Therapeutic Targets for Alzheimer's Disease: Insights from In Vitro and In Vivo Models of Inflammation

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THERAPEUTIC TARGETS FOR ALZHEIMER’S DISEASE: INSIGHTS FROM IN VITRO AND IN VIVO MODELS OF INFLAMMATION

By

Magdalena J Kiprowska

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Magdalena J. Kiprowska
Alzheimer’s disease (AD) is a neurodegenerative disorder characterized by progressive neuronal loss that over the years spreads from the hippocampus to the neural cortex and impairs memory and cognitive functions. At the cellular level AD is linked to the presence of β-amyloid plaques and neurofibrillary tangles but despite decades of research little is known about their contribution to neurodegeneration and whether they are a cause or rather a result of the disease. It is well established that proteasome activity is impaired in AD brains and some studies suggest that this could be one of the initial factors leading to development of this disorder. Defective ubiquitin proteasome pathway (UPP) leads to accumulation of truncated or misfolded, ubiquitinated proteins (Ub-proteins) that can further form insoluble aggregates such as β-amyloid plaques and neurofibrillary tangles. The UPP involves a multitude of components and steps. It starts with the
ATP-dependent E1, E2 and E3 enzymatic cascade that results in tagging targeted proteins with polyubiquitin chains. Subsequently, shuttling factors recognize and deliver tagged proteins to the proteasome for degradation. Once at the proteasome, deubiquitinating enzymes (DUBs) remove the polyubiquitin chain and still other proteasome subunits are involved in unfolding and translocating the targeted protein into the degradation chamber where it is cleaved into small peptide fragments. Several components of this pathway were shown to be dysregulated in neurodegenerative disorders. It has been recognized that enhancing UPP activity could be of therapeutic benefit as it would prevent or diminish accumulation of toxic proteins and possibly prevent neuronal death.

The major GOAL of our studies was to investigate two new potential therapeutic targets for AD related to UPP function:

\textit{a) The Usp14 deubiquitinating enzyme} – we investigated whether its inhibition increases proteasome-dependent degradation and decreases toxicity induced by the endogenous product of inflammation PGJ2.

\textit{b) The product of inflammation PGJ2 that impairs different steps of the UPP} – we investigated whether PGJ2 induces AD-like neuronal and behavioral pathology in vivo, by microinfusing PGJ2 into hippocampi of young and old mice. In addition, we determined the potential of PACAP to overcome the deleterious effects of PGJ2.

\textit{a) Studies with Usp14:} Usp14 is a deubiquitinating enzyme that decreases proteasome-dependent degradation rates of certain substrates by removing their polyubiquitin chain before the substrate is committed to degradation. This leads to premature dissociation of the substrate from the proteasome thus escaping degradation altogether. A recent study showed that downregulating
Usp14 leads to increased degradation of known proteasome substrates implicated in neurodegenerative diseases, such as Tau, TDP-43 and ataxin-3. In addition, IU1 (1-[1-(4-Fluorophenyl)-2,5-dimethyl-1H-pyrrol-3-yl]-2-pyrrolidin-1-yl-ethanone) is a selective Usp14 inhibitor that was shown to increase proteasome-dependent degradation rates and could serve as a potential therapeutic. These studies were conducted with the HEK (human embryonic kidney) cell line and murine embryonic fibroblasts (MEFs), and not with primary neurons.

We investigated the therapeutic potential of targeting Usp14 with IU1 in a more relevant cell model for AD, which is rat E18 cerebral cortical neuronal cultures. The results of our studies were most unexpected. We established that IU1 treatment indeed diminishes the levels of polyubiquitinated proteins but not due to enhanced proteasome-dependent degradation. Instead IU1 prevented the formation of polyubiquitin chains. IU1 blocked mitochondrial complex 1 which resulted in mitochondrial impairment and drastically lowered ATP levels. Such low levels of ATP are insufficient to activate the E1 enzyme of the ubiquitination cascade. E1 carries-out the first step that is responsible for activating ubiquitin. By assessing E1~Ub thioester formation, we confirmed that IU1-treatment lowers E1 activity resulting in low levels of Ub-proteins. Initially it may have appeared that IU1 treatment indeed sped up protein clearance by the proteasome. However, our observation that IU1-treatment lowered 26S proteasome levels with a concomitant increase in 20S proteasomes, suggested that there was not enough ATP in the cell to assemble 26S proteasomes and ensure their optimal activity.

Overall, we showed that IU1, a potential therapeutic that was intended to prevent the accumulation of ubiquinated proteins, did not increase proteasome-dependent degradation. Instead, IU1-treatment blocked the ubiquitination cascade by inhibiting the mitochondrial complex 1 and depleting ATP levels.
Our additional studies demonstrated that downregulating Usp14 with siRNA in rat cortical cultures, or Usp14 loss in cortical cultures from \textit{Usp14}\textsuperscript{axj} mice, did not improve the rates of Ub-protein degradation. Our results are of high importance to the development of potential therapeutics that target the UPP because of its relevance to AD.

\textit{b) Studies with the product of inflammation PGJ2:} Although UPP impairment has been linked to the development of AD, it is still not known what triggers it. Studies point to chronic neuroinflammation as a major contributing factor. Traumatic brain injury (TBI) and stroke both induce neuroinflammation and increase the probability of developing AD. Chronic neuroinflammation, as opposed to its acute version, wreaks havoc in the cells, perpetuating the cycle of synthesis of toxic prostaglandins and continual activation of pathways that lead to neuronal demise. Prostaglandin J2 (PGJ2) is an endogenous product of inflammation and is released by activated microglia, astrocytes and neurons as a part of the immune response. Previous work from our lab confirmed that PGJ2 is the most toxic of the prostaglandins tested and induces AD-relevant neuropathological changes at the molecular and cellular levels. Firstly, PGJ2 activates caspase-3 and leads to caspase-3-dependent Tau cleavage at Asp421, which leads to formation of aggregation prone ΔTau, the major component of neurofibrillary tangles. Secondly, PGJ2 leads to the accumulation and aggregation of ubiquitinated proteins in plaques and tangles, which are AD hallmarks. Thirdly, PGJ2 perturbs the UPP (a) by causing 26S proteasome dissociation of the regulatory and core particles due to carbonylation of the Rpt5 subunit, thus decreasing the levels of 26S proteasomes, and (b) by inhibiting DUBs such UCHL-1. PGJ2 can exert its function via two mechanisms. The first mechanism involves covalent modification of exposed cysteine residues in proteins leading to the formation of Michael adducts. The second mechanism is mediated by receptor binding, including the DP2 and PPARγ receptors.
PGJ2 is a product of the pro-inflammatory enzymes cyclooxygenases, including cyclooxygenase-2 (COX-2), the levels of which increase in AD brains and negatively affect neuronal function. COX-2 catalyzes the synthesis of prostaglandins, some of which are neuroprotective while others are neurotoxic. Treatment with non-steroidal anti-inflammatory drugs (NSAIDs), which target cyclooxygenases, is one of the therapeutic strategies aimed at minimizing neuroinflammation. However, this anti-inflammatory strategy can produce adverse effects, including renal failure, heart attack and stroke, as well as preventing the synthesis of neuroprotective prostaglandins. Therefore, identifying a therapeutic target that is downstream of cyclooxygenases would be beneficial to prevent the effects of neurotoxic prostaglandins, without interfering with the neuroprotective ones. Targeting PGJ2, shown to induce AD-like pathology in cerebral cortical neuronal cultures, could offer a better strategy to prevent inflammation-dependent neurotoxicity.

We investigated whether or not PGJ2 elicits AD-like pathology in vivo by injecting it into the CA1 hippocampal brain region in mice. Since aging is a major risk factor in sporadic AD (sAD) we treated old (52 weeks of age) and young (12 weeks of age) mice to determine if advanced age can make them more susceptible to PGJ2-linked toxicity. Our studies revealed that PGJ2 impairs learning and memory retention in old but not in young mice as assessed with the radial 8-arm maze (RAM) test. Additionally, PGJ2 induced neurodegeneration that spanned from the CA1 to the CA3 hippocampal region in old but not young mice. Lastly we investigated the levels of dendritic spines as their increased numbers are associated with learning and memory. However, the decrease in dendritic spine levels is linked to memory impairment associated with neurodegeneration. We showed that PGJ2-treatment induced dendritic spine changes in old but not in young mice. Compared to young mice, old mice displayed significantly less plastic, immature spines associated
with learning, which could explain their poor performance during the RAM test. Therefore, our in vivo studies showed that PGJ2 hippocampal injections induced AD-like symptoms and pathology in the mice.

Lastly, we co-injected the group of old mice (53 weeks of age) with PGJ2 and PACAP27 to determine if some of the PGJ2-induced pathological symptoms could be prevented. PACAP is a neuroprotective peptide that acts through activation of the cAMP/PKA pathway. PACAP is abundant in the brain and its levels decrease during neurodegeneration. We subjected the mice to RAM training and found learning improvement in mice co-administered PGJ2+PACAP compared to mice treated with PGJ2 alone, although this change was not statistically significant. These results indicate that PGJ2 is indeed an effective therapeutic target, and that targeting PGJ2 to prevent/treat AD could potentially be more efficient than targeting COX-2.

In conclusion, we investigated two potential therapeutic approaches for AD: a) increasing UPP activity, and b) preventing inflammation-based toxicity by focusing on PGJ2, a product of cyclooxygenases. Our studies showed that IU1 is not an effective therapeutic for neurons due to its off-targeting effects on mitochondrial complex 1. Moreover, genetically downregulating Usp14 is not sufficient to induce significant changes in proteasome-dependent degradation rates. Perhaps combining this strategy with another neuroprotective therapeutic or peptide, such as PACAP, would improve the results. Lastly, we showed that PGJ2 by itself induces AD-like pathology in vivo that could be partially prevented by PACAP. PGJ2 could be a better and more directed therapeutic target than COX-2, to halt the deleterious effects of neuroinflammation with fewer side effects.
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LIST OF ABBREVIATIONS

AAA ATPase: ATPase Associated with diverse cellular Activities
AD: Alzheimer’s disease
ADNP: Activity -dependent neuroprotective protein
AIP: autocamide-related inhibitory peptides
ATP: adenosine triphosphate
CNS: central nervous system
COX: cyclooxygenase
CP: 20S proteasome core particle
CSF: cerebrospinal fluid
DMSO: dimethyl sulfoxide
DUB: deubiquitinating enzyme
E18: embryonic day 18
GluA2: ionotropic glutamate receptor subunit
HEK: human embryonic kidney
IU1: 1-[1-(4-fluorophenyl)-2,5-dimethyl-1H-pyrrol-3-yl]-2-(1-pyrrolidinyl)-ethanone
MCAO: middle cerebral artery occlusion
MPTP: 1-methyl-4phenyl-1,2,3,6-tetrahydropyridine
MTT: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells
NSAID: non-steroidal anti-inflammatory drug
PA: proteasome activator
PAC: proteasome assembly chaperone
PACAP: pituitary adenylate cyclase activating peptide
PBS: phosphate buffer saline
PD: Parkinson’s disease
PGD2: prostaglandin D2
PGJ2: prostaglandin J2POMP: proteasome maturation protein
PSD95: post-synaptic density protein 95
RAM: radial arm maze
ROS: reactive oxygen species
Rpn: 19S regulatory particle, non ATP-dependent
Rpt: 19S regulatory particle ATP-dependent
sAD: sporadic AD
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siRNA: short interfering RNA
SNpc: Substantia nigra pars compacta
TBI: traumatic brain injury
TDP-43: TAR DNA-binding protein 43
UCHL-1: Ubiquitin Carboxyl-Terminal Esterase L1
UPP: ubiquitin/proteasome pathway
Usp14: ubiquitin specific peptidase 14
VIP: vasoactive intestinal peptide
VPAC: vasoactive intestinal peptide receptor
CHAPTER I

INTRODUCTION

Therapeutic targets for Alzheimer’s Disease: Ubiquitin/Proteasome Pathway and the endogenous product of inflammation prostaglandin J2

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1.1. ABSTRACT

Interfering with the UPP has recently provided a promising therapeutic approach for treatment of numerous disorders including neurodegenerative diseases. Targets are varied and include the proteasome, E1, E2 and E3 enzymes as well as many DUBs. Some of the therapeutics directed at the proteasome, usually targeting the β5 and/or β1 subunits, have even found their way into clinical trials in cancer therapy, like carfilzomib (Phase III against relapsed multiple myeloma), MLN9708 (Phase I), CEP18770 (Phase I) and the natural product NPI-0052 (Phase I). The UPP is involved in maintaining cellular homeostasis at several levels, like regulating cell cycle progression, apoptosis, various signaling pathways and finally removal of dysfunctional proteins. Abnormalities in any of the steps involved in the cascade of events leading to proteasome-dependent degradation result in different disorders so the targets vary. With respect to neurodegenerative diseases that result from abnormal accumulation of toxic proteins it would be beneficial to aim at a factor that would preserve or enhance proteasome function. Targeting Usp14 seems to be a promising therapeutic strategy in AD, since inhibiting the deubiquitinating activity of Usp14 in different cell lines results in increased degradation of several proteins including Tau which is relevant to AD. Usp14 exhibits ubiquitin chain trimming activity that can lead to the dissociation of protein substrates from the proteasome, if the ubiquitin removal is faster than the competing steps leading to substrate degradation. The finding that Usp14 inhibition results in increased degradation of Tau, the accumulation and aggregation of which is strongly associated with AD pathology seems to be a very interesting prospect that has to be investigated further in primary neuronal cultures. Inhibition of Usp14 was achieved with the small molecule inhibitor IU1: 1-[1-(4-Fluoro-phenyl)-2,5-dimethyl-1H-pyrrol-3-yl]-2-pyrrolidin-1-yl-ethanone [101]. IU1 seems to act selectively toward Usp14 and to increase degradation of numerous proteasomal
substrates, like cyclin B or TDP-43 presumably by inhibiting chain trimming activity thus preventing proteins targeted for degradation from premature dissociation from the proteasome. Our studies established that high concentrations of IU1 (μIU1, 75μM) reduce the accumulation of Ub-proteins induced by the endogenous product of inflammation, prostaglandin J2 (PGJ2) in primary neuronal cultures while low IU1 concentrations (25μM) had no effect. Additionally, we showed that treatment with μIU1 resulted in (a) decrease in ATP levels, (b) a decline in 26S proteasome activity and levels with a concomitant increase in 20S proteasome, (c) calpain activation and calpain-mediated Tau cleavage. These data suggested that apart from the presumed effect on proteasomal degradation rates, IU1 also affects mitochondria. This was confirmed by testing mitochondrial respiratory chain complex activities which revealed that IU1 specifically inhibits complex I thus leading to overall cellular demise. Investigating mechanisms by which potential therapeutics, like IU1 act on neuronal homeostasis, contributes to AD-relevant therapeutic research.

**Figure 1. Ubiquitination and degradation of proteins by the ubiquitin/proteasome pathway (UPP).** Protein ubiquitination is a complex ATP-dependent process in which ubiquitin (Ub) is sequentially activated by ubiquitin-activating enzymes (E1), transferred to ubiquitin-conjugating enzymes (E2) and ligated to protein substrates by ubiquitin ligases (E3). Polyubiquitin chains are formed by isopeptide bonds between Gly76 and Lys48 on adjacent ubiquitin molecules. Once a protein is polyubiquitinated, it is degraded rapidly by the 26S proteasome. Deubiquitinating enzymes (DUB) remove and disassemble polyubiquitin chains (scheme by Dr. Vita Vernace, a former PhD student in the Pereira lab).
1.2. THE UBIQUITIN-PROTEASOME PATHWAY

The UPP requires most proteins to be tagged by ubiquitin to target them for degradation. Proteolysis by the UPP involves two major steps: ubiquitination and degradation (Figure 1). A de-ubiquitination step also plays important role in this pathway as it edits the ubiquitination state of proteins and removes the ubiquitin tag for recycling. Our studies were aimed at increasing 26S proteasome-dependent degradation rates.

