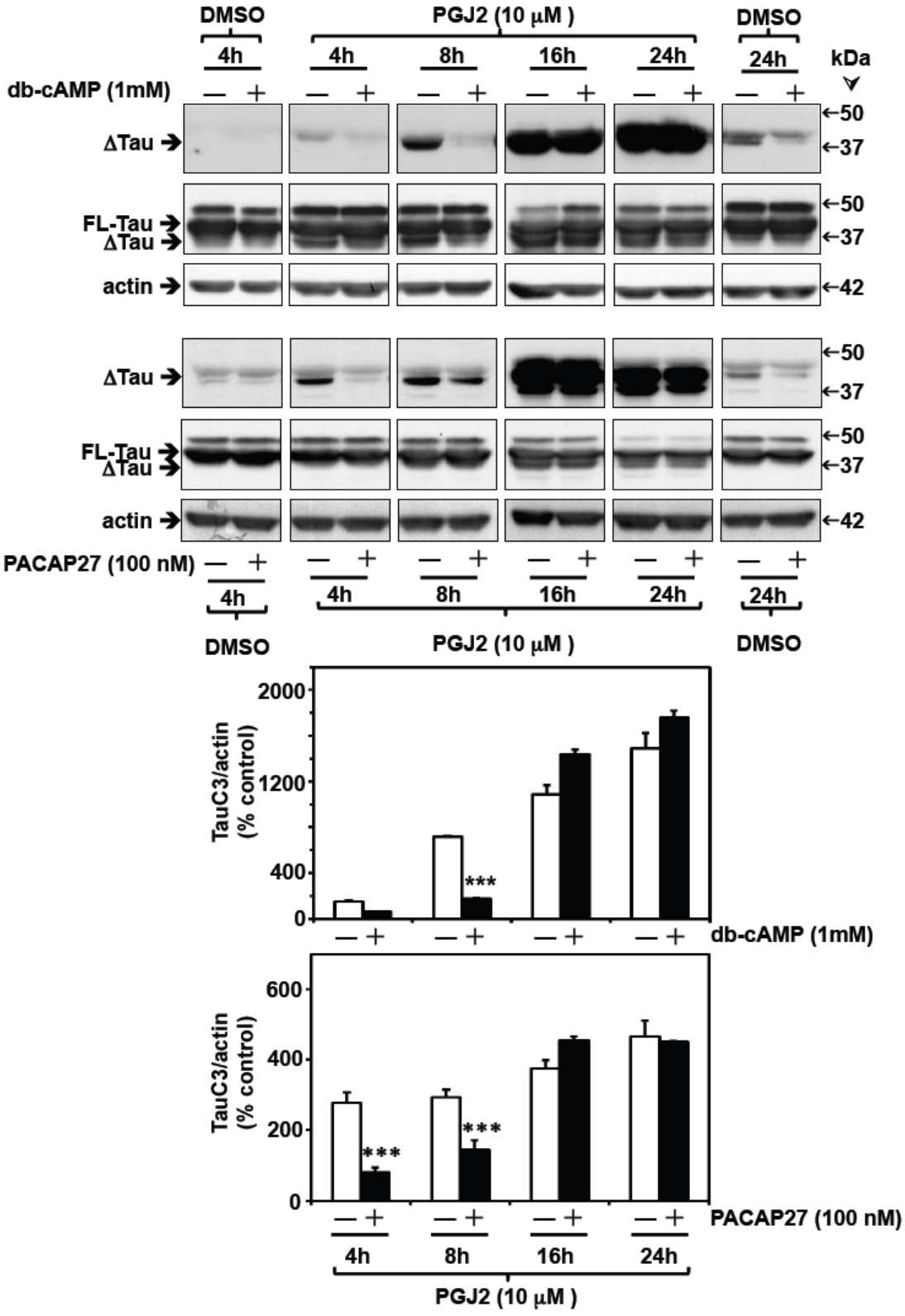

DMSO
PGJ2 (10 μM)
DMSO
4h
4h
8h
16h
24h
24h
kDa
db-cAMP (1mM)
— + — + — + — + — + — +
ΔTau
FL-Tau
ΔTau
actin
←50
←37
←42
PACAP27 (100 nM)
TauC3/actin (% control)
2000
1200
400
0
***
600
200
db-cAMP (1mM)
PACAP27 (100 nM)

Figure 4

Fig. 5 – Db-cAMP (1mM, A) and PACAP27 (100nM, B) diminish the conversion of pro-caspase 8 and pro-caspase 3 to their cleaved forms induced by PGJ2-treatment at early (up to 8h) but not late (24h) time points. Western blot analyses to detect caspases 8 and 3 in their zymogenic (Pro) and cleaved (Cl) forms, and actin (loading control) in cell extracts of rat E18 cerebral cortical cultures (40 µg of protein/lane). The *top panel* (ΔTau) is shown for comparison and was copied from figure 4. The neuronal cultures were pre-treated for 1h with water (minus sign, vehicle, control), db-cAMP (A, plus sign, 1mM) or PACAP27 (B, plus sign, 100nM) prior to DMSO (vehicle, control) or 10µM PGJ2 for different time points (4h, 8h, 16h and 24h). The blots were probed with the respective antibodies. The levels of cleaved caspase 8 (*top graph*) and cleaved caspase 3 (*bottom graph*) were semi-quantified by densitometry. Data represent the percentage of the pixel ratio for each of the cleaved caspases over actin for each condition compared to control. Values are means and s.e. from three experiments for db-cAMP (A) or means and s.d. from two experiments for PACAP27 (B). Molecular mass markers in kDa are shown on the right. The *asterisk* (*) identifies values that are significantly different in a comparison between PGJ2 alone (*white bars*) with PGJ2 in conjunction with db-cAMP or PACAP27 (*black bars*); ***p<0.01; ***p < 0.001*. Pro, zymogenic,

and Cl, cleaved forms of the caspases; ΔTau, Tau cleaved at Asp421.

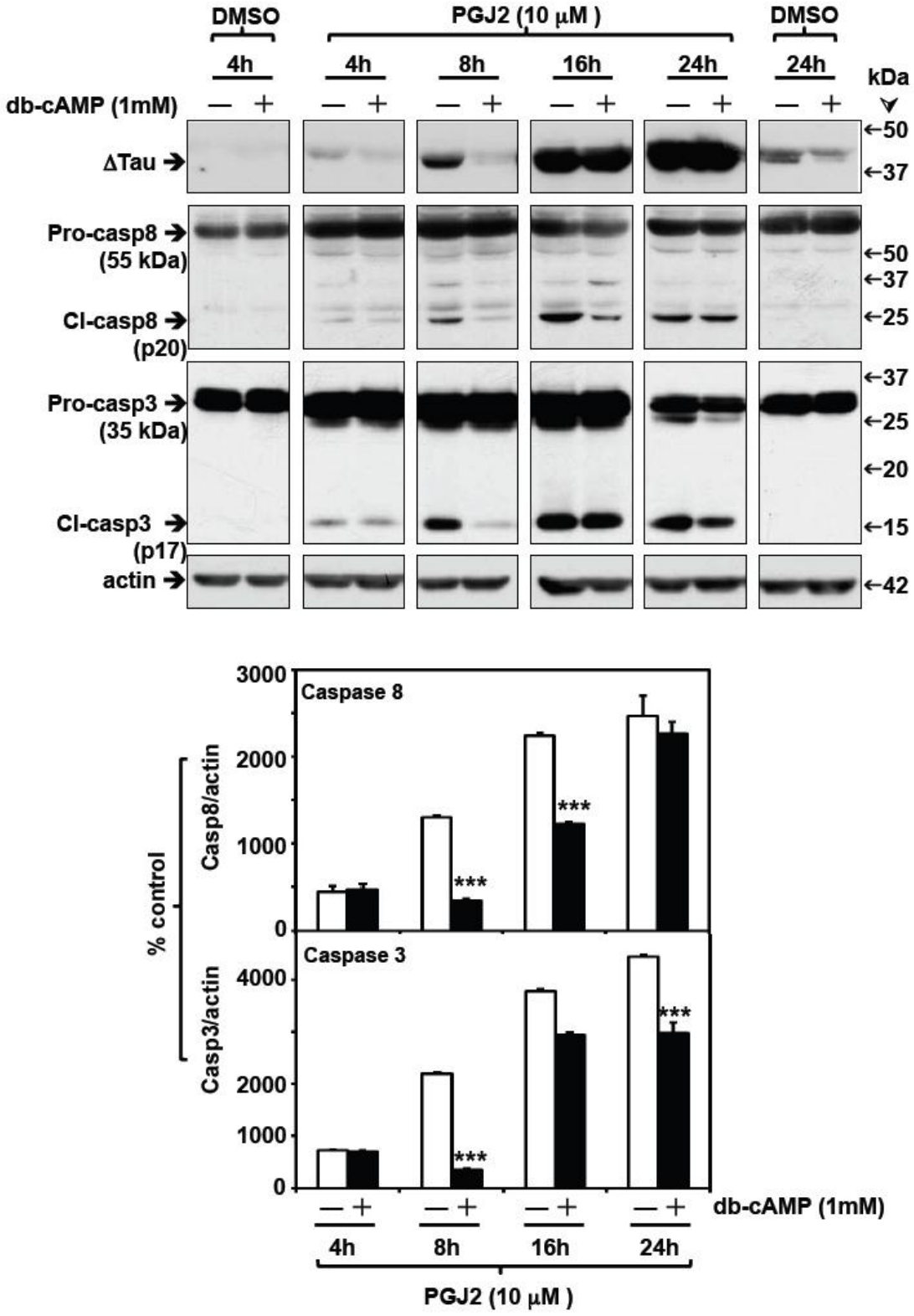

DMSO
PGJ2 (10 μM)
DMSO
4h
4h
8h
16h
24h
24h
kDa
db-cAMP (1mM)
— + — + — + — + — + — +
ΔTau
Pro-casp8
(55 kDa)
Cl-casp8
(p20)
Pro-casp3
(35 kDa)
Cl-casp3
(p17)
actin
←50
←37
←50
←37
←25
←37
←25
←20
←15
←42
Caspase 8
Caspase 3
Casp8/actin
Casp3/actin
% control
3000
2000
1000
0
4000
***
db-cAMP (1mM)
4h
8h
16h
24h
PGJ2 (10 μM)

