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Activation of glycogen synthase kinase-3 beta mediates β -amyloid induced neuritic damage in Alzheimer's disease^{*,**,*}

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Abstract

β -Amyloid (A β) plaques in Alzheimer (AD) brains are surrounded by severe dendritic and axonal changes, including local spine loss, axonal swellings and distorted neurite trajectories. Whether and how plaques induce these neuropil abnormalities remains unknown. We tested the hypothesis that oligomeric assemblies of A β , seen in the periphery of plaques, mediate the neurodegenerative phenotype of AD by triggering activation of the enzyme GSK-3 β , which in turn appears to inhibit a transcriptional program mediated by CREB. We detect increased activity of GSK-3 β after exposure to oligomeric A β in neurons in culture, in the brain of double transgenic APP/tau mice and in AD brains. Activation of GSK-3 β , even in the absence of A β , is sufficient to produce a phenocopy of A β -induced dendritic spine loss in neurons in culture, while pharmacological inhibition of GSK-3 β prevents spine loss and increases expression of CREB-target genes like BDNF. Of note, in transgenic mice GSK-3 β inhibition ameliorated plaque-related neuritic changes and increased CREB-mediated gene expression. Moreover, GSK-3 β inhibition robustly decreased the oligomeric A β load in the mouse brain. All these findings support the idea that GSK3 β is aberrantly activated by the presence of A β , and contributes, at least in part, to the neuronal anatomical derangement associated with A β plaques in AD brains and to A β pathology itself.

Background

Indisputable genetic data point to β -amyloid (A β) as a central player in Alzheimer's disease (AD) pathophysiology. However, how and in which way brain A β accrual leads to neurotoxicity and impaired cognition in AD is not completely understood and identifying the signaling pathways and mechanisms involved may prove critical to prevent or cure this illness. It has been recently proposed that glycogen synthase kinase-3 (GSK-3), a multi-tasking kinase with major roles in brain signaling, may be important in the process leading to A β -mediated neural system collapse and dementia in AD. “The GSK-3 hypothesis of AD” argues that overactivity of GSK-3 β , the most abundant of two isoforms (GSK-3 α and GSK-3 β) expressed in neurons, accounts for cognitive impairment, tau hyperphosphorylation, increased A β production, neuronal death and neuroinflammation in

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AD (Hooper et al., 2008). Remarkable connections between GSK-3 and the hallmarks of AD have been identified. GSK-3 β is a key kinase required for AD-type abnormal hyperphosphorylation of tau (Kosik, 1992) and GSK-3 β overexpression results in tau hyperphosphorylation and microtubule disruption in mice (Lucas et al., 2001). A β activates GSK-3 in vitro, although the exact A β species and mechanism involved remain unknown (Akiyama et al., 2005; Kim et al., 2003; Ryan and Pimplikar, 2005). GSK-3 α promotes A β production (Phiel et al., 2003) and phosphorylates APP and presenilin-1 in vitro (Aplin et al., 1997; Takashima et al., 1998). In vitro and in vivo GSK-3 β overexpression leads to neuronal apoptosis (Beurel and Jope, 2006; Bhat et al., 2000a; Hetman et al., 2000; Kosik, 1992). GSK-3 β also participates in memory and synaptic plasticity, and mice overexpressing GSK-3 β showed impaired memory and long-term potentiation (LTP) (Hernandez et al., 2002). The above evidence has already led to clinical trials of GSK-3 inhibitors in AD, although there are major gaps in knowledge concerning GSK-3 effects in the human brain and uncertainty about whether drugs that specifically target GSK-3 are feasible and safe.

We tested the hypothesis that increased activity of GSK-3 β substantially contributes to A β -induced neuronal damage in AD. We propose that soluble oligomeric A β species trigger GSK-3 β activation, and this in turn mediates the AD neurodegenerative phenotype by promoting phosphorylation of targets critically involved in synaptic stability. In particular, GSK-3 β activation appears to inhibit a transcriptional program mediated by CREB that may compromise synaptic integrity and cognition. We used three different settings to test this idea: primary neurons in culture, transgenic (Tg) mice and AD brains. We find evidence for GSK-3 β activation in cultured neurons exposed to oligomeric A β obtained from either Tg2576 conditioned medium (CM) or AD brains, in the brain of APP^{sw}-tau^{vlw} mice and in AD brains. A β exposure in those three settings resulted in aberrant morphological changes in neurites — dendritic spine loss, dystrophies and altered neurite trajectories — increased GSK-3 β -mediated inhibitory phosphorylation of CREB and subsequent decrease of CREB-target gene expression. Blocking either A β or GSK-3 β activation prevented A β -induced neurite alterations in cultured neurons and normalized CREB-mediated gene expression, while introduction of an uninhibitable form of GSK-3 β recapitulated the neurodegenerative phenotype in the absence of A β . Importantly, in APP^{sw}-tau^{vlw} mice, plaque-related neuritic changes, downregulation of CREB-target gene expression and impaired cognition were rescued by GSK-3 β inhibition. These data suggest that GSK-3 β activation and subsequent inhibition of CREB-mediated transcriptional program are involved in A β -induced neuronal derangement, providing further mechanistic insight into molecular links between A β , synaptic failure and impaired cognition in AD.