The 26S Proteasome: Covalent binding of ubiquitin to proteins marks them for degradation by the ubiquitin/ATP dependent proteinase known as the 26S proteasome. The latter is a multicomponent enzymatic complex with a native molecular mass of approximately 2000kDa [22]. The 26S proteasome complex is composed of two major particles (Figure 2): (1) A cylinder-like structure, known as the 20S proteasome that is located in the center and comprises the proteolytic core of the enzyme, and (2) a regulatory component known as the 19S particle (PA700), which may be attached to each end of the cylinder-like 20S proteasome (reviewed in [33]).

The 19S particle is a multicomponent complex itself containing at least 17 subunits and can be further divided into two subcomplexes: the base and the lid. The base confers ATPase activity and consists of six AAA ATPase subunits (Rpt1-Rpt6) and two non-ATPase subunits
(Rpn1 and Rpn2). The lid is made up of eight non-ATPase subunits (Rpn3, Rpn5-9, Rpn11, and Rpn12) which can specifically bind polyubiquitin-tagged substrates and also deubiquitinate them [33]. Rpn10 helps to tether the lid and the base, and is a receptor for polyubiquitinated substrates [59,59].

The eukaryotic 20S proteasome consists of 28 subunits, which are arranged in four heptameric stacked rings forming a barrel-like structure, each consisting of 7 protein subunits. The α-type subunits, comprising the two outer rings of the 20S proteasome, provide binding sites for regulatory particles and form a gated channel leading to the inner proteolytic chamber. The β-type subunits contain the active sites of the 20S proteasome. The whole particle is a dimer with an α7β7α7 subunit arrangement (reviewed in [33]).

The 20S particle may be alternatively capped by other complexes, including the 11S activator (PA28). PA28 is a cytoplasmic complex formed by equal, stoichiometric amounts of two different 28 kDa subunits, PA28α and PA28β, forming a 200 kDa heterohexamer. PA28 capped 20S proteasomes prefer substrates that are partially degraded proteins and peptides, rather than intact polyubiquitinated molecules [37,61].

**Proteasome Assembly:** The 19S particle guides proteins into the 20S proteasome chamber where unfolded proteins are degraded into short peptides (of 8-9 amino acids). Association between the 19S RP and 20S CP is a dynamic process and requires ATP binding [112]. During degradation of one substrate molecule by the 26S proteasome, it was calculated that 300–400 ATP molecules are hydrolyzed. Substrate binding activates ATP hydrolysis, which promotes three processes: substrate unfolding, gate opening in the 20S particle, and protein translocation [9]. ATP binding
is necessary for the rapid association/dissociation of the 26S proteasome from/into the 19S particle and 20S proteasome [4].

Multiple assembly chaperones are required in different stages of proteasome assembly. During 20S proteasome assembly in mammalian cells, PAC1-PAC4 (Proteasome assembly chaperone 1-4) bind to early-assembly intermediates of proteasome α-subunits and promote α-ring formation [158]. Specifically, the PAC1-PAC2 heterodimer remains attached to the nascent α-ring and suppresses premature α-ring dimerization, thereby promoting attachment of β-subunits to the proper surface of the α-ring. The PAC3-PAC4 complex then dissociates from the α-ring due to steric hindrance with the β3 subunit [73]. The following steps require the assistance of another chaperone, POMP (proteasome maturation protein). POMP inhibits the premature β-ring dimerization until the β7 subunit is properly incorporated [106]. In yeast, it is found that these premature complexes are in association with proteasome activators (PA), either Blm10 (ortholog of human PA200), or with the 19S RP (PA700) [151]. Dimerization of two half-proteasomes is triggered by incorporation of the β7 subunit, which stabilizes the nascent proteasome via its long C-terminal extension together with Blm10. Subsequently, with the autocatalytic maturation of β-subunits, the nascent 20S proteasome is activated, leading to degradation of POMP and PAC1-PAC2. Mature 20S proteasomes function as templates for the initial assembly of the 19S RP.

Compared with 20S proteasome assembly, little is known about the mechanism of 19S RP assembly. The RP is subdivided into lid and base subcomplexes. A recent study [51] demonstrated the role of four conserved assembly factors in the yeast RP base assembly, including Hsm3 (ortholog of human S5b), Nas2, Nas6, and Rpn14. These chaperones interact directly with Rpt1-Rpt6 (Proteasome regulatory particle 1-6) subunits. Nas2 is released after the base precursor is formed. The addition of Rpn10 and the lid contributes to the formation of the RP precursor. Later,
the three other chaperones, Hsm3, Nas6 and Rpn14, are released when 20S CP associates with RP precursors.

As for 26S proteasome assembly, two proteins were proposed to facilitate this process: Blm10 and Nob1. Blm10/PA200, a 200 kDa α-helix-rich repeat protein, is found attached to the 20S α-surface and apparently activates some proteolytic properties by gating the entrance pore [144,164] [83]. Nob1, a nuclear protein in yeast present only during the cell-growing phase, associates with the 19S RP and is degraded just after the doubly capped 26S proteasome is [189]. It is suggested that Nob1 might be required for the nuclear translocation of the 20S [159].

**The Three Peptidase Activities of the 20S Proteasome:** Among the 14 different subunits (7 α and 7 β) of the 20S proteasome, only three of them exhibit active sites for peptide bond hydrolysis, namely β1 (caspase-like), β2 (trypsin-like) and β5 (chymotrypsin-like). The chymotrypsin-like activity cleaves after amino acids with large or hydrophobic side chains, the trypsin-like activity cleaves after basic residues, and the caspase-like activity is a post-glutamyl activity that cleaves after acidic amino acids [142]. The β5-associated chymotrypsin-like activity seems to be the initial and rate-limiting step in protein degradation by the 20S proteasome [90]. The 20S proteasome is a threonine protease in which the nucleophilic attack is mediated by the N-terminal Thr [62]. The three β subunits (β1, β2 and β5) bearing the active sites are first synthesized as precursor proteins each containing a propeptide at the N-terminus which must be cleaved off for the subunits to become catalytically active [165]. Processing of the three catalytically active subunits into mature forms occurs only after their incorporation into 20S proteasomes.

**Proteasome Activation:** One of the major challenges that we are faced with is to figure out how to single out the UPP as a therapeutic target for preventing neurodegeneration. The challenge rests
on developing therapeutic strategies that will enhance degradation of oxidatively-modified and toxic proteins generated by a lifetime’s worth of environmental damage, without compromising the normal function of the UPP. One of the most appealing targets is the proteasome, the biogenesis of which is a highly organized multistep event. It appears that in eukaryotes the cellular level of active proteasomes is regulated at the level of assembly.

(1) Genetic Activation of the Proteasome: The stable overexpression of the β5 subunit in primary human fibroblasts results in elevated levels of other β subunits, and increases the levels of all three proteasome activities [19]. This genetic manipulation results in increased survival against oxidants and a delay in senescence. The same group in a follow up study achieved proteasomal up-regulation via overexpression of the POMP protein, the accessory factor for proteasome assembly in humans [18]. POMP overexpression in fibroblasts led to increased levels of assembled and functional proteasomes, and enhanced the capacity to effectively cope with various oxidative stressors. These data further strengthen the prospect of genetic manipulation of the proteasomal system.

(2) Proteasome activation by natural or synthetic compounds: SDS and some fatty acids were shown to stimulate proteasome activity in vitro by favoring the open conformation of the proteasome [26]. In addition, some proteasome-activating hydrophobic peptides bind as modifiers at non-catalytic sites, thus mimicking the effect of the 11S complex by opening the gate of the α-rings [91]. Katsiki et al., isolated oleuropein, the most abundant phenolic compound in Olea europaea leaf extract, olive oil, and olives, and demonstrated that it has a stimulatory impact on proteasome activity in vitro, probably acting through conformational changes of the gate of 20S α-ring [89]. Some natural antioxidants such as dithiolethione and sulforaphane, were also shown
to enhance mammalian proteasome expression through the Keap1-Nrf2 signaling pathway, resulting in increased protection against various oxidants [99,99].

1.3. IU1 – A SMALL MOLECULE INHIBITOR OF Usp14

Accumulation and aggregation of misfolded proteins is a common feature of many neurodegenerative diseases [138]. Increased levels of ubiquitinated and oxidized proteins including ubiquitinated Tau found in brains of AD patients point to impairment of the ubiquitin proteasome pathway (UPP) [62]. The UPP is essential for maintaining cellular homeostasis as it ensures degradation of dysfunctional proteins and regulates many physiological processes. However, its efficacy declines with age and in neurodegeneration, therefore any means that preserve proteasome activity will serve as a strategy to delay/prevent the onset of neuropathological symptoms. Recent findings show that it can be achieved by inhibiting the deubiquitinating enzyme Usp14 with a small molecule IU1 [101].

Mammalian proteasomes are associated with three DUBs: Rpn11, Uch37 and Usp14. Rpn11 is a stoichiometric subunit of the proteasome unlike Uch37 and Usp14 which are only reversibly associated with it [48]. Rpn11-dependent deubiquitination is linked to translocation and degradation as the substrate will be deubiquitinated only after it has committed to degradation [193]. Such commitment step is absent in Uch37 and Usp14-dependent mode of action [65,100]. Both of them shorten the ubiquitin chain from the distal tip prior to substrate degradation rather than en bloc as is the case with Rpn11. Apparently, tagged substrates can dissociate from the proteasome and thus escape degradation if the rate of chain trimming is faster than the rate leading
to protein degradation. This can contribute to accumulation of dysfunctional proteins. Usp14 slows down degradation of several proteasome substrates, such as Tau, cyclin B, and TDP-43 [104]. Its catalytic mutant shows much less inhibition implicating that it is the chain trimming action that slows down substrate degradation. These observations led to the screening of small molecules that would be capable of inhibiting the catalytic activity of Usp14. 1-[1-(4-Fluoro-phenyl)-2,5-dimethyl-1H-pyrrol-3-yl]-2-pyrrolidin-1-yl-ethanone, known as IU1, seems to specifically inhibit Usp14 when screened against a panel of DUBs [104]. IU1 inhibits ubiquitin chain trimming concomitant with increased degradation of several proteins. The effect of IU1 was tested in vitro and in vivo in murine embryonic fibroblasts (MEFs) and the HEK293 cell line on a number of proteasome substrates including Tau. The results suggest that IU1, a small molecule inhibitor of Usp14, enhances the degradation of proteasomal substrates. Subsequent experiments on usp14-/- MEF cell cultures confirmed that increased degradation rates were due to inhibiting Usp14 with IU1 since treatment of usp14-/- MEF with IU1 stabilized Tau and TDP-43 as well as levels of ubiquinated proteins rather than cause more efficient degradation. IU1 also proved to be effective in increasing clearance of oxidized proteins induced by menadione or H2O2 treatment[104].

Overall these studies strongly support that IU1 effectively enhances proteasome-dependent degradation, a feature that can be applied to eliminating toxic proteins. However, if IU1 is to be used in a clinical setting, its effects need to be assessed in neurons to establish its potential as a new therapeutic approach to intervene in the neurodegeneration process.
1.4. INTERPLAY BETWEEN UPP AND MITOCHONDRIA: ITS ROLE IN NEURODEGENERATION

Cellular homeostasis and viability strongly depend on optimal mitochondrial and ubiquitin-proteasome pathway (UPP) function. Impairment of the UPP, mitochondrial activity and oxidative stress are implicated in aging and neurodegenerative diseases. Recent studies showed that interfering with the UPP or mitochondria results in pathological conditions and can lead to cell death. The UPP depends on mitochondria for ATP production which is needed for (a) ubiquitin activation by the E1 enzyme, the first step of the ubiquitination cascade, and (b) proteasome-mediated protein degradation as substrate unfolding and translocation into the degradation chamber, as well as assembly of the 26S proteasome are energy-dependent processes [112]. Mitochondrial integrity, on the other hand depends on UPP-mediated protein quality control. Among the many proteins targeted for degradation by the proteasome are aberrant mitochondria-destined proteins and proteins involved in maintaining balance between mitochondrial fusion and fission, like Fzo1, a protein involved in mitochondrial fusion or Fis1 and Drp1, essential components of mitochondrial fission machinery [119]. Numerous studies demonstrated that proteasome inhibition results in accumulation of ubiquinated mitochondrial proteins and impaired mitochondrial dynamics like increased/decreased fragmentation, aggregation or mitochondrial swelling which is detrimental to
the cell. UPP is also involved in the turnover of mitochondria by means of the E3 ubiquitin ligase - Parkin, which is recruited to damaged mitochondria to mediate mitophagy [136]. The link is further confirmed by the presence of UPP components in the outer mitochondrial membrane which participate in mitochondrial dynamics [21,133,136]. These include ubiquitin ligases MITOL and MULAN as well as deubiquinating enzyme Ubp16/USP30 [105,134]. There is therefore, a crosstalk between mitochondria and the UPP in a healthy neuron and its maintenance is essential to ensure homeostasis, cellular health and viability. Accordingly, if one component of this system sustains damage it compromises the activity of the other component and this event has a negative impact on the entire cell and can result in apoptosis or necrosis.

The ubiquitin/proteasome pathway (UPP) plays a critical role in aging and in the pathogenesis of AD [155] High levels of oxidized proteins detected in the aging brain as well as in brains of AD patients are an indication of proteasome impairment, since this proteolytic complex degrades the majority of oxidatively modified proteins [63]. Moreover, the accumulation and aggregation of Ub-proteins detected in most neurodegenerative disorders, such as AD, is also a sign of UPP dysfunction, since this pathway degrades Ub-proteins [79]. Dysfunctional mitochondria contribute to oxidative stress by ROS production. Oxidative stress induced by ROS alters the structure of cellular proteins [180] which, if not repaired, must be removed by proteolysis to prevent their accumulation and aggregation. Impaired clearance of oxidatively modified proteins can promote progression of the neurodegenerative process [63]. One of the major roles of the proteasome is to degrade oxidatively modified proteins, but if the oxidative stress persists it leads to accumulation and later aggregation of oxidized proteins which compromise proteasomal activity. The proteasome is also susceptible to oxidation, and oxidative modification of its Rpt3 subunit results in decreased ATP-ase activity of this subunit and an overall decline in
ubiquitin/ATP-dependent proteolysis by the 26S proteasome [81]. This is an important issue since there is ample evidence that neural tissue is especially vulnerable to oxidative stress, which plays an important role in AD [38]. Neurons are especially vulnerable to ROS-induced damage due to high O\textsubscript{2} consumption. Brain processes 20\% of basal O\textsubscript{2} consumption of which 1-2\% are converted to ROS under physiological conditions [41]. Moreover, ROS levels are even higher when mitochondria are damaged. Persistent oxidative stress decreases the level and activity of 26S proteasomes with a concomitant increase in the level of 20S proteasomes. The degradation process that is mediated by 20S proteasomes is ubiquitin/ATP-independent and may act as a cellular strategy to conform to oxidative stress, but the mechanism that leads to the switch from 26S to 20S is unknown. Another deleterious mechanism associated with mitochondrial dysfunction is the limitation in ATP production that can cause an energy crisis in neurons [137]. Degradation of proteins by the 26S proteasome is highly dependent on ATP binding and hydrolysis [112]. Both deleterious consequences of mitochondrial impairment, i.e. restricted ATP generating capacity and ROS production, impair proteasome-dependent proteolysis in neurons and contribute to neurodegeneration. It is clear that there is a mutual dependence between the UPP and mitochondria [119], but the specific mechanisms underlying the functional link between UPP and mitochondrial impairment (Fig. 4) leading to neurodegeneration in AD have not been addressed. New discoveries in the functional links between the UPP and mitochondrial impairment and their roles in overall neuronal homeostasis could lead to novel and successful therapeutic approaches for AD.
1.5. ENDOGENOUS PRODUCT OF INFLAMMATION: PROSTAGLANDIN J2 (PGJ2)

Prostaglandins of the J2 series are derived from PGD2 (Fig. 5 adapted from [190]), the principle cyclooxygenase product synthesized in the mammalian central nervous system (CNS). PGD2 readily undergoes in vivo and in vitro non-enzymatic dehydration to generate PGJ2, which is converted to Δ12-PGJ2 and 15-deoxy-Δ12,14-PGJ2 (15d-PGJ2) [183]. Unlike most other classes of prostaglandins, cyclopentenone PGs like PGJ2 have a cyclopentenone ring with reactive α,β-unsaturated carbonyl groups (red asterisks, left scheme). These highly reactive carbonyl groups form covalent Michael adducts with nucleophiles such as free sulfhydryls in cysteine residues of glutathione and cellular proteins [183]. In neuronal cells prostaglandins of the J2 series induce apoptosis [43,57,129], inhibit mitochondrial activity [95,122,181] as well as 26S proteasome activity [198], and trigger accumulation and aggregation of ubiquitinated proteins [107]. The chemical properties of prostaglandins of the J2 series and their pro-oxidant and UPP disrupting effects render them extremely neurotoxic and capable of inducing neuronal cell death [190].