Figure 5A

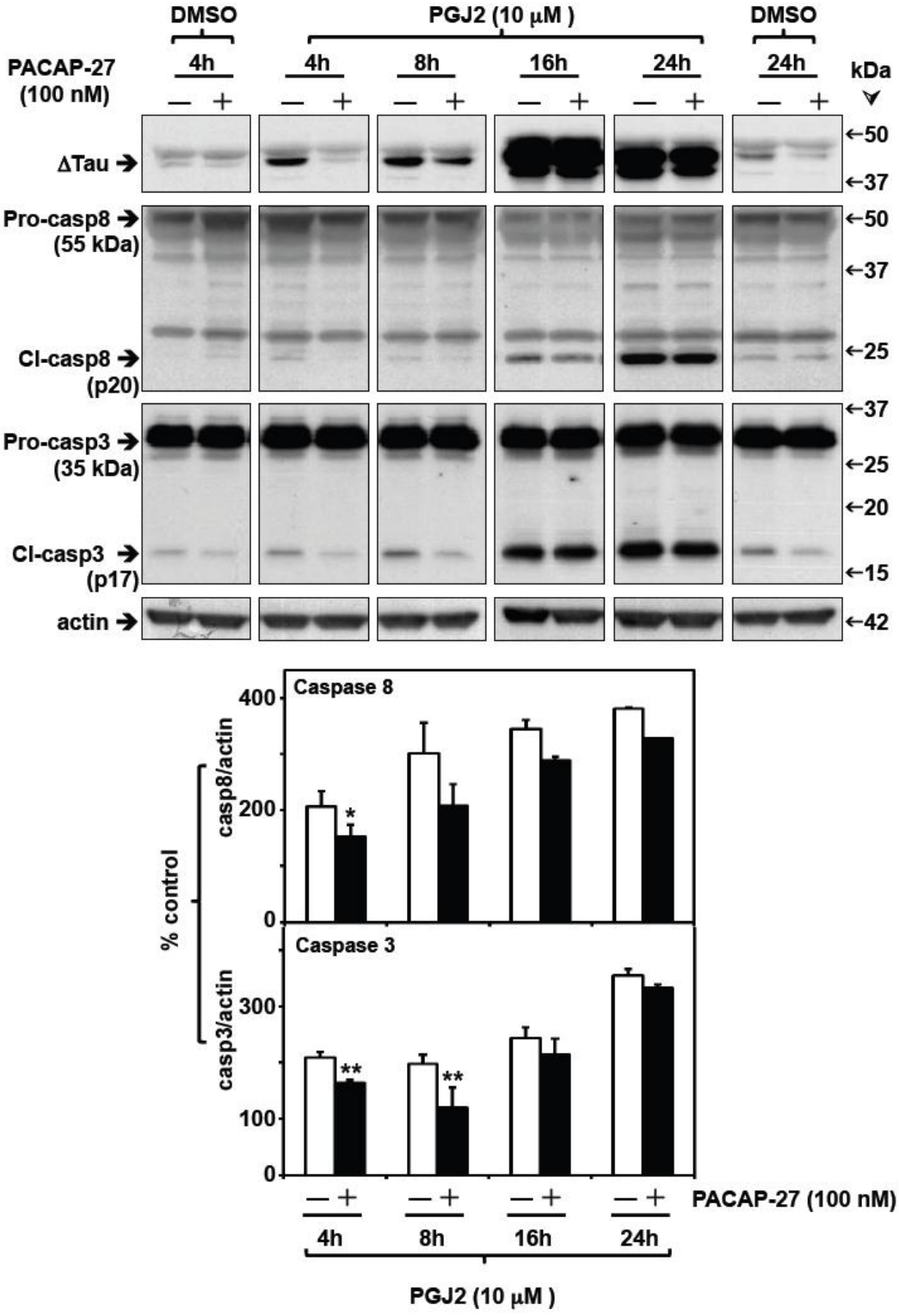

DMSO
PGJ2 (10 μM)
DMSO
PACAP-27
(100 nM)
4h
4h
8h
16h
24h
24h
kDa
— +
ΔTau
Pro-casp8
(55 kDa)
Cl-casp8
(p20)
Pro-casp3
(35 kDa)
Cl-casp3
(p17)
actin
←50
←37
←25
←20
←15
←42
Caspase 8
Caspase 3
% control
casp8/actin
casp3/actin
400
200
0
300
100
*
**
PACAP-27 (100 nM)
PGJ2 (10 μM)

Figure 5B

Fig. 6 - Db-cAMP (1mM) and PACAP27 (100nM) diminish the activation of caspase 3 and 8 induced by PGJ2; caspase 9 activity is not affected by PGJ2. The activities of caspases 3, 8 and 9 were determined in extracts of rat E18 cerebral cortical cultures (150µg of protein/assay) with a colorimetric assay kit as described in "Material and Methods". The neuronal cultures were pre-treated for 1h with water (vehicle, control), db-cAMP (1mM) or PACAP27 (100nM) prior to treatment for 8h with DMSO (vehicle, control, *white bars*) or with 10µM PGJ2 (*black bars*) alone. Caspase activities expressed as OD at 400nM and normalized for protein (150µg/assay) are shown, and represent means and s.e. from four determinations. The *asterisk* (*) identifies values that are significantly different in a comparison between water or each drug alone (*white bars*) with water or each drug in conjunction with PGJ2 (*black bars); **p<0.01 and ***p < 0.001*.

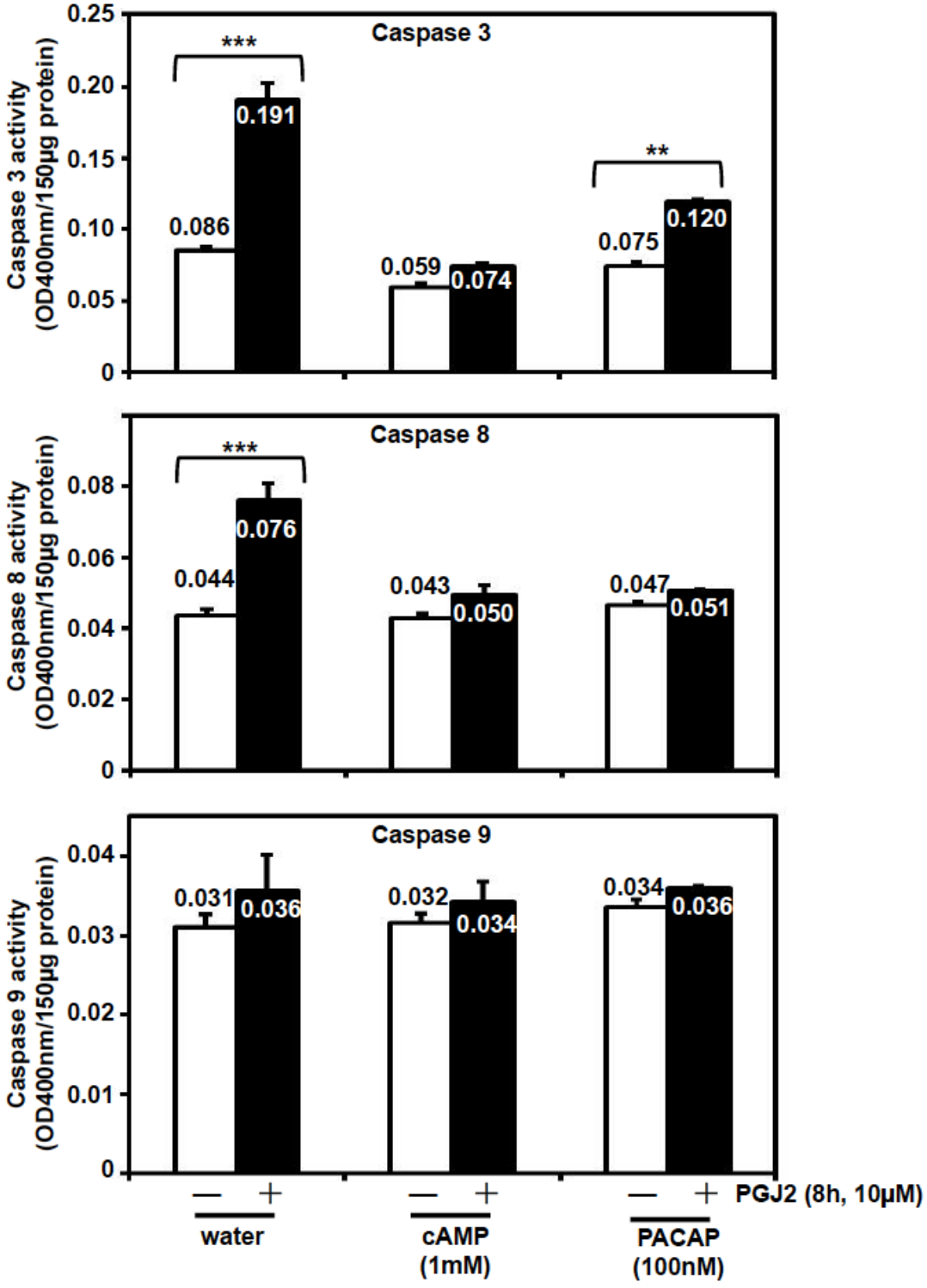

Caspase 3
Caspase 3 activity
(OD400nm/150μg protein)
0.25
0.20
0.15
0.10
0.05
0
***
0.086
0.191
0.059
0.074
**
0.075
0.120
Caspase 8
Caspase 8 activity
(OD400nm/150μg protein)
0.08
0.06
0.04
0.02
0
***
0.044
0.076
0.043
0.050
0.047
0.051
Caspase 9
Caspase 9 activity
(OD400nm/150μg protein)
0.04
0.03
0.02
0.01
0
0.031
0.036
0.032
0.034
0.034
0.036
— +
PGJ2 (8h, 10μM)
water
cAMP
(1mM)
PACAP
(100nM)

Figure 6

Fig. 7 – Db-cAMP (1mM, *top panels*) and PACAP27 (100nM, *bottom panels*) fail to alter the accumulation of ubiquitinated proteins induced by PGJ2-treatment. Western blot analyses to detect ubiquitinated proteins (Ub conjugates) and actin (loading control) in cell extracts of rat E18 cerebral cortical cultures (40 µg of protein/lane). The neuronal cultures were pre-treated for 1h with water (minus sign, vehicle, control), db-cAMP (plus sign, 1mM) or PACAP27 (plus sign, 100nM) prior to DMSO (vehicle, control) or 10µM PGJ2 for different time points (4h, 8h, 16h and 24h). The blots were probed with anti-ubiquitinated proteins and anti-actin antibodies. The levels of ubiquitinated proteins were semi-quantified by densitometry. Data represent the percentage of the pixel ratio for ubiquitinated proteins over actin for each condition compared to control. Values are means and s.e. from three experiments for db-cAMP or means and s.d. from two experiments for PACAP27. Molecular mass markers in kDa are shown on the right. The *asterisk* (*) identifies values that are significantly different in a comparison between PGJ2 alone (*white bars*) with PGJ2 in conjunction with db-cAMP or PACAP27 (*black bars*); *$p<0.05$; ***$p < 0.001$.