Material and methods

Primary neuronal cultures

Tg2576 mice overexpressing human APP containing the Swedish mutation K670NM671L were used for generating the cell cultures (Hsiao et al., 1996). Primary neurons were derived from cerebral cortex of embryonic day 14 as previously described (Wu et al., 2010). Neurons were seeded to a density 6×10^5 viable cells/35-mm culture dishes previously coated with poly-D-lysine (100 μ g/ml) for at least 1 h at 37 °C. Cultures were maintained at 37 °C with 5% CO₂, supplemented with neurobasal medium with 2% B27 nutrient, 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 μ g/ml). Tg2576 and wild type (wt) neuronal cultures were used after 14 days in vitro (DIV). To maintain elevated levels of extracellular A β , the media in the Tg2576 cultures (conditioned media, CM) was not changed during those 14 days, and then was collected to treat 14 DIV wt neurons. CM from 14 DIV wt neurons were used as a control. The genotype of the animals was determined by polymerase chain reaction on DNA obtained from fibroblasts.

Separation of A β oligomers from cultured media and AD brain lysates by size exclusion chromatography (SEC)

5 ml of CM from 14 DIV Tg2576 neurons was centrifuged at 3000 $\times$ g at 4 °C in Amicon Ultra-15ML 3 K (Millipore) to concentrate proteins ~5 fold. AD brain lysates were centrifuged at 260,000 $\times$ g at 4 °C, and the supernatant was used as TBS-soluble fraction (Hashimoto et al., 2002). 750 μ l of concentrated Tg2576 CM and 500 μ l of TBS-soluble fraction from AD brains were separated by SEC on Superdex 75 10/300 GL column (GE healthcare) in 50 mM ammonium acetate pH 8.5 with AKTA purifier 10 (GE healthcare) (Townsend et al., 2006) to characterize the A β species contained in the mixtures based on molecular weights. The concentration of A β in the Tg2576 CM was 1–1.5 nmol/ml. Wt or Tg2576 CM was applied for 24 h to 14 DIV wt neurons at 37 °C. Immunodepletion experiments were utilized to ensure that the active component in the Tg2576 CM media is A β using 3D6 antibody (Johnson-Wood et al., 1997). Confirmatory experiments, using oligomeric A β prepared on the SEC column from AD brains were also conducted.

Pharmacological inhibition of GSK-3 β in vitro

Wt neuron cultures were treated with the selective non-ATP competitive inhibitor of GSK-3 β TDZD-8 (4-Benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione) (Sigma, St Louis, MO) at 1 μ M 30 min before exposure to A β containing Tg2576 CM. GSK-3 activity was measured by phosphorylation of Ser9 of GSK-3 β and Ser21 of GSK-3 α . This inhibitory phosphorylation is known to be the main regulatory mechanism of GSK-3 activity in mammalian cells (Woodgett, 1990). GS3 β /PSer9GSK-3 β and GSK-3 α /PSer21GSK-3 α ratios were determined by using commercially available antibodies (CST, Danvers, MA) and traditional Western Blotting techniques.

Plasmid construction

The open reading frame of the wt mouse GSK-3 β (GSK-3 β wt) and the constitutively active GSK-3 β mut (S9A) were amplified using pcDNA3-GSK3 β -HA (Addgene, Cambridge, MA) and pcDNA3-GSK3 β S9A-HA (Addgene, Cambridge, MA) as templates, respectively. The primers used to amplify GSK-3 β wt were:

GSK3 β wtKpn1-5F 5',
AAAAAAGGTACCACCATGTCAGGGCGGCCCAAGAACC
GSK3 β -Age1-3R 5',
TTTTTTACCGGTTTGGTGGAGTTGGAAGCTGATGCAGAAG

The primers used to amplify GSK-3 β (S9A) were:

GSK3 β mutKpn1-5 F 5',
AAAAAAGGTACCACCATGGCAGGGCGGCCCAAGAACC
GSK3 β -Age1-3R 5',
TTTTTTACCGGTTTGGTGGAGTTGGAAGCTGATGCAGAAG.

The purified restriction fragments of Kpn1-GSK-3 β wt-Age1 and Kpn1-GSK-3 β (S9A)-Age1 were respectively cloned into Kpn1/Age1 sites of vector peGFP-N1 (Clontech, Mountain View, CA) and transfected into 5–7 DIV primary cultured neurons using lipofectamine™ 2000 (Invitrogen, Carlsbad, CA). All the constructs were verified by sequencing before transfection.

Analysis of neuronal morphology and toxicity assays

Neuronal morphology was assessed on 14 DIV neurons transfected with the plasmid peGFP-N1 (Clontech, Mountain View, CA). High-resolution digital images were taken using a

Zeiss LSM510 confocal microscope system and analyzed with NeuronStudio software (CNIC, Mount Sinai School of Medicine). Spine density was defined as the number of spines per micrometer of dendrite length according to modified previously published protocols (Wu et al., 2010). Dendritic spine densities were calculated from 20 neurons per condition. The relative amounts of the three most notable classes of spine shapes “thin”, “stubby” and “mushroom” were quantified.

Toxicity assays were conducted using ToxiLight BioAssay kit from Lonza, Rockland, ME, according to manufacturer protocols.