Our studies focus on PGJ2 because it is an endogenous product of inflammation that causes pleiotropic changes that mimic many of the pathological processes that occur in neurodegenerative disorders that are associated with inflammation, such as AD and Parkinson’s disease (PD). It has been suggested that “formation of cyclopentenone eicosanoids [such as PGJ2] in the brain may represent a novel pathogenic mechanism that contributes to many neurodegenerative conditions”
Moreover, PGJ2 up-regulates the expression and activity of cyclooxygenase-2 (COX-2), a prostaglandin synthesizing enzyme. PGJ2 thus has potential to initiate a series of deleterious cascades leading to self-sustained progressive neurodegeneration. Cyclooxygenases are key players in inflammation and they mediate the conversion of arachidonic acid into an assortment of products. Arachidonic signaling through the cyclooxygenase pathway yields an enormous variety of products, some of them with pro-survival, others with pro-death effects. For instance, prostaglandins are known to be pro-inflammatory under certain conditions and anti-inflammatory under others [reviewed in [201]]. The dual role of prostaglandins is quite complex as a single prostaglandin can bind to multiple receptors. For example, two receptors (DP1 and DP2) were identified for PGD2 the most abundant prostaglandin in the CNS. DP1 activation increases cAMP, activates protein kinase A and is linked to an anti-inflammatory response, while DP2 activation increases the cellular influx of calcium and triggers a pro-inflammatory response [reviewed in [163]]. PGD2-mediated activation of DP1 was found to be neuroprotective while DP2 activation was neurotoxic [109]. Besides binding to different receptors some prostaglandins, such as PGD2, are spontaneously metabolized by non-enzymatic dehydration to cyclopentenone prostaglandins, such as PGJ2. Cyclopentenone prostaglandins may have an anti-inflammatory effect by inhibiting the NFκB pathway or a neurotoxic effect by inducing apoptosis [reviewed in [131]]. In our laboratory we use PGJ2 to induce pathological events relevant to AD in in vitro as well as in vivo studies. PGJ2 causes proteasome impairment, increases the levels of ubiquinated proteins, caspase-3 activation, caspase-3-dependent Tau cleavage at Asp421 that leads to formation of aggregation-prone ΔTau, and formation of ubiquitin aggregates in rat E18 cerebral cortical neurons. PGJ2 also induces loss of dopaminergic neurons in SNpc when injected into mouse brain in a PD mouse model [176].
1.6. NEUROPROTECTION BY PACAP27-INDUCED cAMP SIGNALLING PATHWAY

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a member of the VIP/secretin/glucagon/ hormone superfamily that was first isolated from ovine hypothalamic extracts on the basis of its ability to stimulate cAMP formation in anterior pituitary cells [125]. PACAP is widely distributed throughout the body [191] and has been shown to regulate important biological functions, like control of neurotransmitter release, vasodilation, activation of intestinal motility, increase in insulin and histamine secretion, stimulation of cell proliferation [191]. PACAP is also expressed in the CNS and is known to exhibit neurotrophic and neuroprotective properties [2,13]. PACAP exists in two forms: one contains 38 amino acids (PACAP38), and the other one 27 amino acids (PACAP27).

The action of PACAP is exerted by specific binding to PAC1 as well as to VPAC1 and VPAC2 G proteins coupled receptors. Binding to VPAC receptors is less specific as vasoactive intestinal peptide (VIP) also binds to them with affinity similar to that of PACAP. PAC1 receptors are mainly distributed in the CNS, pituitary and adrenal glands while VPAC receptors are found mainly in lungs, liver and urogenital tract [67,175]. PAC1 receptor is a G-protein coupled receptor which increases intracellular cAMP levels through adenylate cyclase and activates PKA. It can also induce MAP kinase cascades. PACAP and PAC1R expression were mapped to hippocampus, cerebral cortex, basal ganglia, and other brain areas affected in neurodegenerative diseases.

Numerous studies showed that PACAP promotes cell survival in various models of neurodegenerative diseases [152,153,187]. For example, in in vitro PD models of neurodegeneration induced by incubating cultures with 6-hydroxydopamine [186], MPTP [186] or rotenone [196], PACAP enhanced dopamine uptake, increased the number of TH-reactive neurons and prevented apoptotic cell death in a dose dependent manner. In in vivo models of PD
induced by 6-OHDA [153] or MPTP [195], PACAP27 treatment rescued neurons in the substantia nigra pars compacta. It also increased expression of D2 receptors in striatum and improved behavioral deficits. Other beneficial effects against brain damage include neuroprotection in middle cerebral artery occlusion (MCAO) by reducing the infarct volume as well as in traumatic brain injury (TBI) [17]. PACAP levels were shown to be downregulated along with other neurotrophic factors in mouse models of AD which may in part account for increased death of neurons [200]. In vitro studies with AD models using Aβ showed that PACAP protects neurons against the toxic effects of Aβ by reducing caspase-3 activity and thus apoptosis [141]. PACAP also preserved mitochondrial activity and increased alpha-secretase activity. PACAP exerts its neuroprotective effect through multiple pathways. It is strongly anti-apoptotic and anti-inflammatory [31]. It also acts on astrocytes and has anti-oxidant effects. Neurodegenerative diseases, like AD, are characterized by proteasome impairment which strongly contributes to neuropathological symptoms. Therefore means to increase proteasome activity would be useful as potential therapeutic approaches. PACAP provides for such approach, through activation of the cAMP-signaling pathway. Elevating cAMP levels has neuroprotective properties via preventing caspase-3 activation in an in vitro model of AD [124] but also exerts positive effects on the UPP pathway. cAMP activates PKA which in turn phosphorylates Rpt6, a subunit of the 19S regulatory particles that contributes to 26S proteasome stability [35]. cAMP/PKA signaling thus increases 26S proteasome activity and also elevates the expression of proteasome subunits Rpt6 and to a lesser extent β5. Our previous in vitro studies demonstrated that increasing intracellular levels of cAMP via PACAP27 prevented caspase-3-dependent Tau cleavage at Asp421 leading to ∆TAU [124] formation induced by PGJ2, as well as protein aggregation induced by PGJ2. Overall, current
in vitro and in vivo data support the notion that elevating cAMP via PACAP27 prevents AD-like neuropathological symptoms such as PGJ2-induced TAU aggregation and caspase-3 activation.
CHAPTER II

Usp14 inhibitor IU1 is neurotoxic and decreases E1–ubiquitin thioester and ubiquitinated-protein levels concomitantly with lowering ATP levels in rat neurons

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2.1. ABSTRACT

Proteasome activity is reportedly downregulated in Alzheimer’s and Parkinson’s diseases, thus increasing proteasome activity could be therapeutically beneficial. The proteasome-associated deubiquitinase Usp14 disassembles polyubiquitin chains, potentially delaying proteasome-dependent protein degradation. We assessed the protective efficacy of inhibiting or downregulating Usp14 in rat and mouse (Usp14αJ) neuronal cultures treated with prostaglandin J2 (PGJ2). The product of inflammation PGJ2 is neurotoxic and induces accumulation and aggregation of ubiquitinated (Ub)-proteins. We established that: inhibiting Usp14 with high IU1 concentrations (HIU1, >25 µM) reduces the accumulation of Ub-proteins induced by PGJ2, while low IU1 concentrations (LIU1, ≤25 µM) have no impact; HIU1 alone or with PGJ2 is neurotoxic, and induces calpain-dependent cleavage of Tau, caspase and spectrin; HIU1 decreases E1~Ub thioester formation and 26S proteasome assembly, which are energy-dependent processes. We attribute the two latter HIU1 effects to ATP-deficits and mitochondrial Complex I inhibition, as shown herein. These HIU1 effects mimic those of mitochondrial inhibitors, such as oligomycin. Thus, we propose that ATP-depletion is a major mediator of HIU1-actions. Moreover, Usp14 knockdown by siRNA in rat cultures or loss of Usp14 exhibited in cultures from ataxia (Usp14αJ) mice, failed to prevent Ub-protein accumulation induced by PGJ2-treatment. We also show that PGJ2 alone induces Ub-protein accumulation and decreases E1~Ub thioester formation. This seemingly paradoxical result may be explained by PGJ2 inhibiting deubiquitinases resulting in Ub-protein stabilization. In conclusion, we demonstrate that the decline in Ub-protein levels induced by IU1 in neurons correlates with decreased E1~Ub thioester formation rather than with proteasome enhancement.
2.2. INTRODUCTION

The ubiquitin/proteasome pathway (UPP) and autophagy play a critical role in protein quality control and thus have attracted special attention for drug development [39]. Alzheimer’s (AD) and Parkinson’s (PD) diseases are of particular interest since a hallmark of these neurodegenerative disorders is accumulation/aggregation of Ub-proteins in specific areas of the CNS [157]. A potential therapeutic strategy for these disorders could be directed towards deubiquitinases to prevent the accumulation and aggregation of Ub-proteins [24].

In humans there are around one hundred genes encoding deubiquitinases from five different families, four of them being thiol proteases and one a metalloprotease [40]. Deubiquitinases perform a range of functions, including processing newly translated ubiquitin (Ub) to provide monomers for conjugation and chain formation, trimming mono-Ub from the distal end of a poly-Ub chain, disassembling poly-Ub chains, and removing poly-Ub chains from substrates [20]. Overall, these functions provide a means for deubiquitinases to regulate “where, when and why” ubiquitinated substrates are degraded by the 26S proteasome [157].

The deubiquitinase Usp14 is important for development and functioning of the nervous system [23]. Homozygous Usp14 null mice (ataxia mice) exhibit developmental abnormalities including motor impairment, reduced brain mass and death by 2 months of age [25]. Usp14 associates transiently with the proteasome and exhibits Ub-chain trimming activity, thereby replenishing the Ub pool [11]. This deubiquitinase can cause premature dissociation of substrates from the proteasome, if ubiquitin removal is faster than the competing steps leading to substrate degradation [11]. Inhibiting or downregulating Usp14 was proposed as a therapeutic approach for promoting the efficient proteasomal elimination of Ub-proteins by preventing premature trimming.
of their Ub-chains [101].

Selective and reversible inhibition of Usp14 can be achieved with 1-[1-(4-Fluoro-phenyl)-2,5-dimethyl-1H-pyrrol-3-yl]-2-pyrrolidin-1-yl-ethanone, a small molecule known as IU1 [101]. Treatment of murine embryonic fibroblasts (MEFs) and human embryonic kidney (HEK)293 cells with IU1 resulted in the apparent increased degradation of the proteasome substrates Tau, TDP-43 and ataxin-3, which have been implicated in neurodegenerative diseases [101]. These studies suggest that IU1 or IU1-like drugs could be used therapeutically to prevent the neurotoxic build-up of such proteins.

To test if IU1 prevents accumulation of neuronal Ub-proteins, we treated rat and mouse cerebral cortical neurons with the endogenous product of inflammation prostaglandin J2 (PGJ2) [47]. PGJ2 is a product of spontaneous dehydration of prostaglandin D2 (PGD2). The latter is the most abundant prostaglandin in the brain [71,172,190] and the one that increases the most under pathological conditions [109]. In rodents, the in vivo concentration of free PGJ2 in the brain upon stroke and traumatic brain injury increases from almost undetectable to the 100 nM range [115,116]. These values represent average brain levels. Regional brain concentrations of PGJ2 are potentially higher [117], as PGJ2 binds covalently to proteins, and therefore, free PGJ2 does not represent its total amount. Unlike most prostaglandins, PGJ2 and its metabolites have a cyclopentenone ring with reactive α,β-unsaturated carbonyl groups. These carbonyl groups form covalent Michael adducts with cysteine thiols of glutathione or cellular proteins [183]. Electrophiles, such as PGJ2, that bind to specific protein cysteine(s) are regarded to play an important role in determining neuronal survival [162].

We previously established that PGJ2 is the most toxic of four prostaglandins that we tested
in neuronal cells, including PGA1, D2, E2 and J2 [108]. In addition, PGJ2 impairs the UPP by targeting different components of this pathway including the 26S proteasome by perturbing its assembly [139,198], deubiquitinases such as UCH-L1 and Ub isopeptidase activities [93,108,115,130], and by causing the accumulation/aggregation of Ub-proteins [108,116]. Besides its effects on the UPP, we showed that PGJ2 activates caspases and caspase-mediated proteolysis in primary cerebral cortical neuronal cultures, leading to Tau cleavage and pathology [3,124]. In sum, PGJ2 induces a range of pathological processes relevant to neurodegenerative disorders [47].

In the current study we demonstrate that inhibition of Usp14 with high concentrations of IU1 (HIU1, >25 µM) prevents/reduces the accumulation of Ub-proteins in rat cerebral cortical neuronal cultures treated with PGJ2. This effect of HIU1 can be attributed in part to a decline in E1-dependent ubiquitin activation, as we show that IU1 lowers E1–Ub thioester levels. The latter is consistent with the observed drop in ATP levels and mitochondrial Complex I activity induced by HIU1. HIU1 was also neurotoxic and induced calpain-dependent cleavage of Tau, spectrin and caspase. Overall, the effects of HIU1 on neuronal Ub-protein levels, E1- and calpain-activities, Tau cleavage and ATP levels, mimic those of mitochondrial inhibitors, such as oligomycin [78]. Lower IU1 concentrations (LIU1, ≤25 µM), or downregulating Usp14 by siRNA, or loss of Usp14 (Usp14<sup>axl</sup> mouse) had no impact on Ub-protein levels. We also established that PGJ2 lowers E1–Ub thioester levels without directly inhibiting E1 activity. In contrast to IU1, PGJ2 promotes the accumulation of Ub-proteins in neuronal cultures. In conclusion, a deeper understanding of the mechanisms that regulate Ub-protein accumulation, including the balance among E1, deubiquitinase and proteasome activities, is critical for drug development that aims at reducing the accumulation of aberrant proteins in the CNS of patients with chronic neurodegenerative diseases.
2.3. MATERIALS AND METHODS

2.3.1. Materials: Chemicals: IU1 (Life Sensors, Malvern, PA); calpeptin (Calbiochem/EMD Bioscience, Gibbstown, NJ); proteasome substrate Suc-LLVY-AMC (BACHEM Bioscience Inc., King of Prussia, PA); PGJ2 (Cayman Chemical, Ann Arbor, MI); oxidative phosphorylation substrates, creatine phosphokinase, and bovine ubiquitin (Sigma-Aldrich, St. Louis, MO). Primary antibodies: chicken polyclonal anti-Usp14 (1:1,000, cat# AB505) from Life Sensors, Malvern PA; rabbit polyclonal anti-ubiquitinated proteins (1:1,500, cat# Z0458) from Dako North America, Carpinteria, CA; rabbit polyclonal anti-β5 (1:5,000, cat# PW8895), and mouse monoclonal anti-Rpt6 (1:2,000, cat# PW9265), from ENZO Life Sciences, Inc., Farmingdale, NY; mouse monoclonal anti-β-actin (1:10,000, cat# A-2228) from Sigma, St. Louis, MO; mouse monoclonal anti-spectrin α chain (clone AA6, cat# MAB1622) from Millipore, Billerica, MA; mouse monoclonal Tau C3 (1:5,000; detects Tau cleaved at Asp421; ep: a.a. 412–421) and mouse monoclonal Tau C5 (1:50,000; detects all Tau isoforms and ΔTau; ep: a.a. 210–241) were courtesy of Dr. L. Binder (Northwestern University, Chicago, IL, USA); rabbit polyclonal anti-UBE1a (1:1000, cat# 4890) and anti-caspase 3 (1:1000, cat# 9662) from Cell Signaling Technology, Danvers, MA. Secondary antibodies with HRP conjugate (1:10,000) from Bio-Rad Laboratories, Hercules, CA.