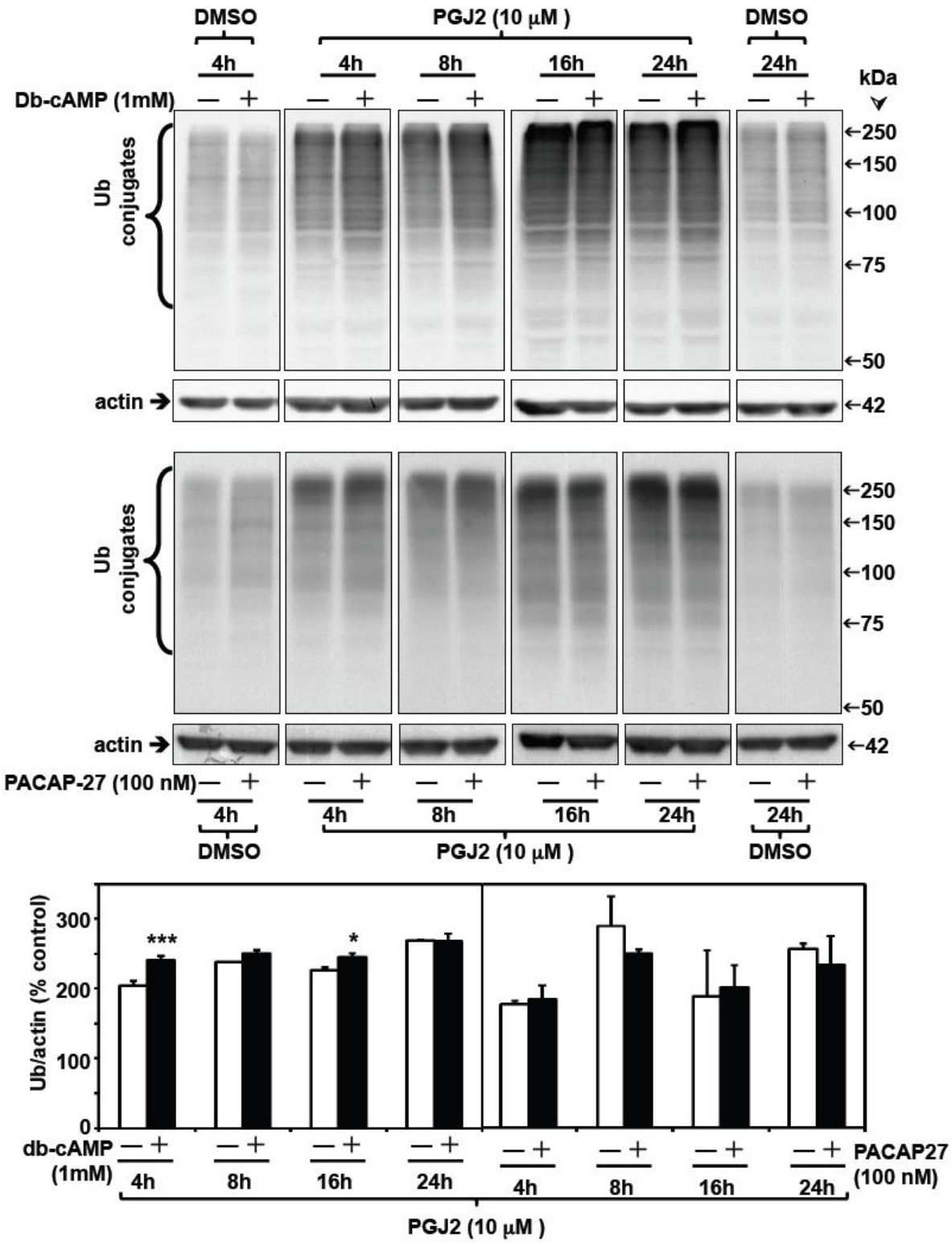

DMSO
PGJ2 (10 μM)
DMSO
4h
4h
8h
16h
24h
24h
kDa
Db-cAMP (1mM)
— + — + — + — + — + — +
Ub conjugates
←250
←150
←100
←75
←50
actin
←42
Ub conjugates
←250
←150
←100
←75
←50
actin
←42
PACAP-27 (100 nM)— + — + — + — + — + — +
4h
4h
8h
16h
24h
24h
DMSO
PGJ2 (10 μM)
DMSO
Ub/actin (% control)
300
200
100
0
***
*
db-cAMP (1mM)
— + — + — + — + — + — + — + — +
PACAP27 (100 nM)
4h
8h
16h
24h
4h
8h
16h
24h
PGJ2 (10 μM)

Figure 7

Fig. 8 - Db-cAMP (1mM) *per se* enhances proteasome activity and weakly prevents the inhibitory effect of PGJ2 (10μM) on the proteasome; PACAP27 (100nM) fails to alter proteasome activity. Cell extracts were prepared from rat E18 cerebral cortical neuronal cultures pre-treated for one hour with water (control, vehicle), db-cAMP (1mM, db-cAMP), or PACAP27 (100nM), in conjunction with DMSO (24h, control, vehicle, *left panels*) or with PJG2 (24h, 10μM, *right panels*). In parallel experiments, cells were pre-treated with Rp-cAMPS (1h, 100μM, Rp) alone or in combination with db-cAMP (1h, 1mM) or PACAP27 (1h, 100nM) with DMSO (24h, control, vehicle, *left panels*) or with PGJ2 (24h, 10μM, *right panels*). Clear lysates (30μg/sample) were subjected to non-denaturing gel electrophoresis as described under "Material and Methods". 26S and 20S proteasomal chymotrypsin-like activity was assessed with Suc-LLVY-AMC by the in-gel assay (*top panels*). 26S (two-capped and one-capped) and 20S proteasomes (*indicated on the left by arrows*) were also detected by immunoblotting with anti-β5 (*bottom panels*). β5 is a subunit of the 20S core particle of the proteasome, thus anti-β5 detects both 20S and 26S proteasomes (*bottom panels*). Activity (*top graphs*) and immunoblot (*bottom graphs*) bands were semi-quantified by densitometry. Arrows point to cells treated with db-cAMP (1mM). Percentages represent the ratio between data for

each condition and control (DMSO) considered to be 100%. Values are from one experiment.

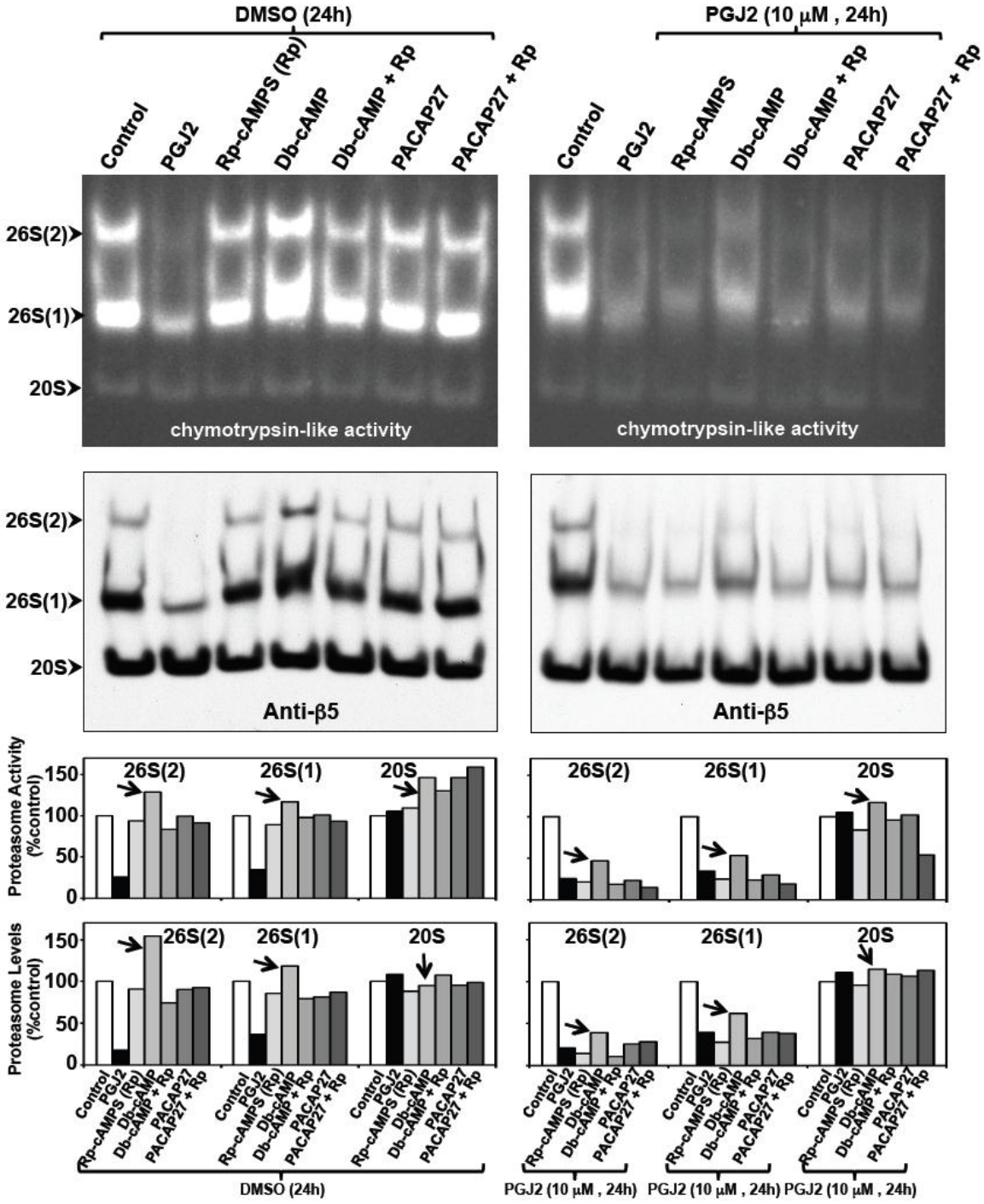

DMSO (24h)
PGJ2 (10 μM , 24h)
Control
PGJ2
Rp-cAMPS (Rp)
Db-cAMP
Db-cAMP + Rp
PACAP27
PACAP27 + Rp
26S(2)
26S(1)
20S
chymotrypsin-like activity
Anti-β5
Proteasome Activity (%control)
Proteasome Levels (%control)
150
100
50
0
DMSO (24h)
PGJ2 (10 μM , 24h)

Figure 8

Fig. 9 – Db-cAMP (1mM) *per se* or in conjunction with PGJ2 (10μM) enhances CREB phosphorylation; PACAP27 (100nM) does not. Western blot analyses to detect Tau cleaved at Asp421 (ΔTau), CREB and phospho-CREB (p-CREB), and actin (loading control) in cell extracts of rat E18 cerebral cortical cultures (40 μg of protein/lane). The neuronal cultures were pre-treated for one hour with water (control, vehicle), db-cAMP (1mM, db-cAMP), or PACAP27 (100nM), in conjunction with DMSO (8h, control, vehicle, *left panels*) or with PJG2 (8h, 10μM, *right panels*). In parallel experiments, cells were pre-treated with Rp-cAMPS (1h, 100μM, Rp) alone or in combination with db-cAMP (1h, 1mM) or PACAP27 (1h, 100nM) with DMSO (8h, control, vehicle, *left panels*) or with PGJ2 (8h, 10μM, *right panels*). The blots were probed with the Tau C3 antibody (Tau cleaved at Asp421, epitope a.a. 412-421), CREB, p-CREB and actin antibodies. The levels of ΔTau, CREB and p-CREB were semi-quantified by densitometry. P-ATF1 is also shown because the p-CREB antibody reacts with it. Data represent the percentage of the pixel ratio for ΔTau over actin or p-CREB/CREB for each condition compared to control. Values are mean from tone experiment. Molecular mass markers in kDa are shown in the middle. ΔTau, Tau cleaved at Asp421; p-CREB, phospho-CREB.