Preparation of total homogenates and nuclear fractions from cultured neurons and human brain samples

Neurons in culture and frozen human temporal cortex samples (20 mg) from AD and control cases were homogenized in 0.32 M sucrose lysis buffer (0.32 M sucrose, 5 mM CaCl₂, 3 mM Mg(Acetate) 2, 0.1 mM EDTA, 10 mM Tris–HCl (pH 8.0) and 0.1% Triton X-100), supplemented with complete protease inhibitor cocktail tablets and centrifuged at 800×g for 15 min at 4°C. Nuclear preparations free of cytosolic proteins were prepared from the pellets from the initial centrifugation step by using Qproteome nuclear protein kit from Qiagen, Germantown, MD, according to manufacturer's protocols. For Western blot analysis, equal amounts of protein (20 µg) from total extracts and nuclear fractions were separated on 10% and 4–12% SDS-PAGE gels. Primary antibodies for GSK-3α, GSK-3β, GSK-3α-pSer21, GSK-3β-pSer9 and CREB (CST, Danvers, MA), 129-pCREB and c-fos (Santa Cruz Biotechnology, Santa Cruz, CA), BDNF (Abcam, Cambridge, UK; Millipore, Billerica, MA; Santa Cruz Biotechnology, Santa Cruz, CA) and ARC (Santa Cruz Biotechnology, Santa Cruz, CA) were used. Primary antibody incubation was followed by the appropriate horseradish peroxidase linked secondary antibody (rabbit polyclonal or mouse monoclonal 1:1000; Bio-Rad, Hercules, CA). Immunoreactivity was visualized using enhanced chemiluminescence reagent (Perkin-Elmer, Wellesley, MA) and exposure on X-ray film. Anti-GAPDH antibody (Millipore, Billerica, MA) and anti-HDAC1 antibody (ABR-Affinity BioReagents, Rockford, IL) were used to verify the cytoplasmic and nuclear fraction separation. BDNF levels in the mouse brain were assessed by ELISA using BDNF Emax ImmunoAssay System (Promega, Madison, WI).

Experimental animals

Transgenic APP^{sw}–tau^{vlw} mice overexpressing human mutant APP (Swedish mutation K670N–M671L) and a triple human tau mutation associated with frontotemporal dementia and parkinsonism linked to chromosome 17 (G272V, P301L and R406W) on a mixed hybrid genetic background C57Bl6j/SJL/CBA were used in this study (Perez et al., 2005; Ribe et al., 2005). In brief, at 9 months these mice exhibit scarce amyloid deposits and infrequent tau filament formation in limbic and cortical areas, as well as incipient neuronal loss in the entorhinal cortex (EC) by about 19%, without significant memory impairment. Severe pathology, including enhanced amyloid deposition, increased levels of tau phosphorylation and aggregation (but no mature NFT formation), glial proliferation, and pronounced neuronal loss in the entorhinal cortex (EC) and the CA1 region of the hippocampus by about 36%, can be seen in mice at 16 months along with overt memory deficits. Mature argyrophilic NFT formation is a late stage event in this mouse line that can be demonstrated in the EC and CA1 regions at 25 months of age. Groups of APP^{sw}–tau^{vlw} mice were administered a daily oral dose of NP12 (Noscira, Madrid, Spain) (n=6) or vehicle (n=6) starting at 9 months during 6 consecutive months and were used for subsequent clinicopathological and biochemical analyses. Groups of age and gender-matched wild-type littermates (n=6) received vehicle alone and were used as controls. All experiments were

conducted in accordance to our institutional Animal Care and Use Committee guidelines and conformed to the European Union Directive 86/609/EEC.

Pharmacological GSK-3 β inhibition in vivo

NP12 (Noscira, Madrid, Spain) is another small heterocyclic thiadiazolidinone (TDZD) derivative, which is an ATP-non competitive inhibitor of GSK-3 β (Martinez et al., 2002). NP12 was reconstituted in 26% peg400 (Polyethylene Glycol 400), 15% Chremophor EL and water, and administered at a daily dose of 200 mg/kg. Drug or vehicle was administered for 6 consecutive months by oral gavage to APP^{sw}-tau^{vlw} mice.

Mouse tissue preparation

Mice were sacrificed under isoflurane administration and brains were immediately removed. One hemisphere was snap frozen in liquid nitrogen for Western blotting and ELISA assays. The other hemisphere was fixed for 24 h in 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.4, and coronally sectioned at 30 μ m on a freezing sledge microtome for histological analyses.

Immunostaining

30 μ m coronal sections were permeabilized with 0.5% Triton-X100 in PBS, blocked with normal goat serum, and sequentially probed with primary antibody (4G8 mouse anti-A β 1:500, Chemicon, Temecula, CA; mouse Nab61 1:100, a kind gift of Dr. Virginia Lee; SMI-312 antibody 1:1000, Covance Research Products, Princeton, NJ) and the appropriate secondary antibody (anti-mouse and anti-rabbit IgG 1:200, Southern Biotechnology, Birmingham, AL; Vector ABC Kit; anti-mouse IgG 1:200 conjugated with cyanine 3 (Cy3), Jackson ImmunoResearch, West Gorge, PA).

A β oligomeric burden quantification

Oligomeric A β deposits were detected using Nab61, an antibody that has been previously shown to recognize a conformational epitope specific to oligomeric A β (Lee et al., 2006), and quantified using the Bioquant Nova Prime V6 90.10 system according to protocols previously published (Gomez-Isla et al., 1996; Irizarry et al., 1997). In brief, video images were captured of each region of interest on 30 μ m-thick sections, and a threshold of optical density was obtained that discriminated staining from background. Manual editing eliminated artifacts. The total “oligomeric amyloid burden”, defined as the total percentage of cortex covered by Nab61 immunostained deposits over three sections were calculated for the CA1 hippocampal subfield, cingulate and entorhinal (EC) cortices.

