

Coordination between proteasome impairment and caspase activation leading to TAU pathology: neuroprotection by cAMP

MJ Metcalfe¹, Q Huang¹ and ME Figueiredo-Pereira^{*1}

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

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Alzheimer's disease (AD) is an age-related neurodegenerative disorder. Little is known about the initial pathology, and when symptoms are detected, neurodegeneration is so advanced that it is seldom reversible. Neurofibrillary tangles (NFTs) are pathological hallmarks of AD. The major component of NFTs is TAU, a microtubule-associated protein that is abundant in neurons and highly soluble; yet TAU aggregates abnormally in AD.¹ Truncation of TAU at Asp421 (Δ TAU) by caspases is an early event in AD tangle pathology.²⁻⁴ In addition, Δ TAU is detected in NFTs, indicating that the apoptotic cascade is involved in NFT formation.⁵

The initial events leading to caspase activation and Δ TAU are poorly defined. We propose that proteasome impairment could be one of the initial critical events that contributes and leads to caspase activation concurring with Δ TAU, protein aggregation and neuronal death. There is a general agreement that proteasome impairment is involved in the pathogenesis of AD. Defective proteasome activity is connected to the early phase of AD characterized by synaptic dysfunction, as well as to late AD stages linked to accumulation and

aggregation of ubiquitinated (Ub) proteins in both senile plaques and NFTs.^{6,7}

To investigate a potential coordination between proteasome impairment and caspase activation leading to TAU pathology, we treated rat cerebral cortical neurons with two drugs that inhibit the proteasome by different means: prostaglandin J2 (PGJ2) and epoxomicin. PGJ2 is an endogenous product of inflammation that inhibits the proteasome by inducing oxidation of its subunit S6 ATPase (Rpt5),⁸ and/or by promoting dissociation of the 26S proteasomes.⁹ By promoting 26S proteasome disassembly, PGJ2 resembles the effects of agents that induce oxidative stress.¹⁰ We recently demonstrated in rat cortical neurons that PGJ2 induces accumulation of Ub proteins, caspase activation, Δ TAU and its aggregation, as well as neuritic dystrophy.¹¹ Epoxomicin is a specific and irreversible inhibitor of the proteasome that forms a covalent adduct with the amino terminal Thr of the 20S proteasome catalytic subunits, generating irreversible morpholino adducts.¹² Other proteasome inhibitors, that is, MG132 and lactacystin, were shown

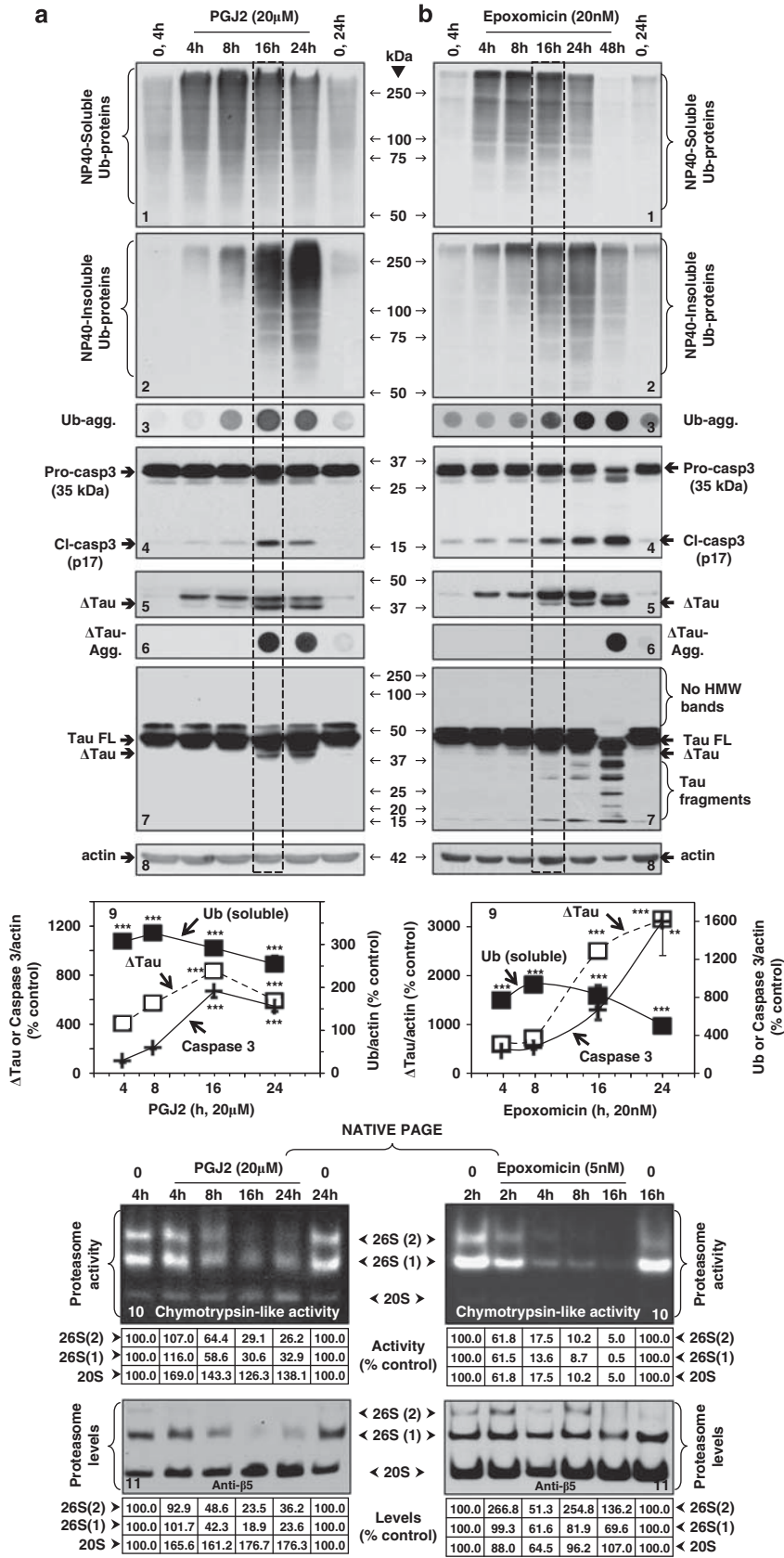
¹Department of Biological Sciences, Hunter College and Graduate Center, City University of New York, New York, NY, USA

*Corresponding author: M Figueiredo-Pereira, Department of Biological Sciences, Hunter College and Graduate Center, City University of New York, 695 Park Avenue, Room 827N, New York, NY 10065, USA. Tel: +212 650 3565; Fax: +212 772 5227; E-mail: pereira@genectr.hunter.cuny.edu

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Abbreviations: Δ TAU, TAU cleaved at Asp421; AD, Alzheimer's disease; db-cAMP, dibutyryl-cAMP; DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescence; Ep, epitope; Epac, exchange protein directly activated by cAMP; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PACAP27, pituitary adenylate cyclase-activating polypeptide-27; PBS, phosphate buffered saline; PGD2, prostaglandin D2; PGJ2, prostaglandin J2; PKA, cAMP-dependent protein kinase; Rp-cAMPS, adenosine 3',5'-cyclic monophosphorothioate, Rp-isomer, triethylammonium salt; SDS, sodium dodecyl sulfate; SUB proteins, detergent (NP40), soluble ubiquitinated proteins; Suc-LLVY-AMC, succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin; Ub proteins, ubiquitinated proteins

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to induce apoptosis via caspase activation in rat cortical neurons, but Ub protein accumulation/aggregation and TAU pathology were not addressed.¹³

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

Results

Detergent (NP40) -SUB proteins accumulate early upon proteasome inhibition, and before caspase activation, as well as Δ TAU formation. We investigated a temporal correlation between proteasome inhibition and caspase activation by treating rat cortical neurons with PGJ2 (Figure 1a) or epoxomicin (Figure 1b). The time-course studies clearly demonstrate that detergent (NP40) -SUB proteins are detected upon a 4 h treatment with 20 μ M PGJ2 or 20 nM epoxomicin (Figures 1a and b, *panel 1*), the earliest time point analyzed. Robust aggregates of Ub proteins, as well as caspase 3 activation, Δ TAU formation and TAU aggregates (Figures 1a and b, *panels 2 to 6, respectively*) were detected much later, that is, after at least 16 h of

treatment. Large ($>0.2 \mu$ m), sodium dodecyl sulfate (SDS)-insoluble Ub- and TAU aggregates were assessed with the filter trap assay. Remarkably, the decline in SUB proteins observed at 16 and 24 h corresponds to a robust increase in Ub aggregates. SUB proteins are considered here to be those that are NP40-soluble, and Ub aggregates those that are NP40-insoluble as well as those that are retained with the filter trap assay.