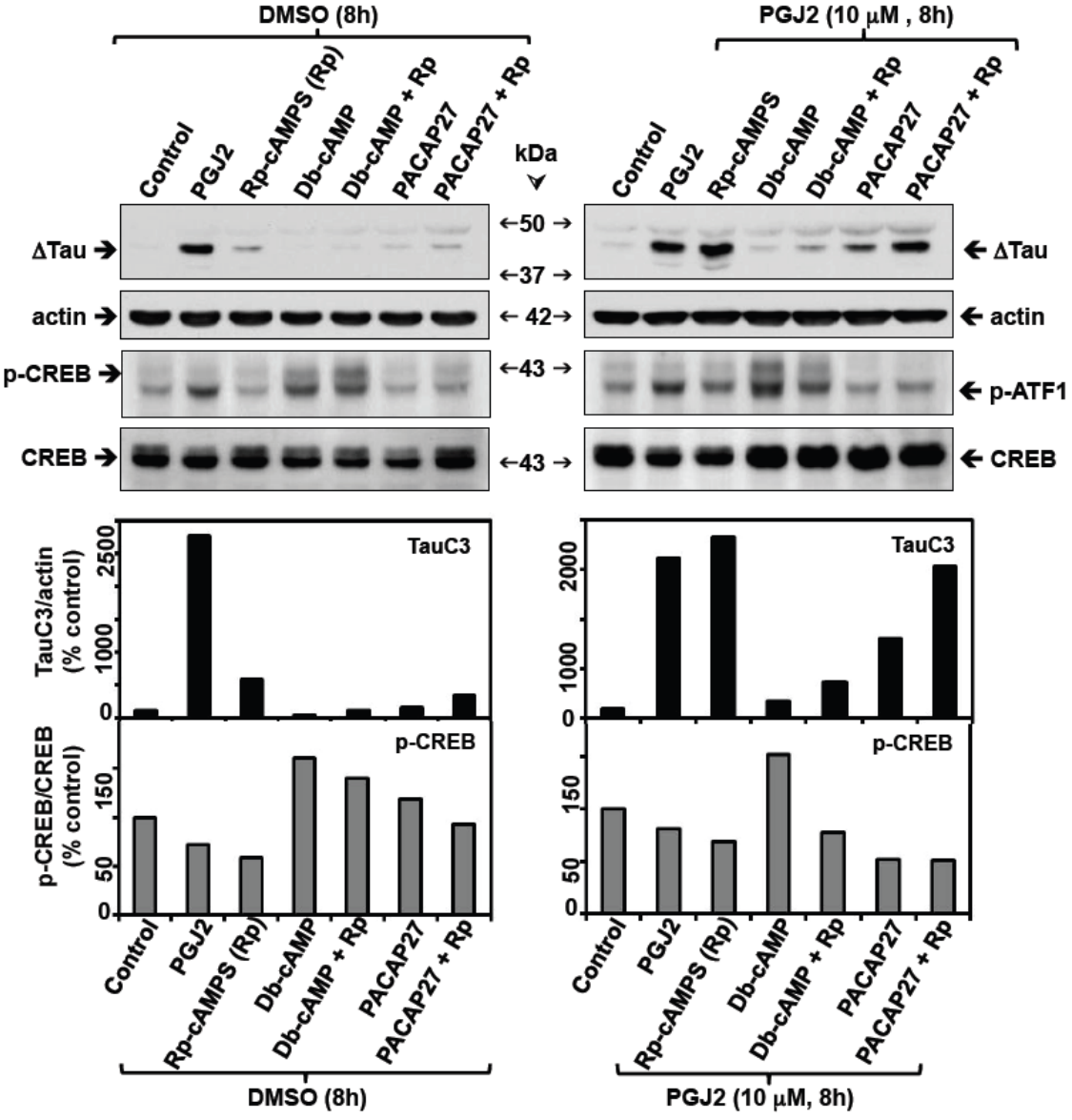

DMSO (8h)
PGJ2 (10 μM , 8h)
Control
PGJ2
Rp-cAMPS (Rp)
Db-cAMP
Db-cAMP + Rp
PACAP27
PACAP27 + Rp
kDa
ΔTau
actin
p-CREB
CREB
p-ATF1
←50→
←37→
← 42→
←43→
TauC3/actin (% control)
TauC3
p-CREB/CREB (% control)
p-CREB
DMSO (8h)
PGJ2 (10 μM, 8h)

Figure 9

Fig. 10 - PJG2 induces the formation of aggregates of ubiquitinated proteins and ΔTau. Protein aggregates were assessed with the filter trap assay in cell extracts of rat E18 cerebral cortical cultures (50 µg of protein/dot) as described in "Material and Methods". The neuronal cultures were treated with increasing concentrations of PGJ2 for 24 hours (*left panels*) or with 20µM PGJ2 for different time points (*right panels*). In parallel experiments (*bottom panels*), cells were pre-treated for one hour with water (vehicle, control), db-cAMP (1mM), or PACAP27 (100nM) alone or in combination with DMSO (8h, vehicle) or with PGJ2 (8h, 10µM). The blots were probed with anti-ubiquitinated proteins antibody or the Tau C3 antibody (Tau cleaved at Asp421, epitope a.a. 412-421). Ub-conjugates, ubiquitinated proteins.

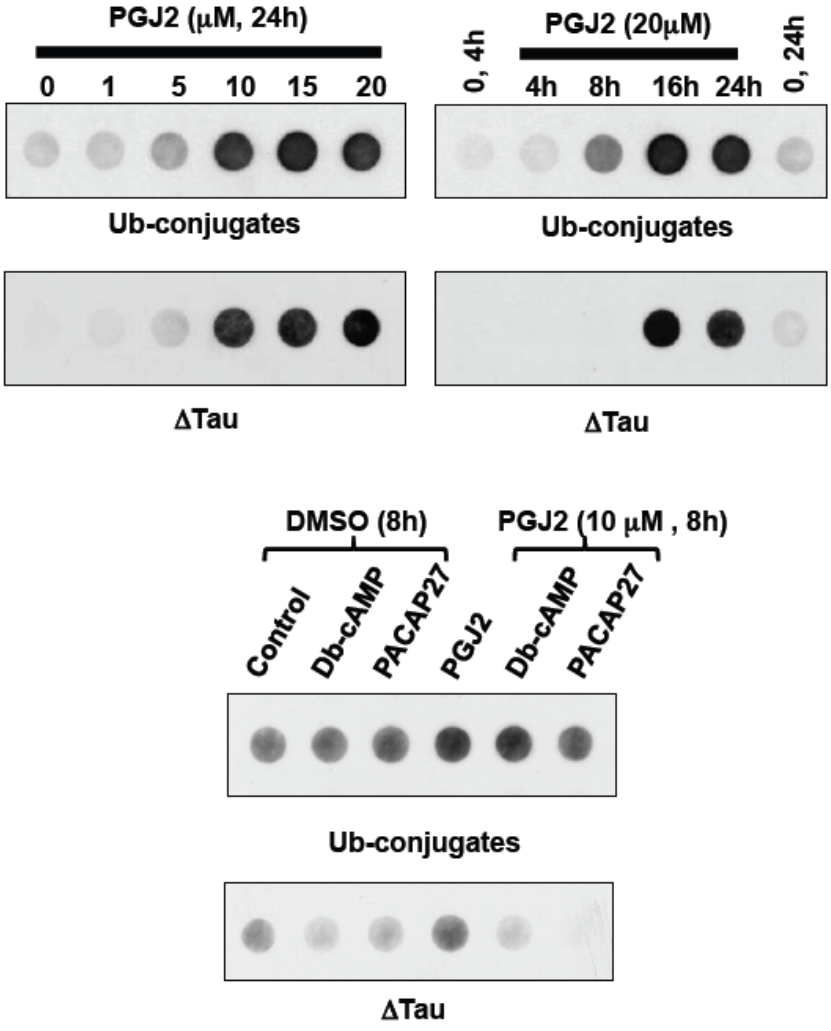

PGJ2 (μM, 24h)
0
1
5
10
15
20
Ub-conjugates
ΔTau
0, 4h
PGJ2 (20μM)
4h
8h
16h
24h
0, 24h
Ub-conjugates
ΔTau
DMSO (8h)
PGJ2 (10 μM , 8h)
Control
Db-cAMP
PACAP27
PGJ2
Db-cAMP
PACAP27
Ub-conjugates
ΔTau

Figure 10

Fig. 11 – Db-cAMP (1mM) and PACAP (100nM) diminish PGJ2-dependent loss of cell viability at early (8h) time points. Cell viability was assessed with the MTT assay as described in "Materials Methods". Rat E18 cerebral cortical cultures in: **(A)** were treated with DMSO (0, control), or increasing concentrations of PGJ2 for 4h, 8h, 16h or 24h; **(B)** were pre-treated for one hour with water (control, vehicle, *minus sign*) or db-cAMP (1mM, db-cAMP, *plus sign*) and then with DMSO (vehicle) or PGJ2 (10µM) for 8h, 16h and 24h; **(C)** were pre-treated for one hour with water (control, vehicle, not shown) or with PACAP27 (25nM, 50nM,100nM or 200nM) and then with DMSO (vehicle, *minus sign*) or PGJ2 (10µM, *plus sign*) for 8h. Percentages represent the ratio between the data for each condition and control (considered to be 100%). Values indicate means and s.e. from at least three experiments. The *asterisks* identify values that are significantly different (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