2.3.2. Mice - C57BL/6J mice (wild type) and Usp14axJ mice maintained on a C57BL/6J background (Jackson Laboratories, Bar Harbor, MA) have been maintained in the breeding colony at the University of Alabama at Birmingham. Homozygous Usp14axJ mice were generated by intercrossing heterozygous axJ siblings.

2.3.3. Cell cultures - Dissociated cultures from Sprague Dawley rat embryonic (E18, both sexes)
cerebral cortical neurons were prepared as in [78]. Dissociated cultures from wild type and homozygous Usp14axJ mouse embryos were prepared as in [28]. The isolated cortices free of meninges were digested with papain (0.5 mg/ml from Worthington Biochemical Corp.) in Hibernate E without calcium (BrainBits LLC.) at 37°C for 30 min in a humidified atmosphere containing 5% CO2. After removal of the enzymatic solution, the tissues were gently dissociated in Neurobasal media (Invitrogen). Dissociated tissues were centrifuged at 300Xg for 2 min. 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). Cells were plated at a density of 6X10^6 cells per 10 cm dish, or 2.5X10^5 cells per well on 24-well plates (cell viability only). Cultures were maintained in Neurobasal media supplemented with 2% B27 and 0.5 mM L-Glutamax (all from Invitrogen) at 37°C in a humidified atmosphere containing 5% CO2. Half of the medium was changed every 4 days. Experiments were run 8-11 DIV. According to manufacturer’s specifications, Neurobasal medium contains several proprietary factors that ensure a mostly pure (> 95%) neuronal culture; glial growth is inhibited without a need for the anti-mitotic agent arabinofuranosyl cytidine [14,135].

2.3.4. Culture treatments – Cortical neurons were treated acutely (4 h, 8 h, 16 h or 24 h) with DMSO or with the different drugs in DMSO added directly to DMEM without serum, supplemented with 0.5 mM L-Glutamax and 1 mM sodium pyruvate (all from Invitrogen). The final DMSO concentration in the medium was 0.5%.

2.3.5. Cell viability assay - Cell viability was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described [128].

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

2.3.7. Mitochondrial respiratory chain complex activities - Bovine heart submitochondrial particles (SMP) were prepared according to standard procedures [53] and stored in liquid nitrogen. Before activity measurements, SMP were resuspended to 5 mg/ml in SET buffer pH 7.5 (0.25 M sucrose, 0.2 mM EDTA, 50 mM Tris-HCl), containing 0.2 mM malonate and incubated at 30°C for 30 min. NADH-dependent enzymatic activities of Complex I were assayed spectrophotometrically (Perkin Elmer Lambda 35) as a decrease in absorption at 340 nm ($\varepsilon_{340}$ nm = 6.22 mM$^{-1}$ cm$^{-1}$) with 150 μM NADH in SET buffer containing 2.5–25 μg of protein/ml SMP. For the measurements of NADH:Q1 or NADH:HAR oxidoreductase activity, SMP were assayed in the presence of 1 mM cyanide with the addition of 80 μM Q1 or 1 mM HAR, respectively. The Complex IV activity was measured at 550 nm ($\varepsilon_{550}$ nm = 21.5 mM$^{-1}$ cm$^{-1}$) with 45 μM ferrocytochrome c in two fold diluted SET buffer containing 0.025% laurilmaltoside and 2.5-5 μg of protein/ml SMP. Since IU1 strongly absorbs in the near UV region, succinate oxidase activity was measured as oxygen consumption using Oroboros oxygraph in SET buffer containing 5 mM succinate and 20 μg of protein/ml SMP. To measure the effect of IU1 on respiratory chain
enzymes, SMP were preincubated directly in the measuring medium for 2 min with the inhibitor before initiating the reaction with a substrate. Separate control studies confirmed the preincubation time was sufficient to reach equilibrium in the enzyme-inhibitor reaction. All activities were measured at 30°C and expressed in μmol of substrate×min⁻¹×mg⁻¹. Protein concentration was determined with the BCA assay. Reported values are the mean ± SD (at least 3 independent experiments).

2.3.8. Western blotting - After treatment, cells were rinsed twice with PBS and harvested by gently scraping into ice-cold lysis buffer [20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 1 mM EGTA, 2.5 mM Na₄P₂O₇, 1 mM β-glycerophosphate, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1% NP40, 1 mM Na₃VO₄, 1% Glycerol and protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO)]. Following lysis (at least 30 min, -80°C), cell extracts were centrifuged (19,000xg for 10 min) at 4°C. Protein concentration of the NP40-soluble supernatants was determined with the BCA assay. Western blot analysis was carried out following SDS-PAGE. Normalized samples were boiled for 5 min in Laemmli buffer and loaded onto gels (30 µg 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 densitometry including image analysis with the ImageJ program (Rasband, W.S., ImageJ, U.S. NIH, Maryland, http://rsb.info.nih.gov/ij/, 1997-2006).

2.3.9. Evaluation of endogenous E1–ubiquitin thioester – Upon treatment with vehicle (control, DMSO) or with the respective drugs, cortical neurons were washed once with PBS, harvested with a thiol stabilizing buffer [5 mM Tris-HCl, pH 7.8, 8.7 M urea, 1% Nonidet P-40, 20 mM N-
ethylmaleimide, 3 mM EDTA, 2% protease inhibitor cocktail (Sigma), and kept on ice for 15 min for lysing, as described [85]. Samples were sonicated for 10 s, centrifuged at 19,000Xg for 15 min at 4°C, mixed (30 μg) 1:2 (volume) with thioester gel buffer (33 mM Tris-HCl, pH 6.8, 2.7 M urea, 2.5% SDS and 13% glycerol), and boiled for 5 min. After determination of the protein concentration with the Bradford assay (Bio-Rad Laboratories), the normalized samples were separated into reducing (with 4% β-mercaptoethanol) and non-reducing (no β-mercaptoethanol) aliquots for SDS-PAGE, followed by western blotting with anti-E1 antibody, as described above.

2.3.10. In vitro E1 activity assay-- Commercial bovine ubiquitin was further purified to apparent homogeneity by FPLC and quantified spectrophotometrically [5]. Ubiquitin was radioiodinated by the Chloramine-T procedure using carrier-free Na\textsuperscript{125}I to yield a specific radioactivity of ~20,000 cpm/pmol [64]. Human Uba1 was purified to apparent homogeneity from outdated erythrocytes and active Uba1 was determined by the stoichiometric formation of \textsuperscript{125}I-ubiquitin thioester [64]. One pmol of Uba1 was pre-incubated with 0.5% DMSO (vehicle) or 15 μM PGJ2 (0.5% DMSO) at 4°C in 50 mM Tris-HCl (pH 7.5) for 0, 1, 2, 4, 6, 12, and 24 hours. Formation of E1 thioester was analyzed at 37°C in 25 μl reactions containing 50 mM Tris-HCl (pH7.5), 1 mM ATP, 10 mM MgCl\textsubscript{2}, 10 mM creatine phosphate, 1 IU of creatine phosphokinase and an aliquot of the time point equivalent to 40 nM Uba1 (original content). Reactions were started by the addition of 4 μM \textsuperscript{125}I-ubiquitin and quenched after 1 min by the addition of 25 μl 2X SDS sample buffer. The E1\textemdash\textsuperscript{125}I-ubiquitin thioesters were resolved from free \textsuperscript{125}I-ubiquitin by 12% SDS-PAGE under non-reducing conditions at 4°C and visualized by autoradiography. Where indicated, E1 was quantified by excising the E1\textemdash\textsuperscript{125}I-ubiquitin thioester band, quantifying associated radioactivity by gamma counting, and calculating absolute thioester formation using the corrected specific radioactivity of the \textsuperscript{125}I-ubiquitin [64].
2.3.11. In gel proteasome activity and levels – Upon treatment with vehicle (control, DMSO) or the respective drugs, cells were washed twice with PBS and harvested for the in gel assay as described in [132]. The native gels loaded with 30 µg protein/lane, were run at 150 V for 120 min. The in gel proteasome activity was detected by incubating the native gel on a rocker for 10 min at 37°C with 15 ml of 300 µM Suc-LLVY-AMC followed by exposure to UV light (360 nm). Gels were photographed with a NIKON Cool Pix 8700 camera with a 3-4219 fluorescent green filter (Peca Products, Inc). Proteins on the native gels were transferred (110 mA) for 2 h onto PVDF membranes. Immunoblotting was carried-out for detection of the 20S and 26S proteasomes with the anti-Rpt6 and anti-β5 antibodies, which react with subunits of the 19S or the 20S particles, respectively. Antigens were visualized by a chemiluminescent horseradish peroxidase method with the ECL reagent.

2.3.12. Caspase-3 and calpain activation – Cell lysates were analyzed by standard western blotting with the anti-caspase-3 antibody to detect caspase-3 cleavage that is indicative of apoptosis. Calpain activation was assessed with the anti-α-spectrin antibody. Calpain cleavage of α-spectrin generates a 150/145 kDa doublet, while caspase cleavage generates a 120 kDa fragment [197].

2.3.13. siRNA – The dicer siRNA substrates targeting the Usp14 gene obtained from IDT (Coralville, Iowa), were encapsulated into lipid nanoparticles using microfluidic technology by Precision NanoSystems (SUB9KITS™; Vancouver, Canada). Cells were treated with 0.1 µg/ml of Neuro9™ RNAi nanoparticles as described in [161] for 72 h prior to treatment with 10 µM PGJ2 for 16 h. During siRNA treatment, cells remained in Neurobasal media supplemented with 2% B27 and 0.5 mM L-Glutamax (all from Invitrogen) at 37°C in a humidified atmosphere containing 5% CO₂. Following the 72 h incubation period with siRNA, the medium was changed.
to DMEM without serum, supplemented with 0.5 mM L-Glutamax and 1 mM sodium pyruvate (all from Invitrogen) and cells were treated with 10 µM PGJ2 for 16h.

2.3.14. Statistical analysis - Statistical significance was estimated using one-way ANOVA (Tukey-Kramer multiple comparison test) or the Welch's t-test (two group comparison) with the Prism 6 program (GraphPad Software, San Diego, CA).

2.4. RESULTS

2.4.1. IU1 blocks PGJ2-induced accumulation of ubiquitinated proteins and triggers calpain-mediated cleavage of Tau, caspase 3 and spectrin – Since IU1 was proposed to promote protein degradation by the proteasome [101] we examined its effects on neuronal Ub-protein levels. IU1 was not previously tested on neurons [101]. To increase the endogenous levels of Ub-proteins, the rat cerebral cortical neuronal cultures were treated with the product of inflammation PGJ2 [174,190]. The neurons were pre-incubated with IU1 (75 µM) for 6 h prior to PGJ2 treatment (15 µM, 16 h). A similar protocol was used to establish protection by IU1 against proteotoxic stress in HEK293 cells treated with menadione, an oxidative stressor [101]. As expected, PGJ2 significantly increased the levels of Ub-proteins but IU1 by itself did not (Fig. 6, lanes 2 and 3, panels 1 and 2). In addition, IU1 blocked the accumulation of Ub-proteins induced by PGJ2 (Fig. 6, compare lanes 2 and 4, panels 1 and 2). Compared to control conditions (lane 1) the levels of free Ub were not decreased by any of the treatments (Fig. 6, panels 1 and 2).
IU1 was previously shown to promote the degradation of a Tau isoform overexpressed in wild type MEFs [101]. Tau is a microtubule associated protein that is abundant in neurons and...
highly soluble, yet Tau forms abnormal aggregates and is the major component of neurofibrillary tangles, one of the hallmarks in AD [121]. Confirming what we previously demonstrated [124], PGJ2 induced caspase-mediated cleavage of Tau (ΔTau, *Fig. 6, panel 4, lane 2*). ΔTau is prone to aggregation and its formation is an early event in AD tangle pathology [29,54,156]. In contrast to PGJ2, IU1 induced calpain-mediated cleavage of Tau (*Fig. 6, panel 3, lane 3*), reminiscent of what we observed in cortical neurons treated with the mitochondrial inhibitor oligomycin [78]. IU1 combined with PGJ2 further increased calpain-mediated cleavage of Tau (*Fig. 6, panel 3, lane 4*).

We established that IU1-treatment induces calpain activation, while PGJ2-treatment triggered mostly caspase 3 activation. As shown in Fig. 6 (*panel 5, lane 3*), IU1 triggered the cleavage of pro-caspase 3 to a ~29 kDa inactive fragment [143] (Cl-caspase 3) and not to its active 17 kDa form (Act-caspase 3). On the other hand, PGJ2 induced the cleavage of pro-caspase 3 (33 kDa) to its active form (17 kDa, Act-caspase 3, *Fig. 6, panel 5, lane 2*), as we previously showed [3]. We further confirmed that calpain was activated upon IU1-treatment by assessing cleavage of α-spectrin, a calpain substrate. IU1-treatment by itself or in combination with PGJ2 clearly induced cleavage of α-spectrin (Pro-α-spectrin, 280 kDa) to 145/150 kDa fragments [Cl-α-spectrin (150)], which are indicative of calpain activation (*Fig. 6, panel 6, lanes 3 and 4*). In contrast, PGJ2-treatment mostly induced α-spectrin cleavage to a 120 kDa α-spectrin fragment [Cl-α-spectrin (120)], which is a marker of apoptotic cell death [197]. Furthermore, pre-treatment with the calpain inhibitor calpeptin (Z-Leu-norleucinal) prevented calpain-dependent Tau and spectrin cleavages induced by IU1 alone (*Fig. 6, panels 3 and 6, compare lanes 3 and 6*). In contrast, treatment with both calpeptin and IU1 stimulated caspase-dependent Tau cleavage the most (*Fig. 6, panel 4, lane 6*), indicating caspase activation under these conditions as discussed below.
We next addressed if lower IU1 concentrations (≤25 µM) also blocked the accumulation of Ub-proteins induced by PGJ2. For these experiments we reduced the PGJ2 concentration to 10 µM, as we also lowered IU1 levels. Adding IU1 to the neuronal cultures prior to PGJ2-treatment had the same effect as co-incubation with the two drugs (*not shown*). Therefore, in all subsequent experiments both agents were added at the same time. Although in some experiments IU1 at a 25 µM appeared to decrease the levels of Ub-conjugates induced by 4 h (*Fig. 7A and 7C*), or 24h PGJ2-treatment, this effect was not statistically significant (*Fig. 7B and 7C*).
Figure 7. Low concentration of IU1 (≤25 µM) did not affect the accumulation of Ub-proteins induced by PGJ2. NP-40 soluble fractions from rat E18 cerebral cortical neurons treated as indicated were analyzed by western blotting (30 µg of protein/lane) and probed with the respective antibodies to assess the effect of the treatments on the level of ubiquitinated proteins (A and B) using actin as loading control. Quantification (C). Molecular mass markers in kDa are shown. In C the levels of ubiquitinated proteins (polyUb/actin) were semi-quantified by densitometry. Data represent the percentage of the pixel ratio for Ub-proteins over the respective loading control for each condition compared to control (100%). Values are means from at least three experiments.
2.4.2. IU1 reduces cell viability and depletes intracellular ATP levels – Calpain-activation is linked to ATP-depletion and necrosis, a cell death pathway associated with a bioenergetic crisis [209]. In fact, calpain-activation was shown to be induced by inhibitors of oxidative phosphorylation, such as rotenone, antimycin and oligomycin [173]. As we established that IU1 induces calpain activation, we reasoned that IU1 may be neurotoxic and lower intracellular ATP levels.