Neurite trajectory analysis

Neurite curvature analyses were conducted in the EC. To visualize axons the tissue was incubated with SMI-312 antibody (1:1000, Covance Research Products, Princeton, NJ) and a secondary anti-mouse antibody conjugated with cyanine 3 (Cy3) (1:200, Jackson ImmunoResearch, West Gorge, PA), and then counterstained with Thioflavine-S (0.05% in 50% ethanol). The analysis of axonal ratio curvature was conducted according to previously published protocols (Spires-Jones et al., 2009). In brief, microphotographs were obtained on an upright Olympus BX51 (Olympus, Denmark) fluorescence microscope with a DP70 camera using DPController and CPManger software (Olympus). Axonal curvature ratio and distance to senile plaques were measured using ImageJ software from the NIH. Axons longer than 20 μ m were analyzed and their curvature ratio was calculated by dividing the curvilinear length of the axon by the straight line length of the process (D'Amore et al., 2003; Knowles et al., 1999; Lombardo et al., 2003). Distance from the measured axon segment to the closest amyloid plaque (if present) was measured at three points — from

each end and the midpoint of the segment — and the average distance was calculated from the three measurements. Axons closer than 50 μm to the plaque ($n=90$ –110 axon segments derived from 6 animals per treatment group) were defined as being close to plaques and the others as being far from plaques ($n=100$ –135 axon segments derived from 6 animals per treatment group).

Statistical analysis

One way ANOVA and Student's *t* test were used to examine main effects of transgenic status and treatment effect on spine density and morphology, oligomeric A β load, GSK-3 phosphorylation, CREB target-gene expression levels and neurite curvature ratio. One way ANOVA was used to compare GSK-3 phosphorylation and CREB target-gene expression levels in human tissue samples from AD patients vs. cognitively normal controls. In all tests the level of significance was at $p<0.05$. Data are presented as mean $\pm$ standard error, unless otherwise indicated.

Results

Exposure to oligomeric A β containing media from Tg2576 cultures and to oligomeric A β isolated by SEC from AD brain decreased spine density and reduced the proportion of thin and mushroom spines in wt primary neurons

Primary cortical neurons derived from Tg2576 embryos at 14 DIV produced high levels of human A β 40 and 42. The concentration of A β 40 was 16 ng/ml and of A β 42 was 1.2 ng/ml as determined by ELISA assay. IP Western blot analysis of CM from Tg2576 cultures at 14 DIV demonstrated the presence of detectable SDS-stable small oligomers, similar to those reported to be synaptotoxic (Shankar et al., 2008). The size exclusion chromatography (SEC) profile of the Tg2576 CM (which contains a mixture of oligomeric A β species — from dimer to about a hexamer, as a source of neuronally produced extracellular oligomeric A β) is in fact quite close to the one in the TBS fraction of AD brain. By Western blot using the ultrasensitive antibody 82E1, we estimated that the total amount of A β in Tg2576 CM is ~ 20 ng/ml. Diluted 1:4, these correspond to concentrations of ~ 5 ng/ml (or ~ 1 nM), and are similar to the amount of A β reported to be present in human CSF (~ 1 –1.5 nM) (Snider et al., 2009). The elution profile from the SEC column further revealed that a large portion of the Tg2576 CM mixture used for the experiments consisted of low-molecular-weight (LMW) A β oligomers, such as dimers and trimers corresponding with fractions 15–18 (8 kDa–12 kDa). We assessed spine density and morphology by transfecting wt cultured neurons with the plasmid peGFP-N1 (Clontech, Mountain View, CA) which encodes a red-shifted variant of wt green fluorescent protein (GFP) to label individual neurons and exposed them for 24 h to Tg2576 CM and to oligomeric A β prepared on the SEC column from TBS extraction of AD brain. During the course of 14 days in vitro (DIV), GFP positive neurons from wt cultures progressively demonstrated intricately branched dendritic arbors studded with protrusions that included mature spines and few filopodia. Neurons exposed to CM from Tg2576 cultures had reduced overall spine density when compared to neurons exposed to wt media ($p=0.0004$) ($n=20$ neurons per condition) (Figs. 1A–B). Of note, confirmatory experiments showed that 24 hour exposure of neurons to oligomeric A β isolated from AD brains induced identical spine density reduction ($p=0.0004$), and immunodepletion using 3D6 antibody completely prevented this neurodegenerative phenotype (Figs. 1A–B). Dendritic spines are small membranous protrusions that provide the anatomical substrate for memory storage and synaptic transmission. The most notable classes of spine shape are “thin”, “stubby” and “mushroom” (Peters and Kaiserman-Abramof, 1970) (Fig. 2A). The variable spine shape and volume is thought to be correlated with the strength and maturity of each spine-synapse and spines with strong synaptic contacts typically have a large spine head. Synaptic enhancement leads to an enlargement of thin spines into mushroom spines

and the mobilization of subcellular resources to potentiated synapses (reviewed in Bourne and Harris, 2007). Our data indicate that exposure to oligomeric A β containing Tg2576 CM is not only associated with a significant decrease in the total number of spines but also with a reduction in the relative proportion of thin spines, thought to be particularly plastic and linked to learning ($p=0.0248$), and mushroom spines, the most stable and mature type of spines and believed to be involved in memory formation ($p=0.0180$) (Fig. 2B). Importantly, all these effects are not due to overt toxicity since ToxiLight assay for cell death showed no significant difference between neurons exposed to Tg2576 or wt CM (data not shown). Taken together, these results indicate that 24 hour exposure to oligomeric A β containing Tg2576 CM is sufficient to induce a significant loss of dendritic spines and to alter spine morphology in cultured neurons.