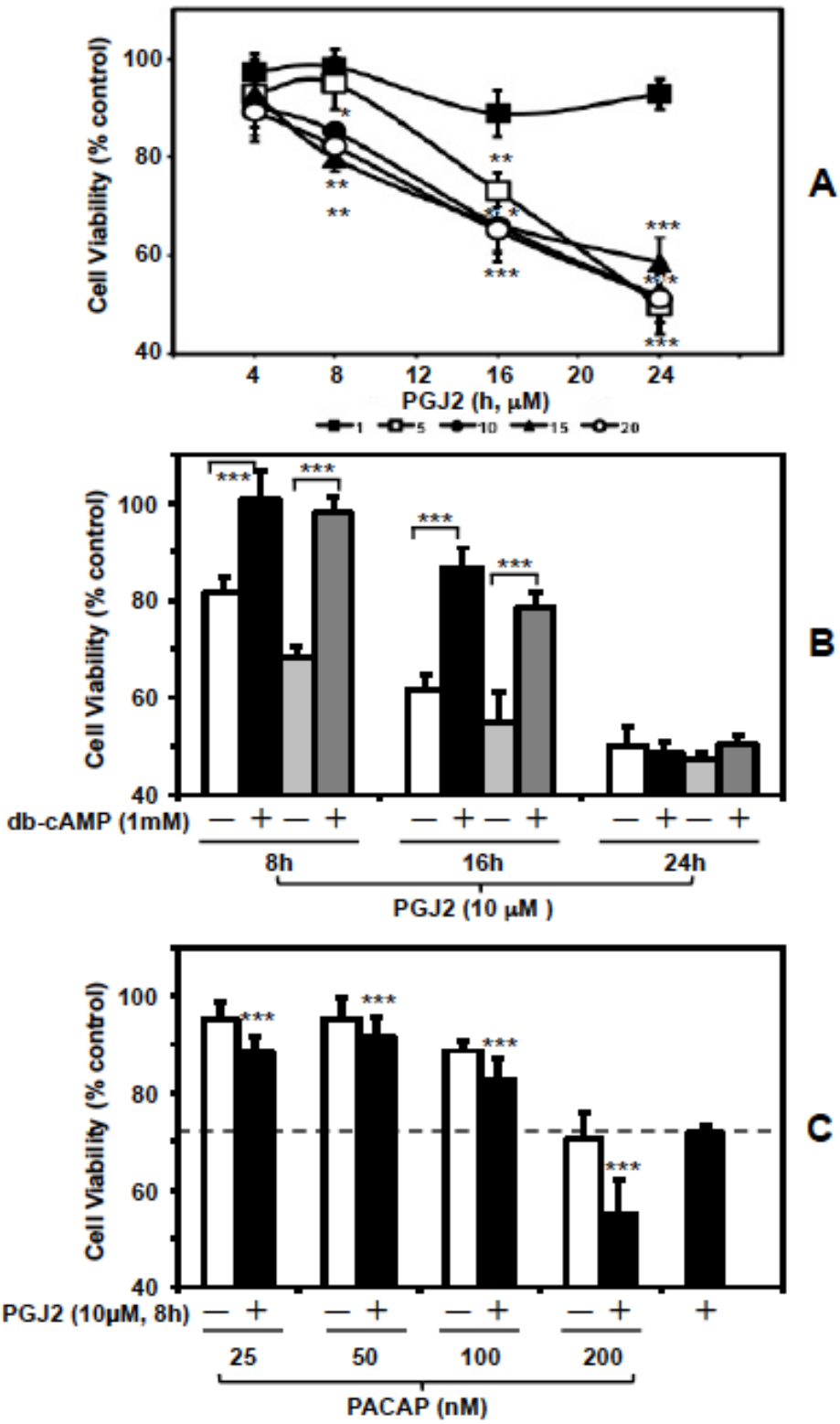

Cell Viability (% control)
100
80
60
40
4
8
12
16
20
24
PGJ2 (h, μM)
1
5
10
15
20
A
Cell Viability (% control)
db-cAMP (1mM)
8h
16h
24h
PGJ2 (10 μM)
B
Cell Viability (% control)
PGJ2 (10μM, 8h)
25
50
100
200
PACAP (nM)
C

Figure 11

Fig. 12 – Transfection of Hsp105 mitigates the decrease in SK-N-SH cell viability induced by PGJ2 treatment. Western blot analysis for Hsp105 following treatment of SK-N-SH cells for different times and with different concentrations of PGJ2. A time- and dose-dependent increase in Hsp105 protein level was observed. Blots were probed with an anti-Hsp105 polyclonal antibody (1:500 from Santa Cruz) *(upper panel)*. Exogenously expressed Hsp105 fused to a V5 tag was detected with an anti-V5 antibody (mouse monoclonal, 1:5000, Invitrogen, CA). SK-N-SH cells were treated with increasing concentrations of PGJ2 for 24h following transfection of 4µg of Hsp105 pDNA (16h) and an MTT cell viability assay was performed. The data represent means and SEM of three determinations. The *asterisks* identify the values that are significantly different (*, $p < 0.05$; **, $p < 0.01$) from untransfected cells *(bottom graph)*.

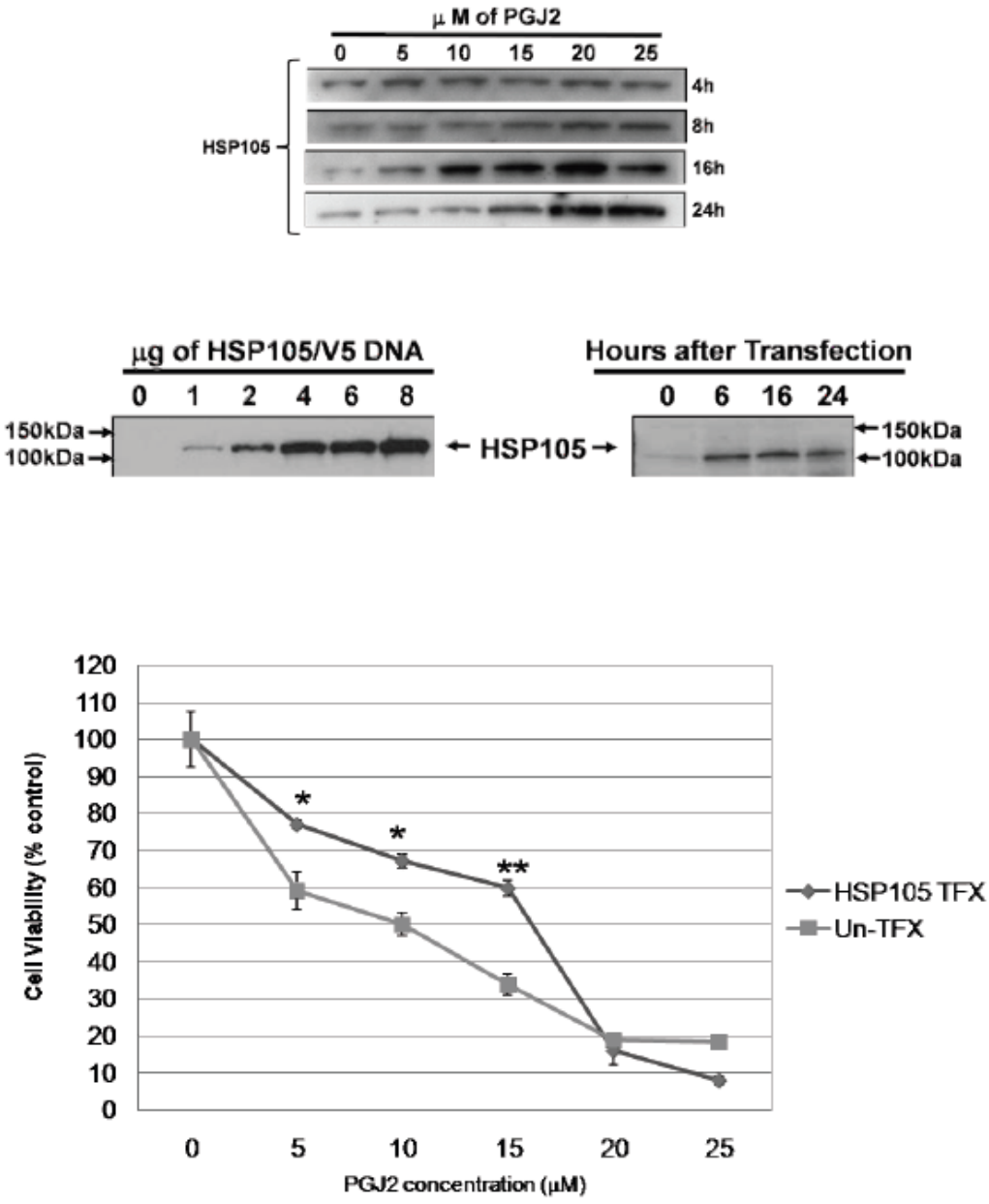

μ M of PGJ2
0 5 10 15 20 25
HSP105
4h
8h
16h
24h
μg of HSP105/V5 DNA
0 1 2 4 6 8
150kDa
100kDa
HSP105
Hours after Transfection
0 6 16 24
150kDa
100kDa
Cell Viability (% control)
120 110 100 90 80 70 60 50 40 30 20 10 0
* * **
0 5 10 15 20 25
PGJ2 concentration (μM)
HSP105 TFX
Un-TFX

Figure 12

Fig. 13 – EC102 pre-treatment reduces PGJ2-dependent loss of cell viability. MTT assay to determine if EC102 pre-treatment reduces PGJ2 toxicity. Prior to 10μM PGJ2 treatment, we treated cells with 0.5, 1, 2, 5 and 10μM of EC102 for 1 hour. We observed that 0.5, 1, 2 and 5μM EC102 prevented PGJ2-induced cell toxicity.

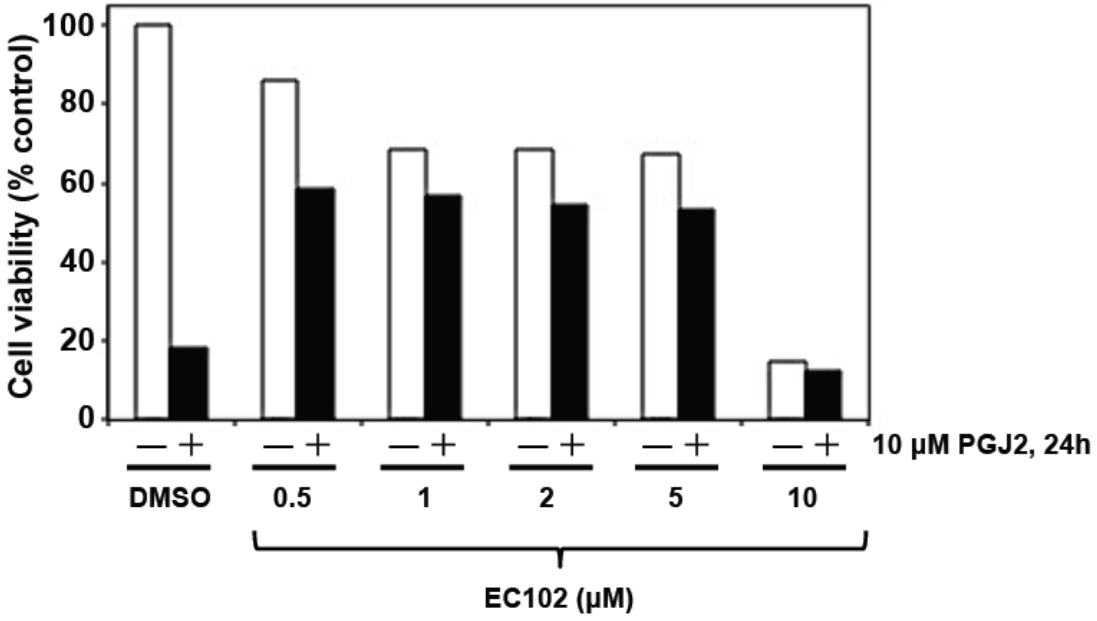

Cell viability (% control)
100
80
60
40
20
0
— +
— +
— +
— +
— +
— +
10 μM PGJ2, 24h
DMSO
0.5
1
2
5
10
EC102 (μM)

Figure 13

Reference List

1. Abdel-Halim MS, Hamberg M, Sjoquist B, Anggard E (1977) Identification of prostaglandin D2 as a major prostaglandin in homogenates of rat brain. Prostaglandins 14:633-643.