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

Notably, proteasome inhibition for 48 h with epoxomicin did not increase the level of full-length TAU nor did it generate high-molecular-weight forms of TAU corresponding to Ub-TAU (Figure 1b, *panel 7*). Instead, epoxomicin caused an accumulation of various TAU fragments ranging in size between 37 kDa and 15 kDa, thus smaller than Δ TAU. These data indicate that upon TAU cleavage at Asp421 by caspases, TAU is further cleaved by unidentified proteases that generate smaller fragments detected upon proteasome inhibition. Actin levels (Figures 1a and b, *panel 8*) were not altered by the treatments.

We assessed with the native in gel assay, the effects of PGJ2 and epoxomicin on proteasome activity and levels in the cortical neurons. This assay detects the three native proteasome forms: 26S with two regulatory caps [26S(2)] or one cap [26S(1)], and the 20S core particle alone (20S). Proteasome chymotrypsin-like activity was determined with the substrate succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-LLVY-AMC; Figures 1a and b, *panel 10*). Under control conditions (*first lanes*), the activity of the 20S proteasome is substantially lower than the 26S, because the 20S is a latent form of the proteasome.¹⁴ Proteasome levels were

Figure 1 Accumulation of NP40-SUB proteins paves the way for caspase 3 activation, caspase-mediated TAU cleavage (Δ TAU) and aggregation of Ub proteins as well as TAU, induced by PGJ2 (a) or epoxomicin (b). Western blot analyses to detect Ub proteins (NP40-soluble and NP40-insoluble), caspase 3, TAU cleaved at Asp421 (Δ Tau), full-length TAU (Tau FL) and actin (loading control) in cell extracts of rat E18 cerebral cortical neurons (40 μ g of protein/lane). The cortical neurons were treated with 20 μ M PGJ2 (a) or 20 nM epoxomicin (b) for different time points. Cell extracts were subjected to centrifugation to separate the NP40-soluble and NP40-insoluble fractions as described under 'Materials and Methods'. The blots were probed with anti-Ub proteins antibody (*panels 1, 2 and 3*), caspase 3 (*panel 4*), TAU C3 antibody (TAU cleaved at Asp421, epitope a.a. 412–421, *panels 5 and 6*), TAU C5 antibody (detects all TAU isoforms and Δ TAU; ep: a.a. 210–241, *panel 7*) and anti-actin antibody (*panel 8*). Molecular mass markers in kDa are shown in the middle. Ub aggregates (Ub-agg.) and Δ TAU aggregates (Δ Tau-agg.) were analyzed with the filter trap assay (50 μ g of protein/dot, *panels 3 and 6*). The levels of NP40-soluble Ub proteins (*solid squares*), cleaved caspase 3 (*crosses*) and Δ TAU (*open squares*) were semi-quantified by densitometry (*panel 9*). Data represent the percentage of the pixel ratio for soluble Ub proteins, cleaved caspase 3 or Δ TAU, over actin for each condition compared with control (100%). Values are means \pm S.D. from at least two experiments. Asterisks identify values that are significantly different from control (** $P < 0.01$, *** $P < 0.001$). Ub proteins, Ub proteins; Δ Tau, TAU cleaved at Asp421; Tau FL, full-length TAU. To assess changes in proteasome activity, cell extracts were prepared from rat E18 cerebral cortical neurons treated with 20 μ M PGJ2 (a) or 5 nM epoxomicin (b) for different time points. Clear lysates (30 μ g/sample) were subjected to non-denaturing gel electrophoresis as described under 'Materials and Methods'. 26S and 20S proteasomal (*indicated in the middle by arrows*) chymotrypsin-like activity was assessed with Suc-LLVY-AMC by the in gel assay (*panel 10*). Proteasome levels were detected by immunoblotting with the anti- β 5 antibody (*panel 11*). The numbers at the bottom of panels 10 and 11 represent 26S and 20S proteasomal chymotrypsin-like activity and levels, respectively, under each treatment condition. Percentages represent the ratio between data for each condition and control (DMSO) considered to be 100%. Values are means from duplicate experiments

established by immunoblotting with an anti- $\beta 5$ antibody (Figures 1a and b, *panel 11*). The $\beta 5$ subunit is a component of the 20S core, thus the antibody detects both 26S and the 20S proteasomes. PGJ2 and epoxomicin inhibited the 26S proteasome in a manner that parallels the accumulation of Ub proteins induced by both drugs. Furthermore, PGJ2 caused 26S proteasome disassembly, whereas 20S proteasome levels increased (Figure 1a, *panel 11*). We used lower epoxomicin concentrations in this assay to be able to determine the gradual time-dependent decrease in proteasome activity, which by 16 h was low (Figure 1b, *panel 10*).

db-cAMP and PACAP27 prevent the decrease in cAMP induced by PGJ2. PGJ2 is derived from prostaglandin D2 (PGD2), the major product of cyclooxygenases in the mammalian central nervous system.¹⁵ PGJ2 signals via one of the PGD2 receptors, that is, the DP2 receptor, which is coupled to inhibitory G proteins thus lowering cAMP.^{16,17} As cAMP has neuroprotective effects,¹⁸ to overcome the decline in cAMP induced by PGJ2, we tested two drugs: (1) db-cAMP, which is more cell permeable and resistant to cyclic phosphodiesterases than cAMP¹⁹ and (2) PACAP27 (pituitary adenylate cyclase-activating polypeptide), which 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.²⁰ PAC1R is expressed in the cerebral cortex, hippocampus and other brain areas.²¹ In our studies, db-cAMP was added to the cultures 30 min before PGJ2, whereas PACAP27 was added in conjunction with PGJ2.

Compared with control conditions, PGJ2 (10 μ M, 4 h) decreased cAMP levels by almost 3-fold, whereas db-cAMP (1 mM) and PACAP27 (100 nM) increased cAMP by 4-fold and 2.5-fold, respectively (Figure 2). In the presence of PGJ2, db-cAMP and PACAP27 still raised cAMP levels, by 8.4-fold and 4.8-fold, respectively, when compared with PGJ2 alone. Remarkably,

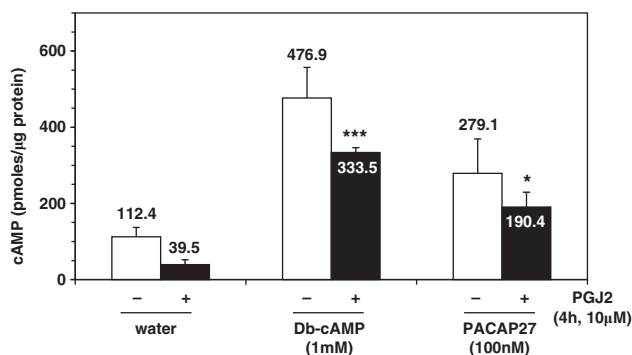


Figure 2 Dibutyl-cAMP (db-cAMP) and PACAP27 prevent the decrease in cAMP induced by PGJ2. Rat E18 cerebral cortical neurons were treated with water (vehicle, control), db-cAMP (1 mM) or PACAP27 (100 nM) in conjunction with DMSO (vehicle, control, *white bars*) or with 10 μ M PGJ2 (*black bars*) for 4 h. Intracellular cAMP was measured with an EIA kit as described under 'Materials and Methods'. Levels of intracellular cAMP in the cortical neurons are expressed as pmoles per μ g of protein. Values represent means and S.E. from three experiments. Asterisks identify values that are significantly different in a comparison between PGJ2 alone and PGJ2 with db-cAMP ($***P < 0.001$) or between PGJ2 alone and PGJ2 with PACAP27 ($*P < 0.05$).