Oligomeric A β containing media from Tg2576 cultures induced aberrant activation of GSK-3 β

It has been recently proposed that aberrant activation of GSK-3 β may play a role in A β mediated neurotoxicity in AD. To examine if A β is capable of inducing an increase in GSK-3 β activity, we studied the effect of Tg2576 CM on the inhibitory phosphorylation of serine 9 of GSK-3 β , the best known control mechanism of the enzyme's activity (Woodgett, 1990), using commercially available antibodies (CST, Danvers, MA) and Western Blot. Because GSK-3 β is dynamically regulated within the nucleus and mitochondria, where it is highly active, readouts were also conducted in nuclear fractions (Bijur and Jope, 2003). Our data indicate that oligomeric A β triggers GSK-3 β activation. The total level of GSK-3 β in whole cell and nuclear extracts did not significantly change in primary cultured neurons after 24 hour exposure to Tg2576 CM compared to wt CM ($p=0.889$) (Figs. 3A–B). However, the GSK-3 β /pSer9GSK-3 β ratio was increased by 25% in whole cell extracts ($p=0.0800$) and by 30% in the nuclear fraction ($p=0.0136$) in neurons exposed to Tg2576 CM indicating that oligomeric A β exposure triggers a significant increase in GSK-3 β activity ($n=24$ dishes per condition). 3D6 immunodepletion completely prevented the elevation of GSK-3 β activity. No significant changes were detected in the level of GSK-3 α or the GSK-3 α /pSer21GSK-3 α ratio in whole cell or nuclear extracts (data not shown).

Pharmacological inhibition of GSK-3 β rescued A β induced spine loss and restored spine morphology while transfection with a constitutively active form of GSK-3 β reproduced the neurodegenerative phenotype in the absence of A β

To examine if pharmacological blockage of GSK-3 β is sufficient to prevent A β induced spine density reduction, we studied the effect of treatment with TDZD-8 (a non-ATP competitive GSK-3 β inhibitor) in neurons exposed to Tg2576 CM and wt CM. We observed that TDZD-8 treatment at 0.5 and 1 μ M concentration had a significant trophic effect in neuronal cultures and significantly increased the overall amount of dendritic spines by over 25% ($p=0.013$) (Figs. 4A–B), and the relative proportion of stubby spines ($p=0.0021$) that are thought to be much more stable than thinner ones (Trachtenberg et al., 2002) (Fig. 2B). Importantly, higher concentrations of TDZD-8 though were toxic and resulted in significant reduction in the number of spines suggesting that excessive inhibition of GSK-3 β endogenous activity might not be safe (Figs. 4A–B). TDZD-8 at 1 μ M fully rescued spine loss triggered by A β exposure (Figs. 5A–B) and restored the normal proportion of both thin and mushroom spines (Fig. 2), strongly supporting that partial blockade of GSK-3 β is sufficient to block the A β induced synaptotoxic phenotype. As expected, TDZD-8 treatment resulted in a significant increase in the phosphorylation of serine 9 of GSK-3 β , indicating that the enzyme is less active ($p=0.0419$) (Figs. 3A–B). No significant changes were observed in the inhibitory phosphorylation of GSK-3 α at Ser21 after exposure to TDZD-8 (data not shown). To test the hypothesis that aberrant activation of GSK-3 β in wt neurons in culture recapitulates the same morphological phenotype in the absence of A β , we next

transfected the wild type mouse GSK-3 β (GSK-3 β wt) and the constitutively active GSK-3 β mut (S9A) into neurons in vitro. Our data indicated that 14 day transfection of wt GSK-3 β into neurons leads to a very robust loss of dendritic spines and a significant reduction in the proportion of thin and mushroom spines recapitulating that observed after exposure to oligomeric A β containing Tg2576 CM ($p < 0.0001$). The phenotype is even more dramatic after transfection of a constitutively active mutant form of GSK-3 β (GSK-3 β mut (S9A)) that leads to a complete loss of all dendritic spines in cultured neurons (Figs. 5A–B). All together the above results suggest that A β induced neurodegeneration is mediated by GSK-3 β activation and can be fully blocked by inhibition of GSK-3 β in vitro.

Activity of GSK-3 β is aberrantly increased in the AD brains

Literature concerning GSK-3 β effects in AD and Tg mouse models is not uniform. Some studies reported increased activity of GSK-3 β in AD brains compared to controls (Blalock et al., 2004; Leroy et al., 2007), but others found no change (Pei et al., 1997). To determine whether the aberrant activation of GSK-3 β observed in neurons exposed to oligomeric A β containing Tg2576 CM could be relevant to the AD human condition, total homogenates and nuclei were prepared from frozen human AD or control temporal cortex tissue samples and analyzed by Western Blotting with antibodies against total GSK-3 β and Ser9PGSK-3 β . GSK-3 β levels in total homogenates and nuclear fractions prepared from the temporal cortex of AD samples were markedly decreased compared to those seen in control samples, as expected due to neuronal cell loss in AD ($p = 0.0091$ and $p = 0.0248$, respectively) (Figs. 6A–D). However, the ratio GSK-3 β /pSer9GSK-3 β was increased in the total homogenates by 260%, even though this difference did not reach statistical significance ($p = 0.160$), and even more so, by 450%, in the nuclear fraction ($p = 0.0019$) indicating that GSK-3 β is in fact more active in AD brains ($n = 11$ – 12 per group). These observations are in agreement with our previous data showing that the activity of GSK-3 β is increased in the brain of APP^{sw}–tau^{vlw} mice in comparison to age-matched littermate controls (Serenio et al., 2009). Thus, A β exposure in both settings, AD brains and Tg mice, results in aberrant activation of GSK-3 β further supporting the notion that increased activity of this enzyme is likely involved in the neurodegenerative phenotype associated to A β exposure not only in vitro but also in vivo.