2. Arnaud L, Robakis NK, Figueiredo-Pereira ME (2006) It may take inflammation, phosphorylation and ubiquitination to 'tangle' in Alzheimer's disease. Neurodegener Dis 3:313-319.

3. Arnaud LT, Myeku N, Figueiredo-Pereira ME (2009) Proteasome-caspase-cathepsin sequence leading to tau pathology induced by prostaglandin J2 in neuronal cells. J Neurochem 110:328-342.

4. Asai M, Tsukamoto O, Minamino T, Asanuma H, Fujita M, Asano Y, Takahama H, Sasaki H, Higo S, Asakura M, Takashima S, Hori M, Kitakaze M (2009) PKA rapidly enhances proteasome assembly and activity in in vivo canine hearts. J Mol Cell Cardiol 46:452-462.

5. Ayala GX, Tapia R (2008) HSP70 expression protects against hippocampal neurodegeneration induced by endogenous glutamate in vivo. Neuropharmacology 55:1383-1390.

6. Babu JR, Geetha T, Wooten MW (2005) Sequestosome 1/p62 shuttles polyubiquitinated tau for proteasomal degradation. J Neurochem 94:192-203.

7. Bennett EJ, Bence NF, Jayakumar R, Kopito RR (2005) Global impairment of the ubiquitin-proteasome system by nuclear or cytoplasmic protein aggregates precedes inclusion body formation. Mol Cell 17:351-365.

8. Bennett EJ, Shaler TA, Woodman B, Ryu KY, Zaitseva TS, Becker CH, Bates GP, Schulman H, Kopito RR (2007) Global changes to the ubiquitin system in Huntington's disease. Nature 448:704-708.

9. Bernardo A, Ajmone-Cat MA, Levi G, Minghetti L (2003) 15-deoxy-delta12,14-prostaglandin J2 regulates the functional state and the survival of microglial cells through multiple molecular mechanisms. J Neurochem 87:742-751.

10. Berry RW, Abraha A, Lagalwar S, LaPointe N, Gamblin TC, Cryns VL, Binder LI (2003) Inhibition of tau polymerization by its carboxy-terminal caspase cleavage fragment. Biochemistry 42:8325-8331.

11. Biederer T, Scheiffele P (2007) Mixed-culture assays for analyzing neuronal synapse formation. Nat Protoc 2:670-676.

12. Biedler JL, Roffler-Tarlov S, Schachner M, Freedman LS (1978) Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones. Cancer Res 38:3751-3757.

13. Billingsley ML, Kincaid RL (1997) Regulated phosphorylation and dephosphorylation of tau protein: effects on microtubule interaction, intracellular trafficking and neurodegeneration. Biochem J 323 (Pt 3):577-591.

14. Borsello T, Forloni G (2007) JNK signalling: a possible target to prevent neurodegeneration. Curr Pharm Des 13:1875-1886.

15. Brown MR, Bondada V, Keller JN, Thorpe J, Geddes JW (2005) Proteasome or calpain inhibition does not alter cellular tau levels in neuroblastoma cells or primary neurons. J Alzheimers Dis 7:15-24.

16. Cho SG, Choi EJ (2002) Apoptotic signaling pathways: caspases and stress-activated protein kinases. J Biochem Mol Biol 35:24-27.

17. Chong YH, Shin YJ, Lee EO, Kayed R, Glabe CG, Tenner AJ (2006) ERK1/2 activation mediates Abeta oligomer-induced neurotoxicity via caspase-3 activation and tau cleavage in rat organotypic hippocampal slice cultures. J Biol Chem 281:20315-20325.

18. Corsetti V, Amadoro G, Gentile A, Capsoni S, Ciotti MT, Cencioni MT, Atlante A, Canu N, Rohn TT, Cattaneo A, Calissano P (2008) Identification of a caspase-derived N-terminal tau fragment in cellular and animal Alzheimer's disease models. Mol Cell Neurosci 38:381-392.

19. Cotman CW, Poon WW, Rissman RA, Blurton-Jones M (2005) The role of caspase cleavage of tau in Alzheimer disease neuropathology. J Neuropathol Exp Neurol 64:104-112.

20. Crowther T, Goedert M, Wischik CM (1989) The repeat region of microtubule-associated protein tau forms part of the core of the paired helical filament of Alzheimer's disease. Ann Med 21:127-132.

21. Cyr DM (2008a) Swapping nucleotides, tuning Hsp70. Cell 133:945-947.

22. David DC, Layfield R, Serpell L, Narain Y, Goedert M, Spillantini MG (2002) Proteasomal degradation of tau protein. J Neurochem 83:176-185.

23. de Wit RJ, Hekstra D, Jastorff B, Stec WJ, Baraniak J, Van DR, Van Haastert PJ (1984) Inhibitory action of certain cyclophosphate derivatives of cAMP on cAMP-dependent protein kinases. Eur J Biochem 142:255-260.

24. De SB (2010) Proteases and proteolysis in Alzheimer disease: a multifactorial view on the disease process. Physiol Rev 90:465-494.

25. Dickey CA, Dunmore J, Lu B, Wang JW, Lee WC, Kamal A, Burrows F, Eckman C, Hutton M, Petrucelli L (2006) HSP induction mediates selective clearance of tau phosphorylated at proline-directed Ser/Thr sites but not KXGS (MARK) sites. FASEB J.

26. Dickey CA, Kamal A, Lundgren K, Klosak N, Bailey RM, Dunmore J, Ash P, Shoraka S, Zlatkovic J, Eckman CB, Patterson C, Dickson DW, Nahman NS, Jr., Hutton M, Burrows F, Petrucelli L (2007) The high-affinity HSP90-CHIP complex recognizes and selectively degrades phosphorylated tau client proteins. J Clin Invest 117:648-658.

27. Dickey CA, Koren J, Zhang YJ, Xu YF, Jinwal UK, Birnbaum MJ, Monks B, Sun M, Cheng JQ, Patterson C, Bailey RM, Dunmore J, Soresh S, Leon C, Morgan D, Petrucelli L (2008d) Akt and CHIP coregulate tau degradation through coordinated interactions. Proc Natl Acad Sci U S A 105:3622-3627.

28. Dickson DW (2004) Apoptotic mechanisms in Alzheimer neurofibrillary degeneration: cause or effect? J Clin Invest 114:23-27.

29. Dogrukol-Ak D, Tore F, Tuncel N (2004) Passage of VIP/PACAP/secretin family across the blood-brain barrier: therapeutic effects. Curr Pharm Des 10:1325-1340.

30. Dorval V, Fraser PE (2006b) Small ubiquitin-like modifier (SUMO) modification of natively unfolded proteins tau and alpha-synuclein. J Biol Chem 281:9919-9924.

31. Dou F, Netzer WJ, Tanemura K, Li F, Hartl FU, Takashima A, Gouras GK, Greengard P, Xu H (2003) Chaperones increase association of tau protein with microtubules. Proc Natl Acad Sci U S A 100:721-726.

32. Drews O, Tsukamoto O, Liem D, Streicher J, Wang Y, Ping P (2010) Differential regulation of proteasome function in isoproterenol-induced cardiac hypertrophy. Circ Res 107:1094-1101.

33. Easton DP, Kaneko Y, Subjeck JR (2000) The hsp110 and Grp1 70 stress proteins: newly recognized relatives of the Hsp70s. Cell Stress Chaperones 5:276-290.

34. Eckert A, Keil U, Marques CA, Bonert A, Frey C, Schussel K, Muller WE (2003) Mitochondrial dysfunction, apoptotic cell death, and Alzheimer's disease. Biochem Pharmacol 66:1627-1634.

35. Espinoza M, de SR, Dickson DW, Davies P (2008) Differential incorporation of tau isoforms in Alzheimer's disease. J Alzheimers Dis 14:1-16.

36. Essayan DM (2001) Cyclic nucleotide phosphodiesterases. J Allergy Clin Immunol 108:671-680.

37. Evans CG, Wisen S, Gestwicki JE (2006) Heat shock proteins 70 and 90 inhibit early stages of amyloid beta-(1-42) aggregation in vitro. J Biol Chem 281:33182-33191.

38. Fuentes JJ, Pritchard MA, Planas AM, Bosch A, Ferrer I, Estivill X (1995) A new human gene from the Down syndrome critical region encodes a proline-rich protein highly expressed in fetal brain and heart. Hum Mol Genet 4:1935-1944.

39. Gamblin TC, Chen F, Zambrano A, Abraha A, Lagalwar S, Guillozet AL, Lu M, Fu Y, Garcia-Sierra F, LaPointe N, Miller R, Berry RW, Binder LI, Cryns VL (2003) Caspase cleavage of tau: linking amyloid and neurofibrillary tangles in Alzheimer's disease. Proc Natl Acad Sci U S A 100:10032-10037.

40. Goedert M, Spillantini MG, Jakes R, Rutherford D, Crowther RA (1989) Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease. Neuron 3:519-526.

41. Gomez-Ramos A, Smith MA, Perry G, Avila J (2004) Tau phosphorylation and assembly. Acta Neurobiol Exp (Wars) 64:33-39.

42. Goryunov D, Liem RK (2007) CHIP-ping away at tau. J Clin Invest 117:590-592.

43. Gotz J, Schild A, Hoerndli F, Pennanen L (2004) Amyloid-induced neurofibrillary tangle formation in Alzheimer's disease: insight from transgenic mouse and tissue-culture models. Int J Dev Neurosci 22:453-465.

44. Guillozet-Bongaarts AL, Cahill ME, Cryns VL, Reynolds MR, Berry RW, Binder LI (2006) Pseudophosphorylation of tau at serine 422 inhibits caspase cleavage: in vitro evidence and implications for tangle formation in vivo. J Neurochem 97:1005-1014.

45. Guillozet-Bongaarts AL, Garcia-Sierra F, Reynolds MR, Horowitz PM, Fu Y, Wang T, Cahill ME, Bigio EH, Berry RW, Binder LI (2005) Tau truncation during neurofibrillary tangle evolution in Alzheimer's disease. Neurobiol Aging 26:1015-1022.