PACAP27 elevated intracellular cAMP at drastically lower (nanomolar) concentrations than db-cAMP (millimolar).

Db-cAMP and PACAP27 prevent activation of caspases 8 and 3 induced by PGJ2. PGJ2 treatment promotes activation of the initiator caspase 8 (extrinsic pathway) in a time-dependent manner (Figure 3a) similar to activation of the effector caspase 3 (Figure 1a, *panel 4*). Caspase activation was assessed by western blotting that detects conversion of pro-caspase 8 and 3 into their cleaved active forms, and with a colorimetric assay (Figure 3). Caspases 3 (Figure 3c) and 8 (Figure 3d) were significantly (\sim twofold, $P < 0.001$) activated by PGJ2 treatment (8 h, 10 μ M). Db-cAMP and PACAP27 stopped/reduced caspase activation triggered by PGJ2 (Figures 3c and d). Caspase 9 was not affected by PGJ2 (Figures 3b and e).

Db-cAMP and PACAP27 prevent Δ TAU formation induced by PGJ2. The formation of Δ TAU triggered by short-term (up to 8 h) incubations with PGJ2 was blocked/reduced by db-cAMP (1 mM) and PACAP27 (100 nM; *rows 1*, Figures 4a and b), respectively. A similar trend was observed for activation of caspase 8 and 3. Both cAMP-elevating drugs prevented/diminished the conversion of procaspase 8 and 3 to the respective cleaved forms upon short-term (up to 8 h) treatment with PGJ2 (10 μ M, *rows 2 and 3*, Figures 4a and b).

Upon long-term (16 and 24 h) incubations with PGJ2, the protective effect of a single dose of db-cAMP or PACAP27 related to Δ TAU and caspase activation was dissipated. PACAP27 was less efficient than db-cAMP at all times. The lanes from each gel were separated into consecutive pairs to facilitate comparing the effect of PGJ2 alone ($-$) with the combination of PGJ2 plus db-cAMP or PACAP27 ($+$).

These data demonstrate that elevating intracellular cAMP prevents caspase-dependent Δ TAU formation triggered by short-term incubations (up to 8 h) with PGJ2. The protective effect of one single dose of db-cAMP or PACAP27 against longer PGJ2 treatments (16 and 24 h) faded away.

Db-cAMP stimulates proteasomal activity in a PKA-dependent manner and reduces 26S proteasome inhibition by PGJ2. One single dose of db-cAMP (1 mM) increased the activity and levels of both forms of the 26S proteasome in the cortical neurons (Figures 5a and b, *panel 1, lane d*). The activity of the 20S particle was also elevated without major changes in its levels. cAMP is known to exert its action by targeting PKA and/or Epac (exchange protein directly activated by cAMP) (reviewed in Cheng *et al.*²²). We show that stimulation of the proteasome by db-cAMP was PKA dependent, as it was abolished by pretreatment with adenosine 3',5'-cyclic monophosphorothioate (Rp-cAMPS; Figures 5a and b, *panel 1, lanes d and e*). Rp-cAMPS is a PKA inhibitor that acts as a competitive antagonist of the cyclic-nucleotide-binding domains on PKA.²³

Db-cAMP reduced the inhibitory effect of PGJ2 on the 26S proteasome, albeit proteasome activity and levels were not completely preserved (Figures 5a and b, *panel 2, compare lanes b and d*). In addition, db-cAMP alone increases proteasome activity via PKA.

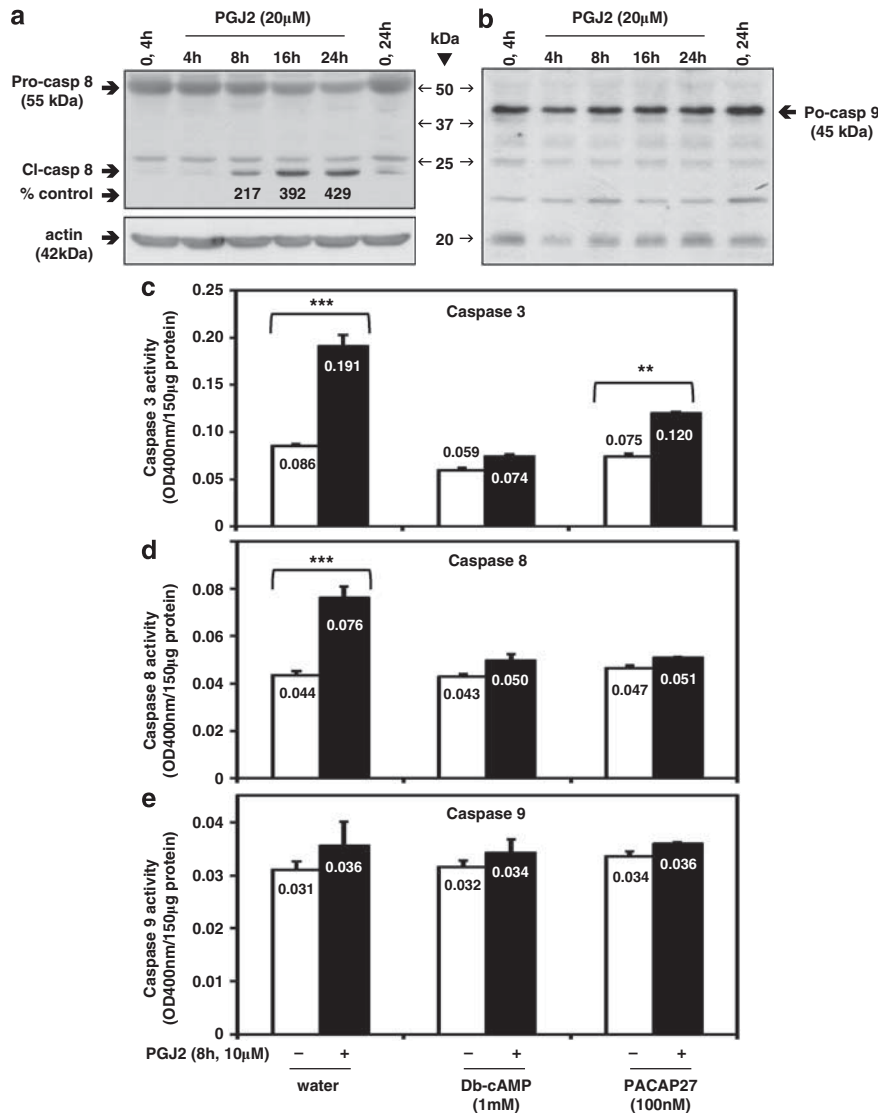


Figure 3 PGJ2-treatment activates caspase 8 (a) in a time-dependent manner, but not caspase 9 (b). Db-cAMP (1 mM) and PACAP27 (100 nM) prevent/reduce caspase 3 and caspase 8 activation induced by PGJ2. Western blot analyses to detect caspases 8 (a) and caspase 9 (b) in their zymogenic (Pro) and cleaved (Cl) forms, and actin (loading control), in extracts (40 μg of protein/lane) of rat E18 cerebral cortical neurons treated with 20 μM PGJ2 for different time points. The blots were probed with the respective antibodies. The numbers in (a) represent the % increase in cleaved caspase 8 compared with control (100%). Values are from triplicate experiments. The control value was calculated as the average between controls for 4 and 24 h, reflecting the average of six values. Activities of caspases 3, 8 and 9 (c, d and e, respectively) were also determined in extracts of rat E18 cerebral cortical neurons (150 μg of protein/assay) as described under 'Materials and Methods'. The cortical neurons were treated with water (vehicle, control), db-cAMP (1 mM) or PACAP27 (100 nM) in conjunction with DMSO (vehicle, control, white bars) or with 10 μM PGJ2 (black bars) for 8 h. Caspase activities expressed as OD at 400 nm and normalized for protein (150 μg/assay) represent means and S.E. from four determinations. Asterisks identify values that are significantly different in a comparison between water, db-cAMP or PACAP27 alone (white bars) and water, db-cAMP or PACAP27 with PGJ2 (black bars), ** $P < 0.01$ and *** $P < 0.001$

Although PACAP27 elevates cAMP, we did not observe any effect of the peptide on 26S proteasomes upon 24 h treatment (Figures 5a and b, all panels, lanes f and g). This is most likely due to rapid hydrolysis of cAMP generated by treatment with the peptide (see Discussion).