Sustained pharmacological inhibition of GSK-3 β successfully rescued A β associated neuritic changes in APP^{sw}–tau^{vlw} mice

If our overall hypothesis that aberrantly increased activity of GSK-3, and GSK-3 β isoform in particular, substantially contributes to A β mediated neural system degeneration in AD is right, then GSK-3 β inhibition gains substantial importance as potentially disease modifying strategy in AD. Our recently published data from 3 month exposure to a novel non-ATP competitive GSK-3 inhibitor (compound NP12) had shown that NP12 treatment resulted in a significant decrease of both tau and A β alterations and, perhaps more importantly, was sufficient to arrest neuronal cell loss in AD vulnerable brain regions and fully prevented memory deficits in APP^{sw}–tau^{vlw} mice (Serenio et al., 2009). We asked whether GSK-3 β pharmacological inhibition also had a significant impact in oligomeric A β accrual in the brain of APP^{sw}–tau^{vlw} mice and was sufficient to restore A β associated neuritic changes. Our previous data indicated that the amount of Nab61 oligomeric A β but not total A β plaque burden closely correlated with the amount of neuronal cell loss in the brain of these mice, favoring the view that oligomeric A β species may play a much larger role in neuronal damage than A β plaques in vivo (DaRocha et al., 2011). The total amount of Nab61 reactive oligomeric A β deposits was very significantly decreased in mice treated with NP12 in comparison to those treated with vehicle (Figs. 7A–C) ($p = 0.0024$). The regional exam further confirmed the robust treatment impact on Nab61 accumulation (cingulate cortex $p = 0.0277$, EC $p = 0.0050$). These data are in agreement with our previous observations that NP12 treatment significantly decreases the total amount of A β plaques in the brain of

APP^{sw}-tau^{vlw} mice (Serenio et al., 2009) strengthening the idea that inhibition of GSK-3 β results in substantial decrease of amyloid pathology in vivo and further supporting the potential therapeutic use of TDZD compounds in AD. The mechanism/s by which NP12 ameliorates A β pathology need now to be clarified. Of note, we did not observe either a significant decrease in the activity of GSK-3 α or changes in the levels of BACE (data not shown) in the brain of mice treated with NP12 in comparison to vehicle-treated animals.

Previous studies have demonstrated that the Thioflavin-S reactive dense-cored subpopulation of plaques in particular are associated with severe neurite changes, including axonal disrupted trajectories and reductions in dendritic spine density, that likely contribute to altered neural system function and behavioral impairments observed in APP mice (Coma et al., 2010; D'Amore et al., 2003; Spires et al., 2005). Of note, our prior work showed that the oligomeric conformational specific antibody Nab61 labeled the core and a halo surrounding the core of most of the Thioflavin-S reactive plaques in the brain of APP^{sw}-tau^{vlw} mice (DaRocha et al., 2011). In the present study we analyzed the axonal curvature ratio in the EC in 30 μ m SMI-312 immunostained coronal sections counterstained with Thioflavin-S. We observed that APP^{sw}-tau^{vlw} mice have significantly curvier axons at 15 months compared to similarly aged non-transgenic littermate controls ($p < 0.0001$) (Figs. 8A–B). When distance from a dense-cored plaque was taken into account, axons were significantly more curved close to plaque ($< 50 \mu$ m) compared to far from plaque ($> 50 \mu$ m) ($p < 0.0001$). Treatment with NP12 significantly reduced axonal curvature both near and far from plaques ($p < 0.0001$). Increased number of neurons with neuritic dystrophies (size of dystrophies defined as areas of swelling $> 2.5 \mu$ m in diameter in neurites) can also be seen in the brain of APP^{sw}-tau^{vlw} mice at 15 months of age in association with amyloid deposits (Figs. 8C–D). NP12 treatment significantly decreased the number of dystrophies near plaques in the brain of APP^{sw}-tau^{vlw} compared to vehicle treated mice ($p = 0.0011$). These results demonstrate that the neuritic changes (neurite trajectory alterations and neuritic dystrophies) that accompany amyloid deposition in the brain of APP^{sw}-tau^{vlw} mice can be successfully rescued by this GSK-3 β inhibitor. The relative contribution of A β (aggregated vs. oligomeric species), inflammation and/or oxidative stress, among others, to the observed morphological axonal changes will now need to be further clarified by future studies.

GSK-3 β activation appears to promote synaptotoxicity through inhibition of CREB dependent gene expression

Inhibitory control of GSK-3 β is important in promotion of cell survival, and hyperactive GSK-3 β contributes to apoptotic cell death (Bijur et al., 2000; Li et al., 2000; Pap and Cooper, 1998). The proapoptotic action of GSK-3 β has been attributed, at least in part, to the regulation by GSK-3 β of an array of transcription factors that control the expression of numerous genes with prominent roles in cell fate and survival. One of the transcription factors regulated by GSK-3 β is CREB. GSK-3 β negatively regulates CREB by phosphorylation of its Ser129 (Grimes and Jope, 2001). Multiple studies in different models have extensively established a critical role for the cAMP signaling pathway and CREB-mediated gene expression in cell survival and also in different forms of synaptic plasticity related to learning (reviewed in Bito and Takemoto-Kimura, 2003; Pittenger and Kandel, 1998). These data provide the rational basis for the hypothesis posed here where oligomeric A β triggers GSK-3 β activation and GSK-3 β in turn contributes to A β -triggered synaptic derangement (spine loss and formation of varicosities) and neuronal toxicity through negative regulation of CREB and its transcriptional targets. We measured P-Ser129 CREB by Western blotting and the levels of protein expression of three of its transcriptional targets (c-fos, ARC and BDNF) in cultured neurons, brain tissue from APP^{sw}-tau^{vlw} mice and AD brains. Our data indicate that the increased activity of GSK-3 β triggered by exposure of neurons to Tg2576 CM media results in a significant increase in the inhibitory