46. Harris SG, Padilla J, Koumas L, Ray D, Phipps RP (2002) Prostaglandins as modulators of immunity. Trends Immunol 23:144-150.

47. Hata AN, Zent R, Breyer MD, Breyer RM (2003) Expression and molecular pharmacology of the mouse CRTH2 receptor. J Pharmacol Exp Ther 306:463-470.

48. Herbst M, Wanker EE (2007) Small molecule inducers of heat-shock response reduce polyQ-mediated huntingtin aggregation. A possible therapeutic strategy. Neurodegener Dis 4:254-260.

49. Herrup K (2010) Reimagining Alzheimer's disease--an age-based hypothesis. J Neurosci 30:16755-16762.

50. Hirai H, Tanaka K, Yoshie O, Ogawa K, Kenmotsu K, Takamori Y, Ichimasa M, Sugamura K, Nakamura M, Takano S,

Nagata K (2001) Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. J Exp Med 193:255-261.

51. Hylander BL, Chen X, Graf PC, Subjeck JR (2000) The distribution and localization of hsp110 in brain. Brain Res 869:49-55.

52. Illenberger S, Zheng-Fischhofer Q, Preuss U, Stamer K, Baumann K, Trinczek B, Biernat J, Godemann R, Mandelkow EM, Mandelkow E (1998) The endogenous and cell cycle-dependent phosphorylation of tau protein in living cells: implications for Alzheimer's disease. Mol Biol Cell 9:1495-1512.

53. Iqbal K, Alonso AC, Grundke-Iqbal I (2008) Cytosolic abnormally hyperphosphorylated tau but not paired helical filaments sequester normal MAPs and inhibit microtubule assembly. J Alzheimers Dis 14:365-370.

54. Ishihara K, Yamagishi N, Saito Y, Adachi H, Kobayashi Y, Sobue G, Ohtsuka K, Hatayama T (2003) Hsp105alpha suppresses the aggregation of truncated androgen receptor with expanded CAG repeats and cell toxicity. J Biol Chem 278:25143-25150.

55. Ishii T, Sakurai T, Usami H, Uchida K (2005) Oxidative modification of proteasome: identification of an oxidation-sensitive subunit in 26 s proteasome. Biochemistry 44:13893-13901.

56. Ishii T, Uchida K (2004) Induction of reversible cysteine-targeted protein oxidation by an endogenous electrophile 15-deoxy-delta12,14-prostaglandin J2. Chem Res Toxicol 17:1313-1322.

57. Johnson ES (2004) Protein modification by SUMO. Annu Rev Biochem 73:355-382.

58. Johnson GV, Stoothoff WH (2004) Tau phosphorylation in neuronal cell function and dysfunction. J Cell Sci 117:5721-5729.

59. Joo KM, Chung YH, Kim MK, Nam RH, Lee BL, Lee KH, Cha CI (2004) Distribution of vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide

receptors (VPAC1, VPAC2, and PAC1 receptor) in the rat brain. J Comp Neurol 476:388-413.

60. Keck S, Nitsch R, Grune T, Ullrich O (2003) Proteasome inhibition by paired helical filament-tau in brains of patients with Alzheimer's disease. J Neurochem 85:115-122.

61. Keller JN, Hanni KB, Markesbery WR (2000) Impaired proteasome function in Alzheimer's disease. J Neurochem 75:436-439.

62. Klegeris A, McGeer EG, McGeer PL (2007) Therapeutic approaches to inflammation in neurodegenerative disease. Curr Opin Neurol 20:351-357.

63. Kojro E, Postina R, Buro C, Meiringer C, Gehrig-Burger K, Fahrenholz F (2006) The neuropeptide PACAP promotes the alpha-secretase pathway for processing the Alzheimer amyloid precursor protein. FASEB J 20:512-514.

64. Kondo M, Shibata T, Kumagai T, Osawa T, Shibata N, Kobayashi M, Sasaki S, Iwata M, Noguchi N, Uchida K (2002) 15-Deoxy-Delta(12,14)-prostaglandin J(2): the endogenous electrophile that induces neuronal apoptosis. Proc Natl Acad Sci U S A 99:7367-7372.

65. Kosik KS, Qiu WQ, Greenberg S (1996) Cellular signaling pathways and cytoskeletal organization. Ann N Y Acad Sci 777:114-120.

66. Lam YA, Pickart CM, Alban A, Landon M, Jamieson C, Ramage R, Mayer RJ, Layfield R (2000) Inhibition of the ubiquitin-proteasome system in Alzheimer's disease. Proc Natl Acad Sci U S A 97:9902-9906.

67. Li Z, Jansen M, Ogburn K, Salvatierra L, Hunter L, Mathew S, Figueiredo-Pereira ME (2004a) Neurotoxic prostaglandin J2 enhances cyclooxygenase-2 expression in neuronal cells through the p38MAPK pathway: a death wish? J Neurosci Res 78:824-836.

68. Li Z, Melandri F, Berdo I, Jansen M, Hunter L, Wright S, Valbrun D, Figueiredo-Pereira ME (2004b) Delta12-Prostaglandin J2 inhibits the ubiquitin hydrolase UCH-L1 and elicits ubiquitin-protein aggregation without proteasome inhibition. Biochem Biophys Res Commun 319:1171-1180.

69. Liang X, Wu L, Wang Q, Hand T, Bilak M, McCullough L, Andreasson K (2007) Function of COX-2 and prostaglandins in neurological disease. J Mol Neurosci 33:94-99.

70. Lu H, Zong C, Wang Y, Young GW, Deng N, Souda P, Li X, Whitelegge J, Drews O, Yang PY, Ping P (2008) Revealing the dynamics of the 20 S proteasome phosphoproteome: a combined CID and electron transfer dissociation approach. Mol Cell Proteomics 7:2073-2089.

71. Lu KP, Liou YC, Vincent I (2003) Proline-directed phosphorylation and isomerization in mitotic regulation and in Alzheimer's Disease. Bioessays 25:174-181.

72. Lu KP, Zhou XZ (2007) The prolyl isomerase PIN1: a pivotal new twist in phosphorylation signalling and disease. Nat Rev Mol Cell Biol 8:904-916.

73. Lu PJ, Wulf G, Zhou XZ, Davies P, Lu KP (1999) The prolyl isomerase Pin1 restores the function of Alzheimer-associated phosphorylated tau protein. Nature 399:784-788.

74. Lucin KM, Wyss-Coray T (2009) Immune activation in brain aging and neurodegeneration: too much or too little? Neuron 64:110-122.

75. Luo W, Dou F, Rodina A, Chip S, Kim J, Zhao Q, Moulick K, Aguirre J, Wu N, Greengard P, Chiosis G (2007) Roles of heat-shock protein 90 in maintaining and facilitating the neurodegenerative phenotype in tauopathies. Proc Natl Acad Sci U S A 104:9511-9516.

76. Mandelkow E, von BM, Biernat J, Mandelkow EM (2007) Structural principles of tau and the paired helical filaments of Alzheimer's disease. Brain Pathol 17:83-90.

77. Mandelkow EM, Thies E, Trinczek B, Biernat J, Mandelkow E (2004) MARK/PAR1 kinase is a regulator of microtubule-dependent transport in axons. J Cell Biol 167:99-110.

78. Maudsley S, Mattson MP (2006) Protein twists and turns in Alzheimer disease. Nat Med 12:392-393.

79. Mayer MP, Bukau B (2005) Hsp70 chaperones: cellular functions and molecular mechanism. Cell Mol Life Sci 62:670-684.

80. McClellan AJ, Tam S, Kaganovich D, Frydman J (2005) Protein quality control: chaperones culling corrupt conformations. Nat Cell Biol 7:736-741.

81. McGeer PL, McGeer EG (2007) NSAIDs and Alzheimer disease: epidemiological, animal model and clinical studies. Neurobiol Aging 28:639-647.

82. Meares GP, Zmijewska AA, Jope RS (2008a) HSP105 interacts with GRP78 and GSK3 and promotes ER stress-induced caspase-3 activation. Cell Signal 20:347-358.

83. Miller JP, Shuman DA, Scholten MB, Dimmitt MK, Stewart CM, Khwaja TA, Robins RK, Simon LN (1973) Synthesis and biological activity of some 2' derivatives of adenosine 3',5'-cyclic phosphate. Biochemistry 12:1010-1016.

84. Mondragon-Rodriguez S, Mena R, Binder LI, Smith MA, Perry G, Garcia-Sierra F (2008) Conformational changes and cleavage of tau in Pick bodies parallel the early processing of tau found in Alzheimer pathology. Neuropathol Appl Neurobiol 34:62-75.

85. Monneret G, Li H, Vasilescu J, Rokach J, Powell WS (2002) 15-Deoxy-delta 12,14-prostaglandins D2 and J2 are potent activators of human eosinophils. J Immunol 168:3563-3569.

86. Moody TW, Ito T, Osefo N, Jensen RT (2011) VIP and PACAP: recent insights into their functions/roles in physiology and disease from molecular and genetic studies. Curr Opin Endocrinol Diabetes Obes 18:61-67.

87. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55-63.

88. Myeku N, Figueiredo-Pereira ME (2011) Dynamics of the degradation of ubiquitinated proteins by proteasomes and autophagy: its association with sequestosome 1/P62. J Biol Chem.

89. Nakamura J, Fujimoto M, Yasuda K, Takeda K, Akira S, Hatayama T, Takagi Y, Nozaki K, Hosokawa N, Nagata K (2008) Targeted disruption of Hsp110/105 gene protects against ischemic stress. Stroke 39:2853-2859.

90. Ogburn KD, Figueiredo-Pereira ME (2006) Cytoskeleton/endoplasmic reticulum collapse induced by prostaglandin J2 parallels centrosomal deposition of ubiquitinated protein aggregates. J Biol Chem 281:23274-23284.

91. Pandey UB, Batlevi Y, Baehrecke EH, Taylor JP (2007) HDAC6 at the Intersection of Autophagy, the Ubiquitin-Proteasome System and Neurodegeneration. Autophagy 3.

92. Park SY, Tournell C, Sinjoanu RC, Ferreira A (2007) Caspase-3- and calpain-mediated tau cleavage are differentially prevented by estrogen and testosterone in beta-amyloid-treated hippocampal neurons. Neuroscience 144:119-127.

93. Pei JJ, Braak E, Braak H, Grundke-Iqbal I, Iqbal K, Winblad B, Cowburn RF (2001) Localization of active forms of C-jun kinase (JNK) and p38 kinase in Alzheimer's disease brains at different stages of neurofibrillary degeneration. J Alzheimers Dis 3:41-48.

94. Pentreath VW, Rees K, Owolabi OA, Philip KA, Doua F (1990) The somnogenic T lymphocyte suppressor prostaglandin D2 is selectively elevated in cerebrospinal fluid of advanced sleeping sickness patients. Transactions of the Royal Society of Tropical Medicine and Hygiene 84:795-799.