Db-cAMP and PACAP27 prevent the formation of Ub protein aggregates induced by PGJ2. Both cAMP-elevating drugs prevented Ub-protein aggregation induced by 8 h treatment with PGJ2 (Figure 6a, upper panel). However, this effect was dissipated upon longer (24 h) PGJ2 incubations

(Figure 6a, lower panel). Db-cAMP reduced 26S proteasome inhibition induced by 8 h as well as 24 h treatments with PGJ2, but proteasome activity was never recovered to 100% (Figure 6b). Thus, the beneficial effect of one single dose of db-cAMP on the proteasome effectively prevented protein aggregation upon short-term but not long-term treatment with PGJ2. For simplicity, only the activity of the 26S proteasome with one cap [26S(1)] was quantified, as it is the strongest of the three proteasome forms detected.

The levels of NP40-SUb proteins induced by PGJ2 were not altered by db-cAMP (Figures 6c and d) or PACAP27 (not shown).

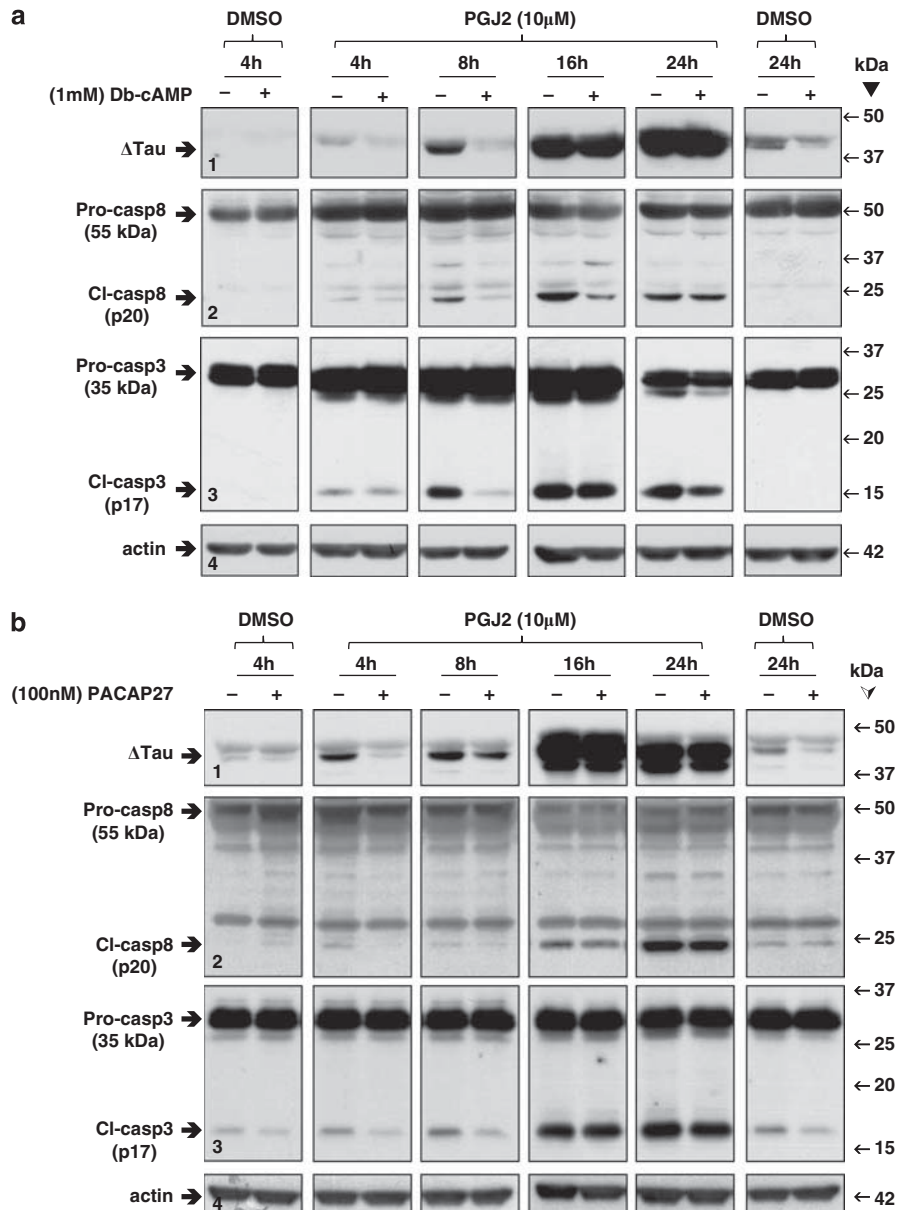


Figure 4 Db-cAMP (1 mM, **a**) and PACAP27 (100 nM, **b**) prevent/reduce caspase-mediated cleavage of TAU at Asp421 (Δ TAU) induced by short-term (up to 8 h) but not long-term (at least 16 h) incubations with PGJ2. Western blot analyses to detect TAU cleaved at Asp421 (Δ TAU, rows 1), caspase 8 (rows 2), caspase 3 (rows 3) and actin (rows 4, loading control) in extracts of rat E18 cerebral cortical neurons (40 μ g of protein/lane). The cortical neurons were treated with water (*minus sign*, vehicle, control), db-cAMP (**a**) *plus sign*, 1 mM) or PACAP27 (**b**) *plus sign*, 100 nM) in conjunction with DMSO (vehicle, control) or 10 μ M PGJ2 for different time points (4, 8, 16 and 24 h). The blots were probed with the respective antibodies. Molecular mass markers in kDa are shown on the right. Similar results were obtained in duplicate experiments. Δ Tau, TAU cleaved at Asp421; Pro, zymogenic; and Cl, cleaved forms of the caspases

We conclude that improvement of proteasome activity by one single dose of db-cAMP was not enough to completely overcome accumulation/aggregation of Ub proteins. Although db-cAMP reduced Ub aggregates formed upon 8 h treatment with PGJ2, in longer treatments the effect of PGJ2 prevailed over the cAMP analog.

Db-cAMP and PACAP27 prevent the loss of cell viability induced by PGJ2. PGJ2 treatment is neurotoxic in a time- and concentration-dependent manner (Figure 7a). Db-cAMP (1 mM, Figures 7b and c) and PACAP27 (25–100 nM,

Figure 7d) significantly ($P < 0.001$) reduced the loss of viability induced by short-term (8 h) treatment with PGJ2. However, neuroprotection by a single dose of db-cAMP, the more effective of the two cAMP-elevating drugs, was lost against longer (24 h) incubations with PGJ2.