![Figure 8. IU1 reduces neuronal viability](image)

As shown in Fig. 8, IU1 caused a decline in cell viability in a concentration (panel A) and time-dependent-manner (panel B), the latter assessed for 25 µM. In addition, treatment with PGJ2 (10 µM) and IU1 (25 µM) combined was even more neurotoxic than any of the drugs alone (panel C).
C). The loss of neuronal viability was assessed with the MTT assay, which is reduced largely within the cytoplasm [10,118].

As we predicted, IU1 decreased ATP levels also in a concentration (Fig. 9A) and time dependent manner (Fig. 9B). Moreover, treatment with PGJ2 (10 µM) combined with IU1 (25 µM) decreased ATP levels the most, compared to the effectiveness of either compound alone (panel C).

The decline in ATP levels induced by the 24h treatment with 25 µM IU1 varied between 70% (Fig. 9B) and 90% (Fig. 9A) of control. The loss of cell viability under the same conditions fluctuated between 40% (Fig. 8 A and B) and 80% (Fig. 8C) of control. This variation could reflect somewhat the heterogeneity of the primary neuronal cultures for each experiment that are prepared from a different set of rat embryos. The finding that neurons that exhibit a 70% to 90% loss of ATP still exhibit low levels of viability is in line with other studies. For example, we showed that 30% of oligomycin-treated rat cortical neurons exhibiting a loss of ~78% of their ATP where still viable [78]. In our studies, the neurons in culture are post-mitotic and not engaged in synaptic activity or cell division thus may require low ATP levels for survival [145].
2.4.3. IU1 inhibits mitochondrial Complex I - (These studies were carried-out by Alexander Galkin, School of Biological Sciences, Queen's University Belfast, Belfast, BT9 7BL, United Kingdom, Feil Family Brain and Mind Research Institute, Weill Cornell Medical College, New York, NY 10065, USA; Anna Stepanova, N.K. Koltzov Institute of Developmental Biology, Russian Academy of Sciences, Moscow 119334, Russia). As shown in Fig. 10, NADH-oxidase (A) and NADH:Q1 reductase (B) activities of Complex I were inhibited by IU1, the latter with an IC50 of around 40 µM. This indicates a direct effect of IU1 on physiological oxidation of NADH by ubiquinone in mitochondrial Complex I. At a much higher concentration IU1 also inhibited oxidation of NADH by the artificial acceptor hexaammineruthenium (HAR, C). In addition, at this concentration range IU1 had almost no effect on succinate-oxidase (Complex II-IV, D) or on cytochrome c oxidase alone (Complex IV, E).
2.4.4. IU1-induced decline in Ub-protein accumulation correlates with E1 failure - To address a mechanism mediating the IU1-induced decline in Ub-protein accumulation, we focused on the E1 ubiquitin activating enzyme. E1 activity requires ATP for formation of a thioester adduct with ubiquitin. In principle, if E1 activity is impaired, protein ubiquitination should be diminished. To assess E1~Ub thioester levels, which are sensitive to reducing agents, the samples were run on SDS-PAGE under reducing (with β-mercaptoethanol) and non-reducing (without β-mercaptoethanol) conditions. As shown in Fig. 11A (panel 1), in the control sample under non-reducing conditions E1~Ub thioester (~126 kDa) migrated ~9 kDa above native E1 (117 kDa),
consistent with the additional mass of ubiquitin. PGJ2 (15 µM) or HIU1 (50 µM)-treatment abolished the E1~Ub thioester, reflecting the loss of the ubiquitin monomer linked to E1 (Fig. 11A, panel 1). The effect of lower IU1 concentrations (≤25 µM) alone on E1~Ub thioester was minimal. As expected, when PGJ2 was combined with increasing concentrations of IU1 (Fig. 11A, panel 1), the E1~Ub thioester was almost undetectable.

Figure 11 A. IU1-induced decline in Ub-protein accumulation correlates with E1 failure

Under reducing conditions (Fig. 11A, panel 2) only native E1 was detected in all treatments. PGJ2 combined with 50 µM IU1, decreased the levels of E1~Ub thioester and native E1. As shown
on the graphs on the right, total E1 levels (E1–Ub thioester + native E1) assessed under reducing conditions were not altered significantly (p>0.05), except in the PGJ2 plus IU1 (50 µM) treatment (p = 0.0014, quantification shown in the graphs on the right). These data demonstrate that in neurons HIU1-mediated ATP depletion prevents E1 from forming thioester intermediates with ubiquitin. The latter correlates with a major down-regulation of Ub-proteins in PGJ2-treated neurons (Fig. 11A panel 3). Unconjugated (free) ubiquitin and actin levels (Fig. 11A, panels 3 and 4, respectively) were not decreased by IU1.

2.4.5. PGJ2 induces a time-dependent decline in E1~Ub thioester formation while raising the levels of Ub-proteins – We characterized the temporal effect of PGJ2 on E1~Ub thioester formation and compared it with the accumulation of Ub-proteins in the rat neurons. As shown in Fig. 11B (panel 1), 15 µM PGJ2 prevented E1~Ub thioester formation by 8 h of treatment. Moreover, PGJ2 increased the accumulation of Ub-proteins by 8 h of incubation (Fig. 11B, panel 3), despite the absence of E1~Ub thioester formation (Fig. 11B, panel 1). This seemingly paradoxical result is addressed below in the discussion.

To determine if the PGJ2-dependent ablation of cellular E1~Ub formation was a direct effect, such as from covalent adduct formation at the active site cysteine of the activating enzyme, a biochemically-defined assay for E1 thioester formation in the absence or presence of PGJ2 was conducted (Fig. 11C). Human E1 is relatively stable in the presence of DMSO alone or in the presence of 15 µM PGJ2 in DMSO for up to 24 hours (Fig. 11C). These studies (Fig. 11C) were carried-out by Arthur Haas and Dustin R. Todaro, Department of Biochemistry and Molecular Biology, LSU Health Sciences Center, New Orleans, LA 70112, USA.
Figure 11 B, C. PGJ2 induces a time-dependent decline in E1–Ub thioester formation while raising the levels of Ub-proteins. (B) NP-40 soluble fractions from rat E18 cerebral cortical neurons treated as indicated were analyzed by western blotting (30 µg of protein/lane) probed with the respective antibodies to detect E1–Ub (ubiquitin) thiol esters and native E1, run under non-reducing conditions (panel 1), or reducing conditions with β-mercaptoethanol (panels 2); Ub-proteins (panel 3); actin (loading control). (C) E1 thioester formation essay
2.4.6. \( \text{IIU1 induces a decline in 26S proteasomes and a concomitant increase in 20S proteasomes} \) – The activity and assembly/disassembly of 26S proteasomes are highly dependent on ATP binding and hydrolysis [44,114]. We thus assessed with the native in-gel assay, the effects of IU1 on proteasome activity and levels in the cortical neurons. The in-gel assay detects the three assembled forms of the proteasome: 26S proteasomes with either two regulatory caps [26S(2)] or one cap [26S(1)], and the 20S core particle alone (20S). Proteasome activity was determined with the substrate Suc-LLVY-AMC, which assesses the chymotrypsin-like activity (Fig. 12). Under control conditions (lanes marked with “0”), the activity of the 20S proteasome is substantially lower than that of the 26S, because the 20S is a latent proteasome form [60]. Proteasome levels were determined by immunoblotting with the anti-Rpt6 antibody that reacts with an ATPase subunit of the 19S particle (Fig. 12, panel 3), and with the anti-\( \beta5 \) antibody (Fig. 12, panel 4). The \( \beta5 \) subunit is a component of the 20S core, thus its antibody detects assembled 26S and 20S proteasomes. It is clear that \( \text{IIU1 induced a decline in 26S proteasome activity that correlates with lower ATP levels} \) [compare Fig 9A for ATP with Fig. 12 (panel 1) for 26S proteasome activity]. Furthermore, \( \text{IIU1 blocked 26S assembly (or promoted disassembly), as its activity and levels decreased while those of the 20S proteasome increased} \) (Fig. 12, panels 1 and 2 for activity and panels 3 and 4 for levels). PGJ2-mediated inhibition of the 26S proteasome is shown for comparison. Semi-quantification of proteasome activity and levels for each condition is shown in Fig. 12B.
Figure 12. HIU1 induces a decline in 26S proteasomes and a concomitant increase in 20S proteasomes
Neurons were treated for 24h with 10µM PGJ2 or various concentrations of IU1 (A). Lysates (30µg/sample) were subjected to non-denaturing gel electrophoresis as described under “Experimental Procedures”. Fully assembled 26S and 20S proteasomal (indicated in the middle by arrows) chymotrypsin-like activity was assessed with Suc-LLVY-AMC by the in-gel assay (panels 1 and 2). To improve detection of 20S proteasome activity, 0.04% SDS was added to the reaction buffer in panel 2. Proteasome levels were detected by immunoblotting with anti-Rpt6 (panel 3) and anti-β5 antibodies (panel 4). Proteasome chymotrypsin-like activity and levels were quantified by densitometry (B). Percentages represent the ratio between data for each condition and control (DMSO) considered to be 100%. Values are means from at least three experiments. Asterisks identify values that are significantly different from control (*p<0.05, **p<0.01, ***p<0.001).
2.4.7. Usp14 knockdown by siRNA or Usp14 loss (Usp14<sup>axJ</sup> mouse) did not prevent the deleterious effects of PGJ2 – To determine if lowering Usp14 levels counteracts some of the PGJ2 effects on the neurons, we induced Usp14 knock down by siRNA prior to treatment with vehicle (control) or PGJ2 (Fig. 13B, panel 1). Usp14 siRNA had no clear impact on the PGJ2-induced changes on neuronal viability (Fig. 13A), ΔTau, the caspase-dependent Tau fragment (Fig. 13B, panel 2), activated caspase 3 (Fig. 13B, panel 3), or ubiquitinated proteins (Fig. 13B, panel 4).

Likewise, studies with cortical cultures from Usp14<sup>axJ</sup> mice which exhibit a 90-95% loss of Usp14 (Fig 13C, panel 1), show that Usp14 depletion has no benefit against the deleterious effects of PGJ2 tested, i.e. caspase-activation, and accumulation of Ub-proteins (Fig.13C, panels 2 to 3, respectively). These studies (Fig. 13C) were carried-out by Scott M. Wilson, Department of Neurobiology, Civitan International Research Center, University of Alabama at Birmingham, Birmingham, AL 35294.

![Figure 13 A. Usp14 siRNA had no clear impact on the PGJ2-induced changes on neuronal viability.](image-url)

*Figure 13 A. Usp14 siRNA had no clear impact on the PGJ2-induced changes on neuronal viability.* Neurons were treated with indicated concentrations of IU1 and/or 10µM PGJ2. Cell viability was assessed with the MTT assay. Percentages represent the ratio between the data for each condition and control (100%). Values indicate means and s.e.m. from at least three experiments per group. Asterisks identify values that are significantly different from control (* p<0.05; ** p<0.01; *** p<0.001)
Figure 13 B. Usp14 knockdown by siRNA did not prevent the deleterious effects of PGJ2 Neurons were treated with 0.1 µg/ml of Neuro9™ RNAi nanoparticles targeting Usp14 gene as described in ‘Materials and Methods’ section. Cell viability was assessed with the MTT assay (A). Percentages represent the ratio between the data for each condition and control (100%). Values indicate means and s.e.m. from at least three experiments per group. NP-40 soluble fractions from rat E18 cerebral cortical neurons treated as indicated were analyzed by western blotting (20 µg of protein/lane) (B) and probed with the respective antibodies to assess the effect of the treatments on: Usp14 (panel 1), Tau cleaved at Asp412, ΔTau (panel 2), caspase 3 (panel 3), level of ubiquitinated proteins (panel 4) and actin as loading control (panel 4). Molecular mass markers in kDa are shown on the right.
Figure 13C. Usp14 loss (Usp14axJ mouse) did not prevent the deleterious effects of PGJ2. Dissociated cultures from homozygous Usp14<sup>axJ</sup> mice prepared as in [24] were treated as indicated. Loss of Usp14 in the KO mice did not prevent activation of caspase-3 nor accumulation of Ub-proteins. Analysis in progress.
2.5. DISCUSSION

In this study we characterize the mechanism(s) by which the Usp14 inhibitor IU1 affects the ubiquitin/proteasome pathway (UPP) in rat cerebral cortical neurons. In non-neuronal cells, IU1 is proposed to enhance the degradation of a range of proteasome substrates including Tau and oxidized proteins [101], which are implicated in neurodegenerative disorders such as AD and PD. Thus, it was critical to investigate the impact of IU1 on neurons.

We show that 75 µM IU1 prevented the accumulation of Ub-proteins induced by PGJ2 in neurons, while IU1 concentrations ≤25 µM had no impact. IU1 (75 µM) was shown to reduce menadione-induced accumulation of oxidized proteins in non-neuronal HEK293 cells [101]. Like PGJ2 [108,115], the vitamin K analog menadione (K3) induces accumulation of Ub-proteins [199,205]. However, whether or not IU1 blocks menadione-induced accumulation of Ub-proteins was not addressed [101]. This is important, as non-aggregated Ub-proteins are turned over by the 26S proteasome, the activity of which is enhanced by inhibiting Usp14 with IU1 [101]. On the other hand, most (70% to 80%) of all oxidized proteins that are not aggregated are degraded by 20S proteasomes in concert with immunoproteasomes [147]. The latter forms of the proteasome do not associate with Usp14 and are not postulated to be affected by IU1 [101,146]. We discuss the impact of IU1 on 20S proteasome activity in neurons below.

We also demonstrate that 75 µM IU1 induces calpain-dependent cleavage of endogenous Tau, as Tau cleavage is blocked by a calpain inhibitor (calpeptin). This is further supported by the finding that IU1-treatment clearly induces cleavage of α-spectrin (280 kDa) to 145/150 kDa fragments, which are indicative of calpain activation [197]. Moreover, the IU1-induced Tau fragments include a typical “17 kDa” fragment, which represents a marker for enhanced calpain activity in AD [46,55].
Calpain-activation is linked to ATP-depletion and necrosis, a cell death pathway characterized by a bioenergetic crisis [209]. It is well established that calpain-activation is induced by mitochondrial inhibitors, such as oligomycin and antimycin [173]. Of relevance, we find that the pattern of IU1-induced Tau cleavage in neurons resembles that observed in our previous studies with these inhibitors [78]. In a similar manner, IU1 induced calpain-mediated cleavage of caspase 3 to a fragment (29 kDa) associated with caspase inactivation [78,143]. Calpain processing of caspase 3 to the 29 kDa inactive form could be a strategy to prevent execution of the apoptotic pathway under conditions of ATP-deficit, as apoptosis is an energy-dependent death pathway [209]. Together these data show that IU1 (75 µM) treatment in neurons activates calpain-dependent cleavage of Tau, spectrin and caspase 3, and suggest that IU1 may be neurotoxic and diminish intracellular ATP levels.