phosphorylation at serine 129 of CREB promoted by this enzyme ($p=0.0262$) (Figs. 9A–H). Of note, CREB inhibition in this condition is associated with a significant decrease in the amount of BDNF ($p=0.0057$) and TDZD-8 treatment significantly decreased the GSK-3 β mediated inhibitory phosphorylation of CREB and reversed the BDNF deficit in cultured neurons. A concordant and significant increase in p129CREB ($p=0.0262$) and decreases in the levels of c-fos ($p=0.0029$) and ARC ($p=0.0154$) were observed in brain extracts from 15 month-old APP^{sw}-tau^{vlw} mice in comparison to similarly aged wt mice (Figs. 10A–H). An 18% reduction in the levels of BDNF was also noted in the brain of APP^{sw}-tau^{vlw} mice compared to wt mice, a difference that approached but did not reach statistical significance ($p=0.1510$). Importantly, all these deficits in CREB-target gene expression levels were fully rescued by pharmacological inhibition of GSK-3 β in APP^{sw}-tau^{vlw} mice (Figs. 10A–H). In accord with these data, a significant increase in the inhibitory phosphorylation at serine 129 of CREB ($p=0.0004$) and a decrease in the levels of ARC ($p=0.0002$) and BDNF ($p=0.0233$) were also observed in the temporal cortex of AD brains in comparison to cognitively normal controls (Figs. 11A–H). All together these results suggest that aberrant activation of GSK-3 β inhibits a transcriptional program mediated by CREB both in vivo and in vitro, and offer an insight into the molecular mechanisms that may link A β exposure to synaptic and neuronal derangement in AD. The fact that the impact on CREB inhibition seems to be relatively more robust in mouse and human brain samples than in vitro could reflect the different durations of A β exposure in those settings (months in mice and years in humans vs. 24 h in cultured neurons).

Discussion

The idea that brain accumulation of β -amyloid (A β) is the primary influence that triggers the cascade of pathogenic events leading to neuronal damage and dysfunction as the final common pathway in AD is the current leading hypothesis in the field (Hardy and Allsop, 1991; Hardy and Selkoe, 2002). Favoring this idea is the long time standing observation that amyloid plaques are associated with dendritic and axonal changes with local spine loss, axonal swellings, and dysmorphic processes surrounding the plaques. However, the exact molecular mechanisms by which accrual of brain A β leads to neuronal toxicity and morphological disruption of dendrites and axons remain unknown. Here we present data supporting the idea that aberrant activation of GSK-3 β and subsequent inhibition of a transcriptional program mediated by CREB are likely key mechanisms that link A β to the structural damage of neural networks. Exposure to A β results in aberrant activation of GSK-3 β that leads in turn to phosphorylation of targets critically involved in synaptic stability and neuronal survival. In particular, GSK-3 β activation appears to inhibit a transcriptional program mediated by CREB that may compromise synaptic anatomical integrity and transmission by promoting dendritic spine loss and neuritic dystrophies, and distortion of the normal straight patterns of dendrites and axons. Importantly, we show reversal of A β induced morphological neurodegenerative phenotypes in vitro and in vivo by pharmacological GSK-3 β inhibition, providing an important proof that this therapeutic intervention protects neuronal structure in cultured neurons and in a transgenic mouse model.

GSK-3 β is a multi-tasking serine/threonine kinase with crucial roles in many major signaling processes in the brain and has recently proposed to promote most of the key steps involved in the neuronal dysfunction associated with AD including tau hyperphosphorylation and increased β -amyloid production (Hooper et al., 2008). In vitro and in vivo overexpression of GSK-3 β has been shown to result in apoptotic neuronal cell death (reviewed in Beurel and Jope, 2006; Bhat et al., 2000b; Hetman et al., 2000; Lucas et al., 2001). GSK-3 β has also been involved in memory formation through mechanisms controlling synaptic function and synaptic plasticity and transgenic mice overexpressing

GSK-3 β exhibited pronounced impairment in spatial memory and long-term potentiation (LTP) in CA1 and dentate gyrus (Hernandez et al., 2002). CREB-mediated gene expression plays a critical role in the best established form of synaptic plasticity related to learning, long-term potentiation (LTP), (reviewed in Barco et al., 2002; Josselyn and Nguyen, 2005; Lonze and Ginty, 2002). Importantly, CREB DNA binding activity is inhibited by phosphorylation by GSK-3 β and facilitated by lithium, the first known GSK-3 inhibitor (Grimes and Jope, 2001). We had previously demonstrated an age-dependent aberrant increase in GSK-3 β activity in the hippocampi of transgenic mice overexpressing human mutant APP and tau (APP^{sw}-tau^{vlw} line) accompanying learning and memory deficits exhibited by these mice in the Morris water maze and contextual fear conditioning paradigms (Serenio et al., 2009). Moreover, we have recently demonstrated that memory impairment can be completely prevented in these mice by sustained pharmacological inhibition of GSK-3 β (Serenio et al., 2009). These results suggest that the synaptic dysfunction and memory retention deficits that occur in AD patients and animal AD models might result, at least in part, from aberrantly increased GSK-3 β activity and the resulting inhibition of the transcriptional program mediated by CREB.