Three doses of db-cAMP or PACAP27 reduce the changes induced by long-term (24 h) incubations with PGJ2. As shown in Figure 7, the viability of the cells upon long-term (24 h) incubations with PGJ2 decreases to about 50%. To overcome the long-term effects of PGJ2, we

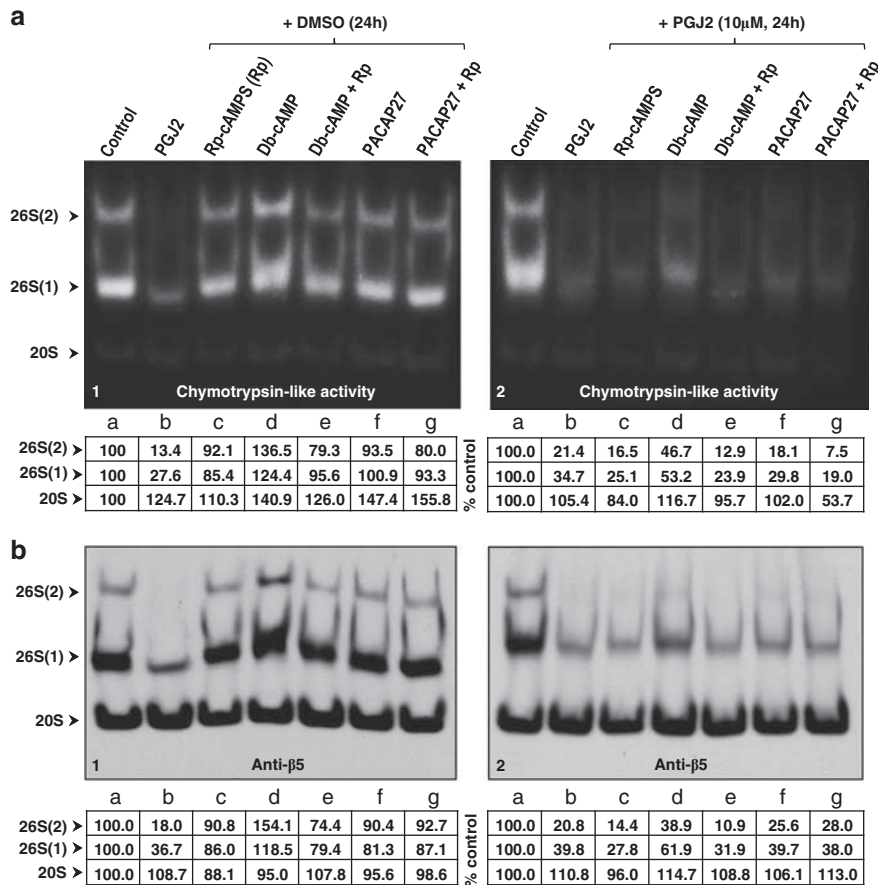


Figure 5 Db-cAMP stimulates 26S proteasomes via PKA, and reduces 26S proteasome inhibition by PGJ2; no changes in 26S proteasome activity were detected with PACAP27. Extracts were prepared from rat E18 cerebral cortical neurons treated with water (control, vehicle), db-cAMP (1 mM, db-cAMP) without or with Rp-cAMPS (100 μ M, 1 h pretreatment, Rp), PACAP27 (100 nM) without or with Rp-cAMPS, in conjunction with DMSO (24 h, control, vehicle, *panel 1*) or with PGJ2 (24 h, 10 μ M, *panel 2*). Rp-cAMPS is a PKA inhibitor. Cleared cell lysates (30 μ g/sample) were subjected to non-denaturing gel electrophoresis as described under 'Materials and Methods'. 26S and 20S proteasomal chymotrypsin-like activities (*indicated on the left by arrows*) were assessed with Suc-LLVY-AMC by the in-gel assay (**a panels 1 and 2**). Proteasome levels were detected by immunoblotting with anti- β 5 (**b panels 1 and 2**). The numbers at the bottom of each panel represent 26S and 20S proteasomal chymotrypsin-like activity and proteasome levels under each treatment condition. Percentages represent the ratio between data for each condition and control (DMSO, 100%). Values are from a representative experiment. Similar results were obtained in duplicate experiments

increased the number of treatments with the cAMP-elevating drugs as described under 'Materials and Methods'. As shown in Figure 8, three doses of db-cAMP (1 mM) or PACAP27 (100 nM) clearly diminished the levels of Δ TAU (*panel 1*), Δ TAU aggregates (*panel 2*), caspase 3 cleavage (*panel 3*), soluble Ub proteins (*panel 4*), Ub-protein aggregates (*panel 5*) and loss of cell viability (*panel 7*) induced by 24 h treatment with 5 μ M PGJ2. Incubations with 10 μ M PGJ2 caused more severe changes than 5 μ M PGJ2, and thus were harder to overcome. In the absence of PGJ2, three doses of the cAMP-elevating drugs did not alter the levels of the proteins tested (*not shown*). In addition, treatment with just two doses of the cAMP-elevating drugs was ineffective against PGJ2 (*not shown*).

Discussion

Our current data with rat cerebral cortical neurons demonstrate a temporal correlation between proteasome inhibition and caspase activation that leads to TAU cleavage at Asp421

associated with TAU pathology and cell death. The temporal correlation depicts the accumulation of detergent (NP40)-Sub proteins occurring early upon proteasome impairment. Caspase activation, TAU cleavage at Asp421 and the aggregation of TAU and Ub proteins occur significantly later. Large aggregates (detected with the filter trap assay) also appear late in this toxic cascade. The filter trap assay captures large (> 0.2 μ m) and SDS-insoluble aggregates.²⁴ The sequence of proteolysis-related events was triggered by the product of inflammation PGJ2 and the specific proteasome inhibitor epoxomicin. Although PGJ2 mimics the effect of some oxidative stressors by causing dissociation of 26S proteasomes,^{9,10} epoxomicin forms covalent adducts with the 20S proteasome active sites.¹² The finding that both drugs induce a similar temporal response to proteasome impairment suggests that these proteolysis-related events could be shared by various proteotoxic conditions that induce a decline in proteasome activity in neurons. This temporal response to proteasome inhibition strongly supports the notion that the accumulation of Sub proteins, if not resolved, could be one of