We also establish that high IU1 concentrations (IU1 ≥25 µM) are significantly neurotoxic as expected, since IU1-treatment stimulated calpain-dependent cleavage of a range of substrates. These findings suggest that IU1-treatment induces cell death via necrosis and not apoptosis, since it induces calpain and not caspase 3 activation. Furthermore, IU1 (≥25 µM) concentrations cause mitochondrial dysfunction reflected in significant decreases in intracellular ATP levels. Of relevance, we show that IU1 inhibits mitochondrial Complex I in vitro. Taking into account the high degree of flux control of mitochondrial Complex I over oxidative phosphorylation in neuronal tissues [56,80,98], inhibition of even a small fraction of the enzyme, may lead to a significant decrease in ATP production by mitochondria [192]. It cannot be excluded that IU1 inhibition of NADH oxidation results in a change of redox state of the mitochondrial matrix leading to an imbalance of ROS metabolism. PGJ2-treatment combined with IU1 exacerbated the detrimental effects of IU1 on neuronal viability and ATP-level.
We demonstrate that IU1-treatment (IU1 >25 µM) impairs E1 and 26S proteasome activities, both of which are ATP-dependent [44,114,167,188]. HIU1 hinders the ubiquitination cascade by blocking its first step; i.e., HIU1 prevents ubiquitin-activation by the E1 enzyme, without decreasing free Ub levels. The combined treatment with PGJ2 and IU1 exacerbated E1 dysfunction, compared to IU1 alone. Our data strongly support the view that IU1 prevents PGJ2-induced accumulation of Ub-proteins, not by enhancing their turnover by the 26S proteasome, but instead by blocking the ubiquitination cascade causing lower levels of Ub-proteins to be formed. This would explain why proteasome substrates such as TDP-43 accumulate as ubiquitinated-TDP-43 reflecting 26S proteasome inhibition (as discussed below), while little change was detected in bulk ubiquitin conjugates in IU1-treated non-neuronal cells [101].

HIU1 elicited the demise of the 26S proteasome in neurons by diminishing its assembly, which partially correlates with ATP-depletion. Concomitant with the decline in 26S proteasomes, HIU1-treatment induced a rise in the activity and levels of 20S proteasomes; that is HIU1 mimicked the effects of mitochondrial inhibitors on proteasomal function, just as found for calpain-activation and ATP-depletion [78]. We speculate that the significant increase in 20S proteasome activity induced by HIU1-treatment, contributes to the reduction in menadione-induced accumulation of oxidized proteins in HEK293 cells [101]. Menadione is a highly toxic oxidant that elevates superoxide levels [50]. Upon ATP-depletion triggered by IU1-treatment it is possible that 20S proteasomes are recruited to promote turnover of oxidized proteins independently of ubiquitination [62,87]. Moreover, the 26S proteasome and the ubiquitination machinery are more vulnerable to oxidative damage than 20S proteasomes [154]. Autophagy is not likely to be involved in the degradation of menadione-induced oxidized proteins, as IU1 was shown to have no effect on autophagy [74].
As shown in the current studies, lowering Usp14 levels by siRNA or loss of Usp14 exhibited by neuronal cultures from Usp14axJ mice, did not counteract or exacerbate the detrimental effects of PGJ2 on neurotoxicity, caspase 3-activation, caspase-dependent Tau cleavage and accumulation of Ub-proteins. These results are in agreement with studies with HeLa cells showing that siRNA for Usp14 alone had no effect on, for example, cell growth [94,96]. The combined siRNA approach to knockdown Usp14 and another DUB UCH37, was necessary to observe accumulation of Ub-proteins [94,96]. The functional relationship among multiple deubiquitinases in terms of substrate hydrolysis and protein homeostasis, in particular in neurons, requires further investigation.

Finally, we confirm that PGJ2 alone induces the accumulation of Ub-proteins, which is consistent with previous studies showing that PGJ2 lowers 26S proteasome levels and activity [81,124], and inhibits some of the thiol deubiquitinases including UCH-L1 and UCH-L3 [108,115], as well as Ub-isopeptidase activity [130]. We also show, for the first time, that PGJ2 lowers E1-Ub thioester levels. This result is surprising since we have shown that drugs that lower E1-Ub thioester levels, such as IU1 (current study) and mitochondrial inhibitors [oligomycin and antimycin in [78]] do not cause accumulation of Ub-proteins in neurons. It is likely that the accumulation of Ub-proteins detected in PGJ2-treated neurons reflects its ability to inhibit deubiquitinases that may stabilize the Ub-proteins formed prior to E1-Ub thioester depletion.

In summary, our findings show that IU1 is neurotoxic and lowers neuronal ATP levels by inhibiting mitochondrial Complex I, leading to a decrease in E1-Ub thioester, Ub-protein, and 26S proteasome levels, as well as calpain activation. In addition, pharmacologic (with IU1) or genetic (with siRNA or by a spontaneous mutation in Usp14axJ mice) inhibition of Usp14 fails to protect neurons against the detrimental effects of PGJ2. However, our data do not exclude the possibility
that inhibiting Usp14 (aside from IU1) is beneficial against other conditions that disturb protein homeostasis. For example, inhibiting Usp14 with three specific RNA aptamers [single-stranded synthetic RNA molecules that act like “chemical antibodies” [45]] facilitated the degradation of Tau conditionally expressed in HeLa cells [103]. In agreement with our data, the latter study with HeLa cells established that IU1 (~167µM) is significantly cytotoxic [103]. We also established that neurons accumulate IU1 to a higher intracellular concentration than MEFs. Using mass spectrometry we determined that neurons accumulate IU1 to ~90% of the extracellular concentration, while MEFs were determined to accumulate IU1 to only 26% of the extracellular concentration. The reason for this discrepancy remains to be established, but it may explain why the neurons are more sensitive than the MEFs to IU1. In conclusion, due to the magnitude and diversity of the enzymes involved in the UPP, a detailed understanding of their reaction mechanisms and relationships in neurons is of the utmost therapeutic interest, since reducing the levels of aberrant proteins in neurons is highly relevant to neurodegenerative disorders, such as AD and PD [77].
CHAPTER III

Infusion of the cyclooxygenase product of inflammation – Prostaglandin J2 into mouse hippocampi induces AD-like pathology in an age-dependent manner

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3.1. ABSTRACT

Upregulation of cyclooxygenase-2 (COX-2) has emerged as an important determinant of the cytotoxicity associated with neuroinflammation in AD. Prostaglandins (PGs) are major products of cyclooxygenases, but their role in neurodegeneration is poorly understood. PGD2 is the most abundant prostaglandin in the brain and increases the most under pathological conditions. Specifically, cortical neurons in AD brains were shown to exhibit accelerated PGD2 synthesis. PGD2 is unstable and is converted to the highly neurotoxic PGJ2 by spontaneous dehydration. In preliminary studies to address the in vivo effects of PGJ2 we established a mouse model of neuroinflammation relevant to AD. PGJ2 (16.7ug/2ul/week, for 3 weeks) was bilaterally microinfused into the CA1 hippocampal brain region of aged (53 weeks of age) and young (12 weeks of age) mice. Following one week of post-surgery recovery, all mice underwent a 12-day training period on the radial 8-arm maze (RAM) to assess spatial learning and memory. Our data revealed significant spatial learning and long-term memory deficits in aged PGJ2-treated mice, compared to the other three groups. Hippocampal Fluoro-Jade C staining identified a significant increase in degeneration neurons in the CA3 region of aged PGJ2-treated mice, indicating a progressive spread of damage from the site of injection to the adjacent sub-region. Golgi-immunohistochemical analysis identified a significant deficit in the expression of plasticity-related spine types, stubby and filopodia, within CA1 dendrites of aged-PGJ2 treated mice compared to the young-PGJ2 condition. This deficit occurred concomitantly with a significant increase in colocalization of synaptic markers GluA2/PSD95 within these spine types, indicating a deficit in maturation of spines via disrupted molecular trafficking and turnover. Our findings indicate that PGJ2, as a product of inflammation, can initiate neurodegeneration as well as facilitate its progression within the hippocampus in an age-dependent manner, thus mimicking processes that
are highly relevant to AD pathology. We also assessed the efficacy of elevating endogenous cAMP as a therapeutic intervention in our model. Accordingly, a group of aged mice received PGJ2 injections at the same time as PACAP27, a lipophilic neuroprotective peptide that raises intracellular cAMP. PACAP27 treatment ameliorated PGJ2-mediated learning and memory deficits on the RAM. We propose that this pre-clinical mouse model is highly valuable to identify and optimize therapeutics that suppress the neurotoxic effects of inflammation as a strategy to prevent or delay the progression of AD.
3.2. INTRODUCTION

Neuroinflammation is a defense process activated upon CNS injury to initiate repair mechanisms acutely, while chronic neuroinflammation can exacerbate, spread and prolong CNS injury. Chronic neuroinflammation is implicated in a variety of neurological and neurodegenerative disorders including AD [58,70,113]. Neuroinflammation is an active process detectable in the earliest and latest stages of AD [110,204,207].

Cyclooxygenases (COX -1, constitutive and COX-2, inducible), which are key enzymes in the conversion of arachidonic acid into bioactive prostaglandins (PGs) play a central role in the inflammatory cascade. Inhibiting cyclooxygenases with non-steroidal anti-inflammatory drugs (NSAIDs) is being explored as a therapeutic strategy to mitigate chronic neuroinflammation and prevent the onset/progression of AD pathology [92,194]. NSAIDs’ effectiveness could be compromised as they block the generation of all prostaglandin products of cyclooxygenases. Prostaglandins act as potent local regulators of physiologic and pathologic pathways linked to CNS inflammation. We focused our studies on PGJ2 because it is highly neurotoxic compared to PGA1, D2 and E2 [108]. J2 prostaglandins including PGJ2, are endogenous toxic products of cyclooxygenases actively involved in neuronal dysfunction [47].

PGJ2 is a product of spontaneous non-enzymatic dehydration of PGD2, which is the most abundant prostaglandin in the brain [190], and undergoes the most changes under pathological conditions [109]. Specifically, cortical neurons in AD brains were shown to exhibit accelerated PGD2 synthesis [82] [202]. PGD2 is unstable as its half-life in the brain is 1.1 min and in the blood 0.9 min, and some of it is converted to PGJ2 [185]. In rodents, the in vivo concentration of free PGJ2 in the brain upon injury such as stroke and TBI, increases from almost undetectable to the
100nM range [115,116], which are levels shown to be neurotoxic *in vitro* [72,97]. These concentrations represent average brain levels, but local cellular and intracellular concentrations of PGJ2 are potentially higher [117], as PGJ2 binds covalently to proteins [190]. Therefore free PGJ2 does not represent its total amounts.

PGJ2 signals in part via one of the PGD2 receptors, i.e. the DP2 receptor [68,126]. DP2 is coupled to inhibitory G-proteins thus lowering cAMP [68], and we confirmed that PGJ2 indeed lowers neuronal cAMP levels [124]. In an effort to overcome the cAMP deficit induced by PGJ2, we showed that raising intracellular cAMP levels with PACAP overcomes some of the neurotoxic effects of PGJ2 in rat cerebral cortical neuronal cultures [124], and in mice exhibiting parkinsonian-like pathology upon microinfusion of PGJ2 into the substantia nigra [176]. PACAP27 is a lipophilic peptide that binds to the seven transmembrane G-coupled receptor PAC1R (pituitary adenylate cyclase 1 receptor) at nanomolar levels, activating adenylate cyclase and elevating cAMP [127]. PAC1R is expressed in the cerebral cortex and hippocampus as well as other brain areas [88]. Overall, PACAP may offer a novel therapeutic approach to treat AD because it not only ameliorates some of the pathological processes observed in AD models, but it also diminishes some of the clinical symptoms of AD [203].

To examine the in vivo effects of PGJ2 relevant to AD, we microinfused PGJ2 into hippocampi of aged (53 weeks of age) and young (12 weeks of age) mice. We included two different age groups in our study because the single greatest risk of developing AD is aging [69], with an exponential increase in cases after the age of 65 [15]. Moreover, steep age-related declines in synaptic density and plasticity make the brain increasingly less efficient. Our findings indicate that PGJ2, as a product of inflammation, can by itself initiate neurodegeneration as well as its progression within the hippocampus in an age-dependent manner, mimicking pathology that is
relevant to AD. Furthermore, PACAP27 diminished some of the memory deficits induced by PGJ2. Like other toxic and genetic models, our new in vivo mouse model has limitations. However, it provides a tool to test neuroprotective strategies with fewer undesirable side effects, like PACAP27, applicable to AD and other neurodegenerative disorders in which the cyclooxygenase pathway of inflammation is involved.
3.3. MATERIALS AND METHODS

3.3.1. Materials - *Drugs*: PGJ2 (cat. # 18500, Cayman Chemical) in DMSO, and PACAP27 (pituitary adenylate cyclase-activating polypeptide, cat. # H-1172, Bachem Bioscience) in sterile water. The final DMSO concentration in PBS was 17% for all microinfusions. The solutions were freshly prepared and stored for a maximum of 2 h at 4 °C and in the dark.

    *Primary antibodies*: GluA2, 1:1000 cat# MAB 397, EMD Millipore, PSD95, 1:1000, cat# AB9708, EMD Millipore. *Secondary antibodies*: Alexa Fluor 568 (1:100, cat.# A11036, rabbit) and Alexa Fluor 488 (1:100, cat.# A11029, mouse) both from Invitrogen. Vectashield Hard Set™ mounting medium with DAPI (cat.# H-1500, Vector Laboratories).

3.3.2. Mice - All procedures were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committees at Hunter College. We included male B6-M (C57BL/6NTac) mice from Taconic farms, ages - young (12 weeks of age) and aged (53 weeks of age), to mimic an aging paradigm. Mice were singly housed on a 12h light/dark cycle with food and water available *ad libitum*, and were habituated for one week before treatment. All mice were microinfused bilaterally into the hippocampal CA1 region. Each mouse was injected once/week (Fig.14) with DMSO or PGJ2 (16.7μg in 2μl DMSO) for three weeks (3 total injections). After behavioral analyses, mice were perfused and the brains removed and processed for immunohistochemical analysis.
3.3.3. Stereotaxic surgery - We followed the same procedures as described in our previous study [148], except that mice received bilateral injections of vehicle (DMSO) or drugs (PGJ2 and PACAP27) into the hippocampal CA1 subregion. Briefly, at the respective ages (12 weeks for the young and 53 weeks for the middle-age), mice were anesthetized by isoflurane inhalation (induction 2–2.5%, maintenance 1.5–2%) administered in 100% oxygen and placed into a stereotaxic frame (Model 51730D, Stoelting Co., Wood Dale, IL) fitted with a gas anesthesia mask (Model 50264, Stoelting Co.). A burr hole was drilled in the skull at coordinates relative to bregma for the hippocampal CA1 subregion: rostral-caudal -2.0mm, medial-lateral +/- 1.0mm, dorsal-ventral +1.5mm, as specified in the mouse brain atlas [49]. A 2 μL microinjection Hamilton syringe (7002 KH) with a 25-gauge needle was slowly inserted into the brain and left in place for five minutes. Thereafter, 2 μL of solution was infused at an injection rate of 0.2 μL/min (Quintessential stereotaxic injector, Model 53311, Stoelting Co.). The needle was left in place an additional five minutes to ensure total diffusion of the solution. Following injection, the needle was slowly removed and the incision was closed with monofilament absorbable sutures (cat. # 033899; Butler Schein Animal Health, Dublin, OH). After surgery, mice were administered a subcutaneous injection of 0.5 cc Lactated Ringer's solution, given wet palatable rodent chow, and
kept in a warm place to recover. Subsequent injections were administered via the same drill hole established during the first surgical procedure.