95. Pettipher R, Hansel TT, Armer R (2007) Antagonism of the prostaglandin D2 receptors DP1 and CRTH2 as an approach to treat allergic diseases. Nat Rev Drug Discov 6:313-325.

96. Piotrowski J, Beal R, Hoffman L, Wilkinson KD, Cohen RE, Pickart CM (1997) Inhibition of the 26 S proteasome by polyubiquitin chains synthesized to have defined lengths. J Biol Chem 272:23712-23721.

97. Rajakariar R, Hilliard M, Lawrence T, Trivedi S, Colville-Nash P, Bellingan G, Fitzgerald D, Yaqoob MM, Gilroy DW (2007) Hematopoietic prostaglandin D2 synthase controls the onset and resolution of acute inflammation through PGD2 and 15-deoxyDelta12 14 PGJ2. Proc Natl Acad Sci U S A 104:20979-20984.

98. Rapoport M, Dawson HN, Binder LI, Vitek MP, Ferreira A (2002) Tau is essential to beta -amyloid-induced neurotoxicity. Proc Natl Acad Sci U S A 99:6364-6369.

99. Reglodi D, Kiss P, Lubics A, Tamas A (2011) Review on the Protective Effects of PACAP in Models of Neurodegenerative Diseases In vitro and In vivo. Curr Pharm Des 17:962-972.

100. Rissman RA, Poon WW, Blurton-Jones M, Oddo S, Torp R, Vitek MP, LaFerla FM, Rohn TT, Cotman CW (2004) Caspase-cleavage of tau is an early event in Alzheimer disease tangle pathology. J Clin Invest 114:121-130..

101. Rohn TT, Rissman RA, Davis MC, Kim YE, Cotman CW, Head E (2002) Caspase-9 activation and caspase cleavage of tau in the Alzheimer's disease brain. Neurobiol Dis 11:341-354.

102. Rosenberg KJ, Ross JL, Feinstein HE, Feinstein SC, Israelachvili J (2008) Complementary dimerization of microtubule-associated tau protein: Implications for microtubule bundling and tau-mediated pathogenesis. Proc Natl Acad Sci U S A 105:7445-7450.

103. Ross CA, Poirier MA (2004) Protein aggregation and neurodegenerative disease. Nat Med 10 Suppl:S10-S17.

104. Sacchetti B, Lorenzini CA, Baldi E, Bucherelli C, Roberto M, Tassoni G, Brunelli M (2001) Pituitary adenylate cyclase-activating polypeptide hormone (PACAP) at very low dosages improves memory in the rat. Neurobiol Learn Mem 76:1-6.

105. Sahara N, Murayama M, Lee B, Park JM, Lagalwar S, Binder LI, Takashima A (2008) Active c-jun N-terminal kinase induces caspase cleavage of tau and additional phosphorylation by GSK-3beta is required for tau aggregation. Eur J Neurosci 27:2897-2906.

106. Sahara N, Murayama M, Mizoroki T, Urushitani M, Imai Y, Takahashi R, Murata S, Tanaka K, Takashima A (2005) In vivo evidence of CHIP up-regulation attenuating tau aggregation. J Neurochem 94:1254-1263.

107. Saito Y, Yamagishi N, Hatayama T (2009) Nuclear localization mechanism of Hsp105beta and its possible function in mammalian cells. J Biochem 145:185-191.

108. Sastre M, Walter J, Gentleman SM (2008) Interactions between APP secretases and inflammatory mediators. J Neuroinflammation 5:25.

109. Satoh J, Yukitake M, Kuroda Y (1998) Constitutive and heat-inducible expression of HSP105 in neurons and glial cells in culture. Neuroreport 9:2977-2983.

110. Satoh T, Lipton SA (2007) Redox regulation of neuronal survival mediated by electrophilic compounds. Trends Neurosci 30:37-45.

111. Saura CA, Valero J (2011) The role of CREB signaling in Alzheimer's disease and other cognitive disorders. Rev Neurosci 22:153-169.

112. Schulte TW, Neckers LM (1998) The benzoquinone ansamycin 17-allylamino-17-demethoxygeldanamycin binds to HSP90 and shares important biologic activities with geldanamycin. Cancer Chemother Pharmacol 42:273-279.

113. Sengupta A, Kabat J, Novak M, Wu Q, Grundke-Iqbal I, Iqbal K (1998) Phosphorylation of tau at both Thr 231 and Ser 262 is required for maximal inhibition of its binding to microtubules. Arch Biochem Biophys 357:299-309.

114. Seo SR, Chung KC (2008) CREB activates proteasomal degradation of DSCR1/RCAN1. FEBS Lett 582:1889-1893.

115. Shibata T, Yamada T, Kondo M, Tanahashi N, Tanaka K, Nakamura H, Masutani H, Yodoi J, Uchida K (2003) An endogenous electrophile that modulates the regulatory mechanism of protein turnover: inhibitory effects of 15-deoxy-delta(12,14)-prostaglandin J2 on proteasome. Biochemistry 42:13960-13968.

116. Shimura H, Schwartz D, Gygi SP, Kosik KS (2004) CHIP-Hsc70 complex ubiquitinates phosphorylated tau and enhances cell survival. J Biol Chem 279:4869-4876.

117. Silveira MS, Linden R (2006) Neuroprotection by cAMP: Another brick in the wall. Adv Exp Med Biol 557:164-176.

118. Snyder H, Mensah K, Theisler C, Lee J, Matouschek A, Wolozin B (2003) Aggregated and monomeric alpha-synuclein bind to the S6' proteasomal protein and inhibit proteasomal function. J Biol Chem 278:11753-11759.

119. Solit DB, Chiosis G (2008) Development and application of Hsp90 inhibitors. Drug Discov Today 13:38-43.

120. Song JJ, Lee YJ (2007) Differential activation of the JNK signal pathway by UV irradiation and glucose deprivation. Cell Signal 19:563-572.

121. Spillantini MG, Goedert M (1998) Tau protein pathology in neurodegenerative diseases. Trends Neurosci 21:428-433.

122. Straus DS, Glass CK (2001) Cyclopentenone prostaglandins: new insights on biological activities and cellular targets. Med Res Rev 21:185-210.

123. Takashima A (2006) GSK-3 is essential in the pathogenesis of Alzheimer's disease. J Alzheimers Dis 9:309-317.

124. Tan JM, Wong ES, Kirkpatrick DS, Pletnikova O, Ko HS, Tay SP, Ho MW, Troncoso J, Gygi SP, Lee MK, Dawson VL, Dawson TM, Lim KL (2008) Lysine 63-linked ubiquitination promotes the formation and autophagic clearance of protein inclusions associated with neurodegenerative diseases. Hum Mol Genet 17:431-439.

125. Tian Q, Wang J (2002) Role of serine/threonine protein phosphatase in Alzheimer's disease. Neurosignals 11:262-269.

126. Trepel J, Mollapour M, Giaccone G, Neckers L (2010) Targeting the dynamic HSP90 complex in cancer. Nat Rev Cancer 10:537-549.

127. Tsukamoto O, Minamino T, Kitakaze M (2010) Functional alterations of cardiac proteasomes under physiological and pathological conditions. Cardiovasc Res 85:339-346.

128. Uchida K, Shibata T (2008) 15-Deoxy-Delta(12,14)-prostaglandin J2: an electrophilic trigger of cellular responses. Chem Res Toxicol 21:138-144.

129. van Leeuwen FW, de Kleijn DP, van den Hurk HH, Neubauer A, Sonnemans MA, Sluijs JA, Koycu S, Ramdjielal RD, Salehi A, Martens GJ, Grosveld FG, Peter J, Burbach H, Hol EM (1998) Frameshift mutants of beta amyloid precursor protein and ubiquitin-B in Alzheimer's and Down patients. Science 279:242-247.

130. Vlad SC, Miller DR, Kowall NW, Felson DT (2008) Protective effects of NSAIDs on the development of Alzheimer disease. Neurology 70:1672-1677.

131. Wang JZ, Grundke-Iqbal I, Iqbal K (2007) Kinases and phosphatases and tau sites involved in Alzheimer neurofibrillary degeneration. Eur J Neurosci 25:59-68.

132. Wang Y, Martinez-Vicente M, Kruger U, Kaushik S, Wong E, Mandelkow EM, Cuervo AM, Mandelkow E (2009) Tau fragmentation, aggregation and clearance: the dual role of lysosomal processing. Hum Mol Genet 18:4153-4170.

133. Wang Z, Aris VM, Ogburn KD, Soteropoulos P, Figueiredo-Pereira ME (2006) Prostaglandin J2 alters pro-survival and pro-death gene expression patterns and 26 S proteasome assembly in human neuroblastoma cells. J Biol Chem 281:21377-21386.

134. Waza M, Adachi H, Katsuno M, Minamiyama M, Tanaka F, Sobue G (2006) Alleviating neurodegeneration by an anticancer agent: an Hsp90 inhibitor (17-AAG). Ann N Y Acad Sci 1086:21-34.

135. Wray S, Saxton M, Anderton BH, Hanger DP (2008) Direct analysis of tau from PSP brain identifies new phosphorylation sites and a major fragment of N-terminally cleaved tau containing four microtubule-binding repeats. J Neurochem.

136. Yamagishi N, Ishihara K, Saito Y, Hatayama T (2003) Hsp105 but not Hsp70 family proteins suppress the aggregation of heat-denatured protein in the presence of ADP. FEBS Lett 555:390-396.

137. Yao TP (2010) The role of ubiquitin in autophagy-dependent protein aggregate processing. Genes Cancer 1:779-786.

138. Yoshiyama Y, Higuchi M, Zhang B, Huang SM, Iwata N, Saido TC, Maeda J, Suhara T, Trojanowski JQ, Lee VM (2007) Synapse loss and microglial activation precede tangles in a P301S tauopathy mouse model. Neuron 53:337-351.

139. Zagol-Ikapitte I, Masterson TS, Amarnath V, Montine TJ, Andreasson KI, Boutaud O, Oates JA (2005) Prostaglandin H(2)-derived adducts of proteins correlate with Alzheimer's disease severity. J Neurochem 94:1140-1145.

140. Zhang F, Hu Y, Huang P, Toleman CA, Paterson AJ, Kudlow JE (2007) Proteasome function is regulated by cyclic

AMP-dependent protein kinase through phosphorylation of Rpt6. J Biol Chem 282:22460-22471.

141. Zhao L, Qian ZM, Zhang C, Wing HY, Du F, Ya K (2008) Amyloid beta-peptide 31-35-induced neuronal apoptosis is mediated by caspase-dependent pathways via cAMP-dependent protein kinase A activation. Aging Cell 7:47-57.

142. Zhao R, Houry WA (2005) Hsp90: a chaperone for protein folding and gene regulation. Biochem Cell Biol 83:703-710.