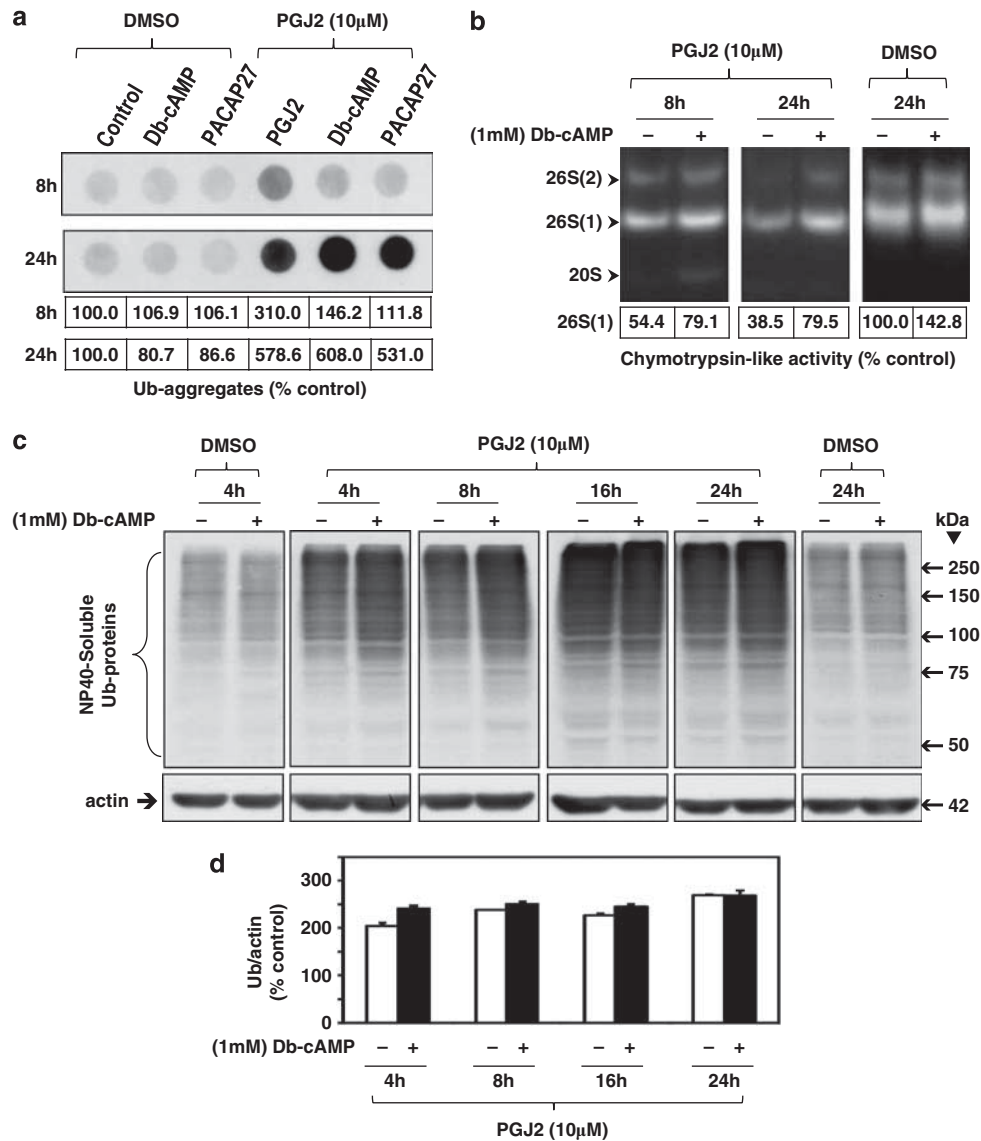


Figure 6 Db-cAMP (1 mM) reduces Ub aggregates and 26S proteasome inhibition induced by PGJ2; accumulation of NP40-soluble Ub proteins triggered by PGJ2 was not affected by db-cAMP. **(a)** Ub aggregates (50 μ g of protein/dot) were analyzed with the filter trap assay as described under 'Materials and Methods'. **(b)** 26S and 20S proteasomal chymotrypsin-like activities were assessed with Suc-LLVY-AMC by the in-gel assay as described under 'Materials and Methods'. The numbers under the panels represent in **(a)** ubiquitin aggregates, and in **(b)** 26S and 20S proteasomal chymotrypsin-like activity. Percentages represent the ratio between data for each condition and control (DMSO, 100%). Values are from a representative experiment. Similar results were obtained in duplicate experiments. **(c)** Western blot analyses to detect NP40-soluble Ub proteins and actin (loading control) in extracts of rat E18 cerebral cortical neurons (40 μ g of protein/lane). The cortical neurons were treated with water (*minus sign*, vehicle, control) or db-cAMP (*plus sign*, 1 mM) in conjunction with DMSO (vehicle, control) or 10 μ M PGJ2 for different time points (4, 8, 16 and 24 h). The blots were probed with the respective antibodies. Molecular mass markers in kDa are shown on the right. The levels of NP40-soluble Ub proteins were semi-quantified by densitometry **(d)**. Data represent the percentage of the pixel ratio for soluble Ub proteins over actin for each condition compared with control (100%). Values are means and S.E. from three experiments

the critical events triggering caspase activation that mediates TAU cleavage and generates aggregation-prone fragments of TAU. Other studies support this notion. For example, 26S proteasome dysfunction was sufficient to trigger neurodegenerative disease in a transgenic mouse model developed by conditionally depleting a 26S proteasome subunit in forebrain neurons.²⁵ The mutant mice exhibited diffuse accumulation of Ub proteins in forebrain neurons at 2 weeks of age. Caspase activation and intraneuronal Ub-positive inclusions were observed later, at 4 weeks of age, indicating extensive

neurodegeneration in the targeted neurons.²⁵ In another study, proteasome impairment was found to occur early in the progression of the pathological events detected in 3 \times Tg-AD mice, leading to A β and TAU accumulation.²⁶ Based on these studies, we propose that elevating proteasome activity to prevent the accumulation of Sub proteins early in the neurodegenerative process could be an effective approach to prevent caspase activation and TAU pathology.

Notably, we found that a single dose of db-cAMP increases 26S proteasome activity via PKA activation in the cortical

neurons. Others established a similar phenomenon in 293 cells²⁷ and in myocardium.²⁸ The latter studies demonstrated that PKA stimulation increased the activity of the 26S proteasome via subunit phosphorylation and/or transcription. In our experiments with the cortical neurons, proteasome stimulation promoted by a single dose of db-cAMP mitigated proteasome inhibition induced by PGJ2. Under the conditions tested, we did not observe proteasome stimulation by PACAP27, although the peptide elevates intracellular cAMP. This is not surprising, as cAMP is significantly more susceptible to hydrolysis by cyclic phosphodiesterases than its analog db-cAMP. Cyclic phosphodiesterases exhibit very rapid kinetics for cAMP degradation, compared with cAMP synthesis by adenylate cyclases.²⁹ It is likely that to maintain proteasome activity via db-cAMP or PACAP27 on a long-term basis under proteotoxic conditions, these drugs have to be delivered more than once, and not as a single dose. PACAP27 may be preferred over db-cAMP, as the latter was shown to have adverse biological effects when administered *in vivo*.³⁰ Once prolonged 26S proteasome stimulation is attained, it could prevent the early accumulation of SUB proteins and avoid later on caspase activation, protein aggregation and neurons from reaching a point of no return.

We also demonstrate, for the first time to our knowledge, that elevating cAMP via db-cAMP or PACAP27 prevents caspase activation and generation of Δ TAU induced by PGJ2. TAU proteolysis is recognized as having an important role in TAU aggregation and neurodegeneration in AD.^{2,3} Thus, blocking TAU cleavage at Asp421 could be a potential therapeutic approach against TAU pathology. We show that only a single dose of db-cAMP or PACAP27 blocked caspase activation and Δ TAU upon short-term (up to 8 h) treatment with PGJ2. As shown in Figure 7, the viability of the cells upon long-term (24 h) incubations with PGJ2 decreases to about 50%. To attempt to overcome the harsh effects of long-term (24 h) incubations with PGJ2, we decided to increase the number of treatments with the cAMP-elevating drugs. Administration of three sequential doses of the cAMP-elevating drugs was necessary to diminish Δ TAU, caspase activation and loss of cell viability promoted by long-term (24 h) incubations with 5 μ M PGJ2. It is notable that PACAP27 mimics the protective effects of db-cAMP at considerably lower concentrations (nanomolar for PACAP27 *versus* millimolar for db-cAMP). The difference in effectiveness could be due to some properties of db-cAMP, such as that it remains inactive until endogenous esterases/amylases remove the butyrate.³¹ Furthermore butyrate, by itself, affects gene transcription and PKC, thus interfering with several cAMP-dependent pathways.³¹ As discussed above in relation to PACAP27, treatment with a single dose of the peptide is unlikely to be optimal and/or maximized for long-term neuroprotection, because its action depends on the production of hydrolysable cAMP. This notion is supported by our data showing that three consecutive doses of the cAMP-elevating drugs diminish long-term (24 h) effects of PGJ2. Interestingly, PACAP was shown to enhance α -secretase activity³² and improve memory in rats.³³ That PACAP27 shows promise in delaying AD is corroborated by a recent study showing that long-term daily intranasal administration of PACAP slowed down AD-like pathology in APP[V717I]