3.3.4. Groups - Mice were randomly assigned to the treatment groups (Fig. 14). Mice in each age group received three bilateral injections (once/week for three consecutive weeks) of DMSO/PBS (n = 5 aged mice, n = 5 young mice) or PGJ2 (16.7μg in 2μl DMSO/PBS, n =5 aged mice, n = 4 young mice) per injection. To assess the therapeutic efficacy of PACAP27, groups of aged mice received the same number of injections including PACAP27 (50 ng/2μL n =5), or PACAP27 + PGJ2 (n = 6) and were compared to those mice that received DMSO or PGJ2 only.

3.3.5. The radial 8-arm maze (RAM) (Fig. 14) – RAM is a behavioral task used to evaluate long-term reference and short-term working memory simultaneously [168,170,171]. Briefly, mice were food restricted to 85% of free-feeding weight. To acclimate mice to the maze prior to training each mouse received three exposures/day (2 days) to the maze with all arms baited (10min intervals, followed by 1h in home cage). During training, four of the 8 arms were baited with wet sweetened oatmeal (Maypo Inc, NJ) as previously described [168,170,171]. The sequence of baited arms remained fixed for each mouse. To avoid the use of internal maze cues, each day the maze was rotated 90° keeping the position of the baited arms stationary with respect to the room cues. Mice received 6 consecutive trials per day lasting no more than 3min per trial. To establish the trial % correct score, we divided the number of food rewards collected by the total number of arms entered. Reference and working memory errors were also scored. Reference memory errors occur when a subject entered an arm that is never baited. Reference memory is also associated with long-term memory performance, thus the more correct arms entered during training, the better long-term memory the mouse has. Working memory errors were committed when a subject re-entered an arm where the food reward had already been collected for that trial.
3.3.6. Fluoro-Jade C analysis - Following behavioral analyses, mice were terminally anesthetized (i.p.) with ketamine (100 mg/kg) and acepromazine (3 mg/kg), and transcardially perfused with 4% paraformaldehyde in PBS. The mouse brains were removed, post-fixed overnight at 4 °C, followed by cryoprotection (30% sucrose/PBS at 4 °C). Brains were sectioned in the coronal plane using a freezing microtome at a thickness of 20 μm, and sections were collected serially along the rostrocaudal axis of the hippocampus. Tissue series were stored at 4 °C (TBS, pH7.4 plus 0.1% sodium azide) until use. Each series was processed as free floating sections for Fluoro-Jade C analysis. Degenerating neurons/terminals were detected with Fluoro-Jade C (cat# AG325, Milipore) as described in [27,166].

3.3.7. Quantification - Fluoro-Jade analysis: Under a wide-field fluorescence microscope (Zeiss AxioImager) the software AxioVision was used to capture whole hippocampal region mosaics (MosaiX capture mode). Exposure time for channel was kept constant between sections. For each captured image, ZVI files were load onto Image J (NIH, Bethesda, MD). Hippocampal subfields were isolated, cropped, and saved as .tif files for use in intensity analysis. Channel was thresholded to extract the positive signal from all [150]. Pixel data above 75th percentile intensity were measured for per square micron in each image crop.

3.3.8. Simultaneous Golgi-Cox Immunohistochemistry (GC-IHC) - Hippocampal brain sections cut at a 50 μm were processed for GC-IHC (dendritic spines, GluA2, PSD95) as described in [179]. LSCM (laser scanning confocal microscopy) with a Zeiss LSM 510 laser-scanning confocal microscope and 3D analysis: Z-stacks (4-6 μm; Z-step size 0.041 μm for CA1) were acquired of CA1 (15-75 μm in length, projecting into striatum radiatum). For 3D analysis, IMARIS 7.5 (Andor Technology; Belfast, Northern Ireland) was used to analyze spines, determine spine morphology, and co-localize synaptic markers (PSD95 and GluA2) and dendritic structure.
3.3.9. **Statistics.** All data are expressed as the mean ± SEM. Statistical analyses were performed with GraphPad Prism 6 (GraphPad Software, San Diego, CA). A p-value < 0.05 was considered statistically significant. For group comparisons, we performed one-way or two-way analysis of variance (ANOVA) followed by *post hoc* Tukey or Bonferroni tests for multiple comparison.

3.4. **RESULTS**

3.4.1. **PGJ2 delays spatial learning in aged compared to young mice** - Previous data from our lab with rat E18 cerebral cortical neuronal cultures showed that PGJ2-treatment causes AD-like molecular pathological events, such as accumulation of ubiquitinated proteins, formation of ΔTau which is the major component of neurofibrillary tangles, formation of Ub-protein aggregates, and caspase-3 activation [124]. We were interested in investigating whether PGJ2 would induce AD-like behavioral deficits in an age-dependent manner in a mouse model.

We observed that aged (53 weeks of age) PGJ2-treated mice performed worse in the radial arm maze (RAM) compared to the other 3 groups tested. The results obtained can be represented as % correct arm entry, and reference memory errors. Reference memory error occurs when a mouse enters an arm that is never baited. Behavioral analysis revealed that aged PGJ2-treated mice scored the lowest amount of correct arms entry of all the 4 groups tested which translates into their decreased learning and memory (Fig. 15 A and B). This correlates well with reference memory error analysis. Aged PGJ2-treated mice had the highest number of reference memory errors meaning that they entered arms that were never baited much more frequently than mice from the other groups (Fig. 15 C and D). This suggests impairment in memory formation.
There was no significant age difference in performance between DMSO-treated animals. As shown in Fig. 15A mice injected with DMSO learned the radial arm maze (RAM) over 12 days of training. Conversely young PGJ2-treated mice learned significantly better than aged PGJ2-treated mice (Fig 15B). While all groups of mice showed significant improvement in their % correct scores over training days, the aged PGJ2-treated mice appear to reach a lower level of asymptotic performance at 65% correct. This learning deficit in aged PGJ2-treated mice is also reflected in the reference memory errors (Fig.15C and D).
Figure 15. PGJ2 delays spatial learning in old compared to young mice. (A) DMSO treated mice learn equivalently showing a significant effect of training ($F_{11,44} = 7.47, p < 0.001$) without an effect of age. (B) PGJ2-treated mice show a significant overall effect of training ($F_{11,110} = 18.92, p < 0.001$), age ($F_{1,10} = 13.76, p = 0.004$), and a training by age interaction ($F_{11,110} = 3.72, p < 0.001$). Posthoc analysis for training days 1, 8, 11 and 12 showed significance (*$p < 0.01$). (C) Average Reference Memory Errors (RME) per trial were not different between young and aged DMSO mice. (D) Aged PGJ2 mice averaged more Reference Memory Errors (RME) per trial compared to young mice.
3.4.2. PGJ2 affects long-term memory retention in aged compared to young PGJ2-treated mice - Long-term memory formation is known to be affected in AD patients. Therefore we investigated if PGJ2 impaired memory retention in mice one week after completion of the 12 days long training period. We found that PGJ2 impaired long-term memory in aged compared to young mice. Six days after the last training trial all mice were given another 6 trials. The days average % correct scores show that PGJ2-treated mice had significantly lower % correct scores than all other groups (Fig. 16). These data reflect the behavioral traits of AD and its age-dependent vulnerability.

![Figure 16. PGJ2 affects long-term memory in aged compared to young mice. Six days following the last training, mice were given an additional 6 trials. (A) The % correct scores show a significant overall effect of age ($F_{1,14} = 5.56$, $p = 0.03$) and an age by drug interaction ($F_{1,14} = 7.56$, $p = 0.015$), with no overall effect of drug treatment. Post-hoc analysis showed significance (*$p = 0.05$). (B) Aged PgJ2-treated mice performed significantly more reference memory errors compared to other groups tested.](image-url)
3.4.3. PGJ2 induces hippocampal damage that spans from CA1 to CA3 only in aged PGJ2-treated mice - We next analyzed changes at the cellular level in the hippocampus. PGJ2 induced a higher level of neurodegeneration in the hippocampus of aged compared to young mice as shown with Fluoro-Jade C staining, which identifies degenerating neurons, glia and terminals (Fig. 17) [27,166].

Figure 17. PGJ2 induces hippocampal damage that spans from CA1 to CA3 only in aged mice. Fluoro-Jade staining analysis (% DMSO) ran only on PGJ2 groups shows an overall significant effect of subregion CA1 vs CA3 ($F_{1,20} = 9.67$, $p = 0.006$). No significant age effect and a significant age by subregion interaction ($F_{1,20} = 5.96$, $p = 0.02$). Significant posthoc analysis within CA3 subregion (*p<0.05) Scale bar, 100 µm.
3.4.4. PGJ2 induces dendritic spines defects in the hippocampal CA1 region of aged mice - Dendritic spines are protrusions in post-synaptic compartments and are sites of synaptic transmission. Each spine receives input from excitatory synapses and due to the presence of neurotransmitter receptors, organelles and signaling cascades it has the ability to establish and maintain the synapse. The presence of dendritic spines is linked to memory formation and defects in their number and types are linked to memory deficits in various neural disorders, such as AD. Spines are dynamic structures; their plasticity and remodeling are associated with learning. There are four main types of spines, stubby, filopodia, long-thin and mushroom) (Fig. 18), characterized by their developmental profile ranging from immature (stubby, filopodia) to mature (long-thin, mushroom) [75,123].

![Figure 18. Dendritic spine types and characteristics](image)

**Figure 18. Dendritic spine types and characteristics** A, Spine types as characterized by [75]: (Left to right) stubby, filopodia, long thin, and mushroom. Spines demonstrate a dynamic developmental profile. Plastic, immature spines (stubby and filopodia) lack post-synaptic density and AMPARs. Stable, mature spines (long-thin, mushroom) express higher levels of synaptic markers. IMARIS XT parameters for spine detection using the spine classification module. B, Parameters were based on relative spine neck and head relationships as in [12,124]. Representative Golgi-Cox IMHC dendrites for control conditions (scale bar = 5 μm for C; 3 μm for D. Golgi-Cox indicated in green, colocalization of synaptic markers in yellow. Red arrowheads indicate long-thin spines, blue arrowheads indicate mushroom spines.
The size of the spine head dictates whether the spine is immature and plastic or mature and stable. Spines that have large mushroom-shaped heads are considered established, “memory” spines. Characterizing spine morphology with Golgi-IHC is an innovative approach that allows the coupling of immunohistochemistry with Golgi-Cox staining visualized with confocal microscopy [179]. Our studies using this technique, identified a significant decrease in the total number of dendritic spines in the hippocampal CA1 region of aged (Fig. 19 A) compared to young PGJ2-treated mice. This result indicates deficits in memory and corroborates the findings of our behavioral analyses. Moreover, aged PGJ2-treated mice have less stubby and filopodia spines in the CA1 dendrites (Fig. 19 B and C), suggesting that PGJ2 impaired spine plasticity that could lead to learning and memory deficits.

**Figure 19 (A). Dendritic spine analysis.** Using a Leitz Diaplan microscope, a Nikon DXM 1200F camera, and Image Pro Plus software (Media Cybernetics, Bethesda, MD, USA), dendritic spine density on pyramidal cells from the CA1 region of the dorsal hippocampus and layer II/III of the mPFC were analyzed. Regions were defined based on *The Mouse Brain in Stereotaxic Coordinates* (Franklin KBJ, Paxinos G: 1997) and counting was done as described by Frankfurt et al (Dev Neurosci. 2009;31(1-2):71-5). Images of tertiary dendrites projecting into the stratum radiatum of CA1 (15-95 μm in length) and in CA3 (20-95 μm in length) were captured using a Leica SP2 confocal microscope. Spine morphology and expression of synaptic proteins were then quantified using IMARIS filament tracer.
Mature spines have well established post-synaptic densities (PSD), which are zones close to the membrane that consists of neurotransmitter receptors, channels and systems involved in synaptic transmission (Fig. 20). One of the markers of a fully developed, mature spine is PSD95, a scaffold protein that determines structural and functional integrity of excitatory synapses [16]. The other marker is GluA2, a subunit of an AMPA glutamate receptor which is critical for synaptic plasticity. We assessed the levels of both of these synaptic markers because their co-localization at the post-synaptic terminal labels an active, mature synapse associated with formed memories. Our analysis revealed increased levels of GluA2 and PSD95 in stubby (immature) spines in the CA1 region of aged compared to young PGJ2-treated mice (Fig. 20). This unexpectedly high level of mature synaptic markers in plastic spines may indicate disrupted protein trafficking resulting in their inability to mature into memory-associated spines, i.e. long-thin and mushroom types, which

**Figure 19. Dendritic spine analysis.** (B) Aged-PGJ2 treated mice display significantly less plastic spines (stubby and filopodia), (C) Aged-PGJ2-treated mice display less mature spine types than young PGJ2-treated mice but this change is not statistically significant.
can explain memory deficits *in vivo*.

**Figure 20.** IHC analysis of dendritic spines (A) GluA2 and (B) PSD95 levels are significantly increased in stubby spines in aged PGJ-treated mice. (C) Significantly increased colocalization of GluA2 and PSD95 in stubby spines in aged PGJ2-treated mice. (D) Representative Golgi-Cox IMHC dendrites for control conditions (scale bar = 5 μm. Golgi-Cox indicated in green, co-localization of synaptic markers in yellow.)
3.4.5. PACAP27 prevents learning and memory deficits induced by PGJ2 in aged mice.

We investigated if neuroprotective peptide PACAP27 could prevent learning and memory retention loss in PGJ2-treated mice (Fig. 21). PACAP did prevent learning deficits in aged PGJ2-treated mice to a certain extent. The % correct arm entry was higher for aged PGJ2/PACAP-treated mice from day 3 to day 12 of RAM training than PGJ2-treated mice. On day 9 the difference between the two groups was statistically significant (p value = 0.0107). Aged mice injected with PACAP alone did not perform significantly different from aged mice injected with Vehicle (DMSO).
Figure 21. PACAP27 prevents learning and memory deficits induced by PGJ2 in aged mice. Mice underwent 12 day learning period on RAM. %correct arm entries for all the mice from all cohorts on a particular day was averaged as represented in (A) and (B). 6 days after completing last training, mice were subjected to additional 6 trials and their performance from that day is represented in (C) and (D).
DISCUSSION

One of the earliest clinical hallmarks of AD is progressive memory impairment. Patients suffering from this debilitating condition exhibit loss of spatial memory and are unable to orient themselves with respect to physical surroundings. This memory symptom correlates with neuronal death in the hippocampus, the brain region responsible for learning, spatial orientation and memory storage. AD-relevant memory loss can be recapitulated in mouse models and is easily examined as there are numerous, well established behavioral assays available to test memory in rodents. Studies showed that a lesion in hippocampal CA1 region in rats is sufficient to produce memory impairment [84]. The causes of initial pathology of AD are not yet understood but there is consensus in the scientific community that chronic neuroinflammation plays an important role in neurodegeneration.

We focused our investigations on the product of inflammation PGJ2 because it is highly neurotoxic [108], and its levels have been shown to increase upon pro-inflammatory stimuli such as brain injury (ex. stroke and TBI) [97],[72], which is a well-established risk factor for AD. Our preliminary studies indicate that microinfusion of the cyclooxygenase product of inflammation PGJ2 into the CA1 region of the hippocampus initiates neurodegeneration as well as its progression within the hippocampus in an age-dependent manner, thus mimicking pathology that are highly relevant to AD.