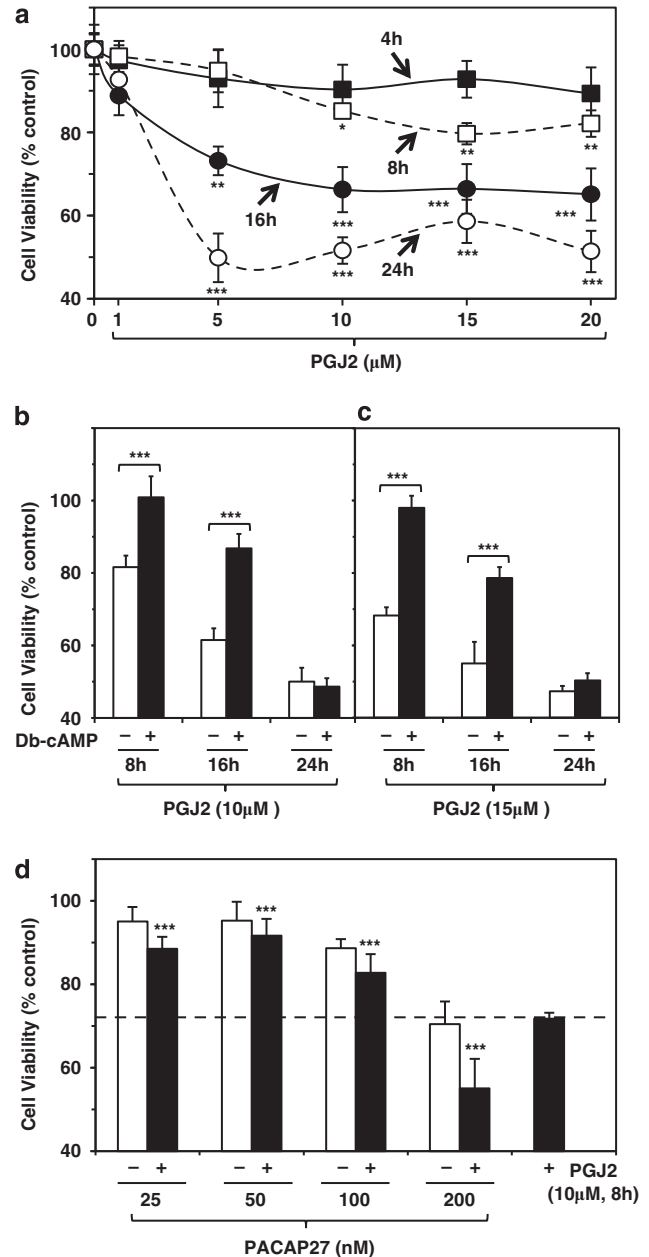


Figure 7 Db-cAMP (1mM) and PACAP (100 nM) prevent/reduce the loss of cell viability induced by short-term (8 h) incubations with PGJ2. Rat E18 cerebral cortical neurons were treated: in (a) with DMSO (0, control), or increasing concentrations of PGJ2 for 4, 8, 16 or 24 h; in (b) and (c) with water (control, vehicle, *minus sign*) or db-cAMP (1 mM, db-cAMP, *plus sign*) in conjunction with DMSO (vehicle, *not shown*) or PGJ2 (b, 10 μ M, c, 15 μ M) for 8, 16 and 24 h; in (d) with water (control, vehicle, *not shown*) or with PACAP27 (25, 50, 100 or 200 nM) in conjunction with DMSO (vehicle, *minus sign*) or PGJ2 (10 μ M, *plus sign*) for 8 h. Cell viability was assessed with the MTT assay as described under 'Materials and Methods'. Percentages represent the ratio between the data for each condition and control (100%). Values indicate means and S.E. from at least three experiments. Asterisks identify values that are significantly different in (a) from control, in (b) and (c) between PGJ2 alone and PGJ2 with db-cAMP, and in (d) between PGJ2 alone and PGJ2 with PACAP27, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

AD transgenic mice.³⁴ In the latter studies, TAU pathology was not addressed. Together these results support the view that, due to its beneficial properties, PACAP27 could be a very

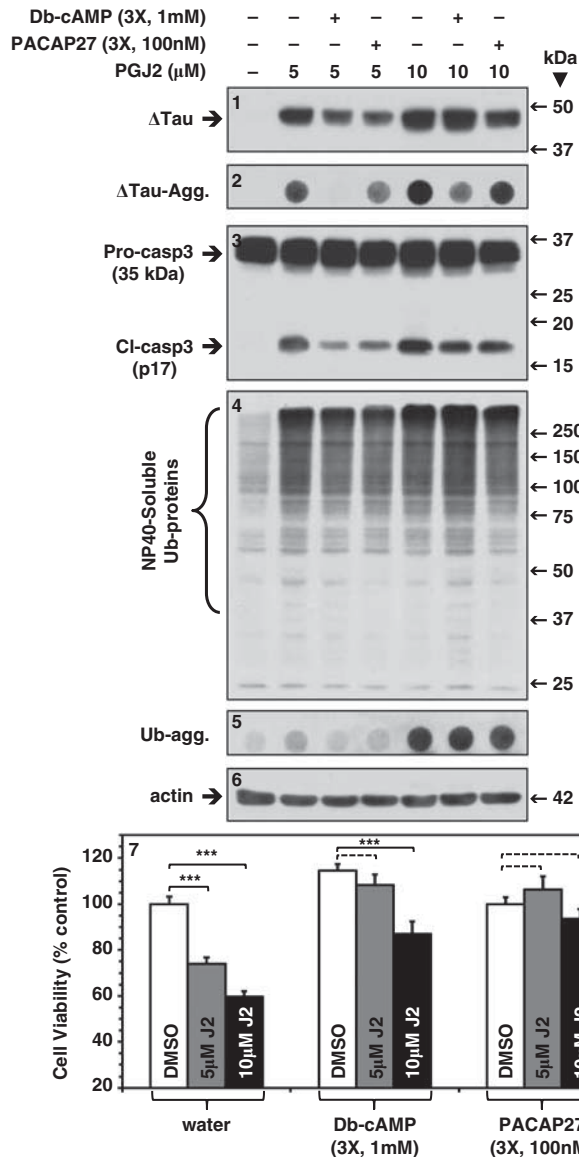


Figure 8 Three doses of db-cAMP (1 mM) or PACAP (100 nM) diminish the effects of long-term (24 h) incubations with PGJ2. Rat E18 cerebral cortical neurons were treated with DMSO (0, control, vehicle for PGJ2) or PGJ2 (5 or 10 μ M) in conjunction with three consecutive doses of water (control, vehicle for db-cAMP and PACAP27), db-cAMP (1 mM, db-cAMP) or PACAP27 (100 nM) over a period of 24 h. The cAMP-elevating drugs were added as described under 'Materials and Methods'. Western blots of the NP-40 soluble fractions (30 μ g of protein/lane) were probed for TAU cleaved at Asp421 (Δ TAU, panel 1), caspase 3 (panel 3), soluble Ub proteins (panel 4) and actin (panel 6, loading control). Δ TAU- and Ub aggregates (panels 2 and 5) were assessed with the filter trap assay (30 μ g of protein/dot). Molecular mass markers in kDa are shown on the right. Similar results were obtained in duplicate experiments. Δ Tau, TAU cleaved at Asp421; Pro, zymogenic; and Cl, cleaved forms of caspase 3. Cell viability (panel 7) was assessed with the MTT assay. Percentages represent the ratio between the data for each condition and control (100%). Values indicate means and S.E. from six determinations. Asterisks identify values that are significantly different from treatment with water alone or db-cAMP alone (white bars, respectively) within each group, with *** P <0.001. Dashed lines compare conditions that are not significantly different

interesting agent for long-term treatment of AD (reviewed in Reglodi *et al.*³⁵). The challenge is to identify which mechanisms PACAP27 is triggering to bring about its protective effects.

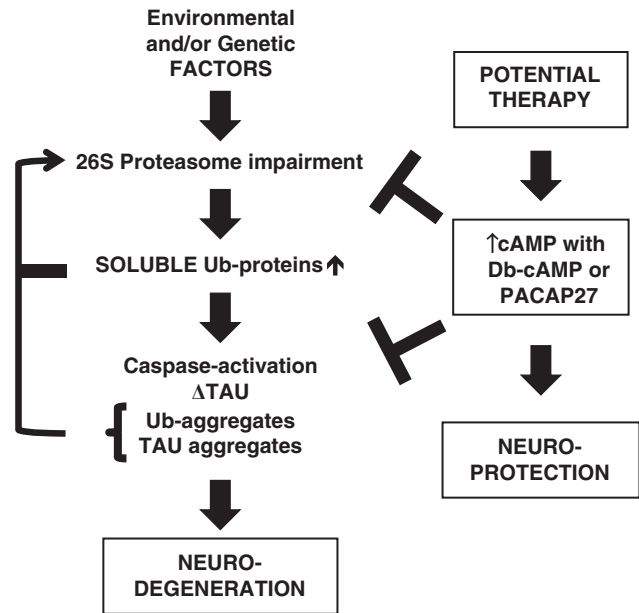


Figure 9 Model for the progression of neurodegeneration and potential therapy. The model is based on proteasome impairment and the early accumulation of detergent soluble Ub proteins. The therapeutic approach focuses on a robust and sustainable stimulation of 26S proteasome activity accomplished by targeting cAMP/PKA signaling. Details are provided in the last paragraph of the discussion.

In conclusion, our data clearly demonstrate that the accumulation of SUb proteins is an early event that occurs after treating cortical neurons with two drugs, that is, PGJ2 and epoxomicin, that inhibit the proteasome by different means. As there is compelling evidence for impairment of proteasome activity in AD³⁶ and aging,³⁷ it is possible that the sequence of proteolysis-related events that we established here and that are triggered by PGJ2 and epoxomicin is similar in the early stages of neurodegeneration in AD (modeled in Figure 9). Initially, low levels of SUb proteins that escape degradation accumulate in the affected areas of the AD brain. These SUb proteins could further exacerbate proteasome malfunction. Several studies demonstrate that increasing the levels of polyubiquitin chains³⁸ and/or aggregation prone proteins, such as PHF-TAU,³⁹ decreases proteasome activity. When these proteins accumulate in the cell, they can bind to proteasomes and block access of other substrates to the proteasomal-degradation machinery, thus further aggravating the proteotoxic situation. With time, proteasome activity continues to deteriorate to a point that the neurons can no longer cope with the proteotoxic stress and reach a point of no return. This feed-forward incremental proteasome inhibition could be a major factor in neurodegeneration. We propose (modeled in Figure 9) that targeting the proteasome to enhance its activity in a robust and sustainable manner via cAMP/PKA signaling could avoid the early accumulation of SUb proteins, followed later on, by caspase activation and protein aggregation. An early intervention strategy could prevent neurons from reaching a point of no return, and provide an effective therapy to avoid/delay neurodegeneration in AD.

Materials and Methods

Materials. PGJ2 was from Cayman Chemical (Ann Arbor, MI, USA) and epoxomicin from Peptides International Inc. (Louisville, KY, USA). Adenosine 3', 5'-cyclic monophosphate dibutyl sodium salt (db-cAMP), Rp-cAMPS, triethylammonium salt (Rp-isomer) and the cAMP colorimetric direct immunoassay Kit were from Calbiochem/EMD Bioscience (Gibbstown, NJ, USA). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was from Sigma-Aldrich (St. Louis, MO, USA). The substrate Suc-LLVY-AMC and the peptide PACAP27 were from BACHEM Bioscience Inc. (King of Prussia, PA, USA). Antibodies: rabbit polyclonal anti-Ub-proteins (1:1,500, cat# Z0458, Dako, North America, Carpinteria, CA, USA); rabbit polyclonal anti- β 5 (1:1,000, cat# PW8895) from BIOMOL (Plymouth Meeting, PA, USA); mouse monoclonal anti- β -actin (1:10,000, cat# A2228, Sigma, St. Louis, MO, USA); rabbit polyclonal anti-caspase 8 (1:500, cat# 3020) and anti-caspase 9 (1:1,000, cat# 3016) from BioVision (Mountain View, CA, USA); 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 Δ TAU; Ep: a.a. 210–241) were courtesy of Dr. L Binder (Northwestern University, Chicago, IL, USA); rabbit polyclonal anti-caspase 3 (1:1000, cat# 9662, Cell Signaling Technology, Danvers, MA, USA). The respective secondary antibodies with HRP conjugate (1:10,000) were from BioRad Laboratories (Hercules, CA, USA).

Cell cultures. Dissociated cultures from Sprague Dawley rat embryonic (E18, both sexes) cerebral cortical neurons were prepared as follows: the isolated cortices free of meninges were digested with papain (0.5 mg/ml from Worthington Biochemical Corp., Lakewood, NJ, USA) in Hibernate E without calcium (BrainBits LLC., Springfield, IL, USA) 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, Carlsbad, CA, USA). Dissociated tissues were centrifuged at 300 \times g for 2 min. The pellet was resuspended in Neurobasal media without antibiotics and plated on 10 cm dishes pre-coated with 50 μ g/ml poly-D-lysine (Sigma). Cells were plated at a density of 6 \times 10⁶ cells per 10 cm dish or 2.5 \times 10⁵ cells per well on 24-well plates (cell viability only). Cultures were maintained in Neurobasal media supplemented with 2% B27 and 0.5 mM glutamine (all from Invitrogen) at 37°C in a humidified atmosphere containing 5% CO₂. Half of the medium was changed every 4 days.

Culture treatments. Cortical neurons were treated for 4, 8, 16 or 24 h with DMSO or ultra pure filtered water (controls) or with different drugs: PGJ2 and epoxomicin in DMSO; db-cAMP, Rp-cAMPS and PACAP27 in ultra pure filtered water added directly to DMEM without serum supplemented with 0.5 mM glutamine and 1 mM sodium pyruvate (all from Invitrogen). The final DMSO concentration in the medium was 0.5%. For a single administration, db-cAMP was added to the cultures 30 min before PGJ2, whereas PACAP27 was added in conjunction with PGJ2. For triple (3 \times) administrations, each of the cAMP-elevating drugs was added firstly as for the single administration, and then 4 and 8 h after that. PGJ2 was added only once at the beginning of the treatment. At the end of the incubations, all cultures were washed twice with phosphate buffered saline (PBS) and processed for the different assays as described below.

Cell viability assay. Cells were treated under various conditions for 4, 8, 16 or 24 h. Cell viability was assessed with the MTT assay as described in Mosmann.⁴⁰

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 Na₂P₂O₇, 1 mM β -glycerophosphate, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1% NP40, 1 mM Na₃VO₄, 1% Glycerol and protease inhibitor cocktail (Sigma-Aldrich)). Following lysis (at least 30 min, –80°C), cell extracts were centrifuged (19,000 \times g for 10 min) at 4°C for separation into two fractions: NP40-soluble (supernatant) and NP40-insoluble (pellet). The pellet was resuspended in a buffer containing 1% SDS and 10 mM Tris-EDTA pH 7.5. Protein concentration of both fractions was determined (BCA kit, Pierce, Rockford, IL, USA). Note that we consider SUB proteins those that are NP40-soluble and Ub aggregates those that are NP40-insoluble as well as detected by the filter trap assay described below. Western blot analysis was carried out following SDS-polyacrylamide gel electrophoresis. Normalized samples were boiled for 5 min in Laemmli buffer and loaded onto gels (40 μ g 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 enhanced chemiluminescence (ECL) reagent. Semi-quantitative analysis of protein detection was done by image analysis with the ImageJ program (Rasband, WS, ImageJ; US NIH, Bethesda, MD, USA; <http://rsb.info.nih.gov/ij/>).

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

In-gel proteasome activity and levels. Upon treatment with vehicle (control, DMSO or water) or the respective drugs, cells were washed twice with PBS and harvested for the in-gel assay as described in Wang *et al.*⁹ The native gels loaded with 30 μ g 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 μ M 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., Beloit, WI, USA). 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- β 5 subunit antibody, which reacts with a subunit of the 20S core particle, therefore detects 26S and 20S proteasomes. The antigen was visualized by a chemiluminescent horseradish peroxidase method with the ECL reagent.

Intracellular cAMP. After treatment, media was removed and 500 μ l of 0.1 N HCl were added to each dish followed by incubation for 5 min. Cell lysates were harvested and centrifuged at 600 \times g for 10 min at room temperature. The supernatant was used directly in the non-acetylated version of the assay. cAMP levels were determined with a non-radioactive assay kit following manufacturer's specifications. Absorbance was measured at 405 nm with correction at 570 nm, with a PowerWave HT Spectrophotometer (Winooski, VT, USA). cAMP concentration (pmol/ μ g protein) for each sample was determined according to the kit's instructions.

Caspase activity assays. Caspase activity assays (for caspases 3, 8 and 9) were carried out with caspase colorimetric assay kits from Biovision (Mountain View, CA, USA) following manufacturer's specifications.

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

Conflict of Interest

The authors declare no conflict of interest.

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