As memory and cognitive impairment are central problems in AD, we used behavioral tests to characterize spatial learning and memory deficits induced by PGJ2 over time in aged (53 weeks of age) and young (12 weeks of age) mice. Our preliminary studies show that PGJ2 impaired long-term memory in aged compared to young mice. Moreover, PGJ2 induced neurodegeneration in the
hippocampus of aged mice compared to young mice as shown with Fluoro-Jade C staining. PGJ2 also decreased synaptic plasticity in aged mice, which could explain learning impairment. The behavioral deficits induced by PGJ2 were correlated with the colocalization of specific synaptic markers within various spine types. Trafficking of the GluA2 receptor subunits to the postsynaptic density increases during episodes of synaptic plasticity, and stabilizing AMPA receptors within the synaptic membrane is important for memory consolidation [169]. Our analysis revealed that GluA2 (subunit of the AMPA receptor) as well as PSD95 (postsynaptic density marker) were both significantly higher in the stubby spines of aged-PGJ2 treated mice. This novel finding could indicate that PGJ2 impairs the ability of plastic spines to mature into memory associated spines, resulting in memory deficits in-vivo.

We also showed that the neuroprotective peptide PACAP27 prevented to a certain extent learning and memory impairment induced by PGJ2 in aged mice. These studies require further analysis, for example assessing whether PACAP could prevent PGJ2-induced neurodegeneration as well as dendritic spine deficiencies. Overall, our data indicate that targeting PGJ2 by increasing cAMP levels with PACAP27 may be an efficient therapeutic approach to prevent PGJ2-dependent neurodegeneration and memory deficits.

Our investigation of the neurotoxic pathway and mechanisms by which PGJ2 leads to neurodegeneration underscores its relevance as a risk factor mediating the long-term effects of neuroinflammation in the progression to AD. Targeting factors downstream of cyclooxygenases, such as PGJ2, offer great promise as a new therapeutic strategy that would not alter the homeostatic balance offered by cyclooxygenase derived prostaglandins.
CHAPTER IV

CONCLUSIONS
Sporadic Alzheimer’s disease (sAD) is an age-related neurodegenerative disorder. Its causes are largely unknown but evidence supports that a combination of environmental and lifestyle factors can cause recurring inflammatory insults that contribute to chronic neuroinflammation and neuronal death. Despite decades of research it is still not known how to prevent, slow down or cure AD. Medicine that is currently available for AD patients offers only temporary, symptomatic relief [52]. Current research on AD focuses on developing new imaging tools that facilitate detection of pathological changes in the brain early enough to start treatment that would prevent neurodegeneration [87]. Another research focus is on developing new therapeutic strategies. These two areas of investigation need to go hand in hand for advancement and efficient treatment of AD.

UPP is essential for maintaining cellular homeostasis as it is involved in a number of cellular processes, such as neuronal homeostasis, synaptic plasticity, signal transduction, and mitochondrial dynamics. Impairment of this proteolytic pathway has long been associated with AD. Agents that increase the efficacy of the UPP could improve proteasomal function and prevent accumulation and aggregation of Ub-proteins, both of which are hallmarks of AD.

Our studies focused on examining two new potential therapeutic targets related to the UPP:

a) The Usp14 deubiquitinating enzyme – we investigated whether its inhibition increases proteasome-dependent degradation and decreases toxicity induced by the endogenous product of inflammation PGJ2.

b) The product of inflammation PGJ2 that impairs different steps of the UPP – we investigated whether PGJ2 induces AD-like neuronal and behavioral pathology in vivo, by microinfusing it into hippocampi of young and aged mice. In addition, we determined the
potential of PACAP to overcome the deleterious effects of PGJ2.

a) Studies with the Usp14 deubiquitinating enzyme:

We attempted to increase proteasome-dependent degradation by inhibiting the Usp14 enzyme using pharmacological and genetic approaches. The Usp14 deubiquitinating enzyme slows down protein degradation by removing ubiquitin tags too soon, allowing the substrate to prematurely dissociate from proteasomes thus avoid degradation. Studies from other groups showed that inhibiting Usp14 in MEFs and HEK cells with the small molecule inhibitor IU1 increased the degradation of proteasome substrates implicated in neurodegenerative diseases, such as Tau, TDP-43 and ataxin-3 [101]. In our studies, we investigated the effects of IU1 on rat E18 cerebral cortical neuronal cultures, a cell model that is much more physiologically relevant to AD than MEFs or cancer cell lines such as HEK cells. Treatment of primary neurons with high concentration of IU1 (75 µM) decreased the levels of Ub-proteins induced by PGJ2, suggesting that inhibiting Usp14 with IU1 indeed enhanced Ub-protein degradation and prevented their accumulation. However, this result was accompanied by other thought provoking data. Our studies showed that IU1 (75 µM) treatment in neurons activates calpain-dependent cleavage of Tau, spectrin and caspase-3 and decreases the levels of 26S proteasomes with a concomitant increase in 20S proteasomes. These findings suggested that IU1 could have off target effects. We confirmed this by demonstrating that IU1 inhibits mitochondrial complex 1 leading to mitochondrial impairment and ATP deficits. In addition, we established that Usp14 knockdown by siRNA in rat neuronal cultures, or Usp14 loss in Usp14axd mouse neuronal cultures did not change the levels of Ub-proteins induced by PGJ2. These data strongly support the view that IU1 prevents PGJ2-induced accumulation of Ub-proteins, not by enhancing their turnover by the 26S proteasome, but instead by blocking the ubiquitination cascade causing lower levels of Ub-proteins to be formed.
While our studies focused on investigating the properties of a new potential therapeutic, we had another unexpected finding. We showed, for the first time to our knowledge, that PGJ2 lowers E1–Ub thioester levels. This result is surprising since we have shown that drugs that lower E1–Ub thioester levels, such as IU1 (current study) and mitochondrial inhibitors (oligomycin and antimycin in [78]) do not cause accumulation of Ub-proteins in neurons. It is likely that the accumulation of Ub-proteins detected in PGJ2-treated neurons reflects its ability to inhibit deubiquitinases that may stabilize the Ub-proteins formed prior to E1–Ub thioester depletion.

In conclusion, the functional relationship between the enzymes and components of UPP requires further investigation as detailed understanding of their interactions are of the utmost therapeutic interest since reducing the levels of aberrant proteins in neurons is highly relevant to neurodegenerative disorders, such as AD.

**Figure 22.**

**Model 1.**

IU1 impairs mitochondria which leads to decrease in ATP levels. This inhibits ubiquitination cascade and results in low levels of Ub-proteins.
b) Studies with the product of inflammation PGJ2 that impairs different steps of the UPP.

Chronic inflammation is emerging as a major factor involved in AD pathogenesis and its progression. Chronic inflammation is manifested by activated microglia and astrocytes that release neuroprotective and neurotoxic factors. Some of these factors are produced by cyclooxygenases, key enzymes in the conversion of arachidonic acid into prostaglandins. Our previous studies showed that PGJ2 is a highly toxic product of COX-2 [108], the levels of which increase upon brain injury and during neurodegeneration [110],[7]. PGJ2 belongs to the cyclopentenone prostaglandin family. These PGs are unique in that they form covalent Michael adducts by binding to free sulfhydryl groups on proteins thus modifying them and potentially impairing their structure and function. This pathogenic process can contribute to neurodegeneration, in particular because PGJ2 impairs the UPP by different mechanisms, such as inhibiting UCH-L1 the most abundant deubiquitinating enzyme in the brain, as well as disrupting 26S proteasome assembly [198].

Epidemiological studies suggest that patients taking non-steroidal anti-inflammatory drugs (NSAIDs) are at a reduced risk (by as much as 50%) for developing AD, although this notion is controversial. NSAIDs target cyclooxygenases thus inhibit formation of all downstream prostaglandins including neuroprotective ones. Additionally, inhibiting COX-1 and COX-2 was shown to have side effects such as renal failure and stroke [140]. Therefore, we postulated that taking a more directed approach and targeting PGJ2, a neurotoxic prostaglandin that is downstream of COX-2 would be of more benefit.

To test this hypothesis PGJ2 (16.7μg in 2μl/week, for 3 weeks) was bilaterally microinfused into the CA1 hippocampal region of aged (53 weeks) and young (12 weeks) mice. Our preliminary data established that PGJ2-treatment caused a) memory deficits, b) degeneration that spanned from the CA1 to the CA3 region of the hippocampus, and c) dendritic spine defects
in the CA1 region. We also found that these pathological changes were age-dependent being detected in aged but not young PGJ2-treated mice. These results strongly support the view that PGJ2 by itself is sufficient to induce AD-like neuronal and behavioral pathology, and suggest that inhibiting its activity would be a beneficial therapeutic approach. The benefit lays in the fact that expression of other neuroprotective prostaglandins would not be affected by this approach, and perhaps side effects stemming from prolonged usage of NSAIDs could be avoided.

Our in vivo studies with PGJ2 are very promising but we still need to address other potential mechanisms by which PGJ2 disturbs neuronal homeostasis to broaden our understanding of the impact that inflammation has on the brain (discussed in Future Directions). In addition, our studies could contribute to developing an AD model of inflammation that could serve as a platform for testing neuroprotective drugs. To this end, we concluded our studies with briefly testing the neuroprotective peptide, PACAP27 as a potential therapeutic for AD. We demonstrated that co-administration of PACAP27 and PGJ2 mitigates some memory impairment induced by PGJ2 treatment, although this change is not statistically significant. PGJ2 signals via the DP2 receptor, which is coupled to inhibitory G proteins that lower intracellular cAMP [68], [76], [126]. We and others previously demonstrated that PACAP treatment of neuronal cultures overcomes the decrease in cAMP levels induced by PGJ2 [124], [178]. This approach requires further investigation to assess whether PACAP27 protects against PGJ2-induced neurotoxicity.

In conclusion, the potential therapeutic strategies we investigated here offer new insights into mechanisms of AD development and if further explored could potentially prevent neurodegeneration in AD.
Figure 23. Model 2. PGJ2 as a potential therapeutic target in AD model of inflammation
CHAPTER V

FUTURE DIRECTIONS
Many neurodegenerative disorders, including AD are related to the abnormal accumulation of symptomatic proteins. For example in AD it is truncated Tau – ΔTau - that is deposited in neurofibrillary tangles. A decrease in protein turnover is associated with impaired UPP. Maintaining protein homeostasis presents a critical task in post-mitotic neuronal cells. Therefore, developing new strategies aimed at enhancing or preserving the function of the UPP as well preventing neurotoxicity is essential for substantial therapeutic efficacy.

Inhibiting Usp14 was not effective in our cell model, i.e. rat E18 cerebral cortical neuronal cultures. Moreover, other investigators showed that Usp14 null mice (ataxia mice) exhibit developmental abnormalities including motor impairment, reduced brain mass and death by 2 months of age [25]. This proves that targeting any component of such a complex pathway like the UPP requires detailed knowledge of its function and understanding of its interactions with other elements. Furthermore, the small molecule inhibitor of Usp14, i.e. IU1, turned out to have an off-target effect by inhibiting mitochondrial complex 1 thereby causing mitochondrial impairment and ATP deficits. Since many exogenous, small molecules have off-target effects that are difficult to predict we suggest employing other strategies.

We propose the following:

1) Investigate increasing UPP function and preventing neurotoxicity by using neuroprotective peptides - Perhaps endogenous, neuroprotective peptides could be a better choice of potential therapeutics as unpredictable off-target effect would be avoided. Additionally a combination therapy involving more than one peptide could act in a variety of ways to prevent AD-related neurodegeneration. Studies from our group previously showed that one such peptide, PACAP27, diminishes PGJ2-induced toxicity in rat E18 cerebral cortical neuronal cultures via activating cAMP/PKA pathway. Conversely, PGJ2 was shown to decrease cAMP [124].
PACAP27 decreased caspase-3 activation, Δtau formation, Ub-proteins levels and aggregation, and the loss of cell viability [124]. PACAP27 was also shown to be protective in vivo [30,32,36,42]. Overall, PACAP27 appears to be a good therapeutic as it alleviates many of the symptoms induced in our cell model of inflammation. However, AD is a multifactorial disease, so combining PACAP with other protective peptides that exert different functions could be of greater benefit. Another potential therapeutic, (VEGF) is a vascular endothelial growth factor that promotes angiogenesis and neurogenesis both in vivo and in vitro [86,102,120]. VEGF's potential as a protective factor has been demonstrated in hypoxia–ischemia, in vitro excitotoxicity, and motor neuron degeneration [184], [182]. There are a number of neuroprotective peptides that could be explored such as AdNP (activity-dependent neuroprotective protein), which is a glial cell modulator of VIP associated neuroprotection and is implicated in maintenance of cell survival through modulation of p53 expression ([8], [177,208]). AIP (autocamtide-related inhibitory peptide) was shown to inhibit Ca\(^{2+}\)/Calmodulin dependent protein kinase II, Aβ-triggered activation of caspase 2 and 3, and Tau phosphorylation, as well as protect neurons against Aβ toxicity [111]. Patients with AD show a marked loss of cholinergic neurons and diminished Substance P expression [149]. Substance P is a short peptide that interacts with the cholinergic ascending system resulting in enhancement effects [1]. In conclusion, there are a number of endogenous neuroprotective peptides the levels of which are decreased in AD. They act in different ways and in different pathways. Therefore increasing their levels and using them in combination could be of great therapeutic value.
2) Our studies would benefit from more detailed evaluation of PGJ2 toxicity in vivo. We propose to:

   a) Assess if PGJ2 causes microglia activation in hippocampus. Activated microglia are a hallmark of inflammation, therefore assessing their level of activation would allow us to determine if PGJ2 administration exacerbates the immune response in the hippocampus. It would also allow us to investigate whether age exacerbates PGJ2-induced microglial activation. This would be carried-out by IHC analysis using IBA-1 as an activated microglia marker.

   b) Assess if PGJ2 induces caspase-dependent Tau cleavage and leads to the formation of ΔTau. Tau is a microtubule associated protein the function of which is to stabilize microtubules. However, during AD-related neurodegeneration it becomes hyperphosphorylated and cleaved by activated caspases 3 and 8 to aggregation-prone ΔTau which is a major component of neurofibrillary tangles. We previously showed that this is the case in rat E18 cerebral cortical neuronal cultures treated with PGJ2 [124]. It would be interesting to investigate whether PGJ2 induces caspase activation and caspase-dependent Tau cleavage to ΔTau in our PGJ2-induced mouse model of neuroinflammation that exhibits AD-like pathology.

   c) Investigate if PGJ2-induced pathology could be prevented by delivering PACAP in vivo. We have already determined that PACAP co-injected with PGJ2 improves learning in aged mice, although this change is not statistically significant. It would be interesting to investigate if PACAP could prevent molecular and cellular pathological changes induced by bilateral injections of PGJ2 into the CA1 region of the hippocampus. We would assess microglial activation, degeneration, dendritic spines abnormalities and other parameters. The next step would be to administer PACAP27 intranasally to mice that have been previously injected with PGJ2. The intranasal route of administration is an established procedure and has been exploited by numerous
investigators to successfully deliver a variety of therapeutics, like neurotrophic factors [185,191], cytokines [160,206], and neuropeptides [6]. Most importantly, intranasal delivery of PACAP has been shown to reduce β-amyloid plaques in an AD transgenic mouse model [152]. Intranasal delivery of therapeutics to the central nervous system (CNS) has the advantage that it bypasses the blood brain barrier (BBB) and systemic adversities [66]. Drugs get absorbed through the nasal mucosa and reach CNS via three potential pathways: a) adsorptive or receptor-mediated endocytosis into olfactory sensory neurons (OSNs) followed by intracellular transport to the olfactory bulb; b) non-specific fluid phase endocytosis into OSNs followed by intracellular transport into the olfactory bulb; c) extracellular diffusion along the open inter-olfactory clefts directly to the olfactory bulb, then to the subarachnoid space and cerebrospinal fluid (CSF) [66] [34]. From the cerebrospinal fluid drugs are distributed throughout the brain. Overall, intranasal delivery of PACAP provides a promising alternative to other routes of administration and is physiologically relevant, since a direct pathway exists between the olfactory epithelium and the brain.
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