In the present study, we have shown that exposure of neurons in culture to Tg2576 CM, which contains high levels of naturally secreted A β , and to oligomeric A β isolated from human brain, trigger aberrant activation of GSK-3 β and this can be blocked by immunodepletion of the Tg2576 CM with an A β -specific antibody and by treatment with TDZD-8 which inhibits GSK-3 β (Fig. 3). In this model, GSK-3 β activation results in turn in decreased expression of one of the CREB targeted genes involved in synaptic plasticity, BDNF (Fig. 9). Importantly, aberrant activation of GSK-3 β and decreased levels of two of the CREB transcriptional targets extensively implicated in synaptic plasticity and neuronal survival, ARC and BDNF, were also found in AD postmortem temporal cortex, indicating that the increase in the activity of this enzyme and the compromise of CREB signaling pathway also occurs in AD. Moreover, in cultures, GSK-3 β inhibition provided very significant neuroprotection from Tg2576 CM and restored BDNF levels (Figs. 5 and 9).

GSK-3 β activation has profound effects on spine density and morphology. We show in cultures that overexpression of GSK-3 β is sufficient to mimic the A β induced neurodegenerative phenotype in the absence of A β (dendritic spine loss and reduction in the proportion of thin and mushroom spines) (Figs. 2 and 5), and transfection of neurons with a constitutively active form of GSK-3 β results in a complete loss of dendritic spines (Fig. 5) further favoring the idea that aberrant activation of this enzyme has a dramatic detrimental effect on neuron sculpture. GSK-3 β inhibition in cultured neurons was associated with increases in spine density and the proportion of stubby spines and these effects may be mediated by BDNF signaling, as it has been shown in CA1 pyramidal neurons of hippocampal slice cultures (Chapleau et al., 2008), reinforcing the notion that CREB-dependent transcription plays a key role in spine remodeling. GSK-3 β inhibition in the mouse brain completely rescued the A β -related neurite morphological changes that occur both far from and near plaques, decreased the level of the inhibitory phosphorylation of CREB at Serine 129 and restored the level of expression of two of CREB target genes, c-fos and ARC (Figs. 8 and 10) confirming in vivo relevance of our in vitro observations. All together these data point to a pivotal role of GSK-3 β activation and CREB signaling pathway in A β -related neurite alterations, providing a mechanistic link between aberrant activation of GSK-3 β , inhibition of CREB transcriptional targets, and A β -induced morphological disruption of neural systems both in vitro and in vivo. GSK3 β is reportedly the predominant NFAT kinase, and inhibits NFAT activity (Beurel et al., 2010). Our prior work (Wu et al., 2010) suggested that increased (rather than decreased) NFAT activity mediated by calcineurin activation disrupts neurite structure around plaques. We have confirmed that oligomeric A β exposure increases the levels of nuclear NFAT in cultured

neurons (data not shown), despite concomitant increase in GSK-3 β activity, and this is consistent with the idea that calcineurin/NFAT is one of the signaling pathway involved in A β toxicity. The finding reported here that GSK-3 β activation alone, even in the absence of A β , is sufficient to produce a phenocopy of A β -induced dendritic spine loss in neurons in culture provides evidence for the involvement of multiple mechanisms (e.g. calcineurin/NFAT activation, GSK-3 β activation/CREB inhibition) in A β mediated neurotoxicity. Interestingly, nuclear NFAT levels did not increase after treatment with TDZD-8, as it could be predicted a priori. We argue that this is most likely mediated by the reported agonistic effect of TDZD compounds on the peroxisome proliferator-activated receptor gamma (PPAR-gamma) (Luna-Medina et al., 2007). It has been previously shown that PPAR-gamma agonists and overexpression of PPAR-gamma inhibit the nuclear translocation of NFAT in different human cell types, pointing to a cross-talk between PPAR-gamma and calcineurin/NFAT (Yang et al., 2000.; Bao et al., 2008). Thus, some of the effects of TDZDs on neurite changes may be mediated in part by PPAR-gamma agonism in addition to GSK-3 β inhibition, and this reinforces the potential usefulness of these compounds in AD.

Interestingly, sustained pharmacological GSK-3 β inhibition robustly decreases the amount of total A β plaques and oligomeric A β deposits in the brain of APP^{sw}-tau^{vlw} mice, (Fig. 7) favoring a model where, on the one hand, activation of GSK-3 β lies downstream of soluble A β neurotoxic effects and, on the other hand, inhibition of this enzyme impacts oligomeric brain A β accrual in vivo. These observations are in agreement with recent data showing reduction of A β 42 levels upon GSK-3 inhibition in a *Drosophila* model of AD (Sofola et al., 2010), and point to a direct role of GSK-3 in the regulation of A β levels. The mechanism/s responsible for the reduction of brain A β deposition observed after pharmacological inhibition of GSK-3 β in the APP^{sw}-tau^{vlw} mouse model need now to be elucidated.

One important implication of our results is that they confirm that A β induces aberrant activation of GSK-3 β (Akiyama et al., 2005; Kim et al., 2003; Ryan and Pimplikar, 2005) and we now show that aberrantly increased activity of GSK-3 β in neurons causes loss of dendritic spines and spine morphological alterations. Although activation of GSK-3 has multiple effects, including tau phosphorylation, regulation of APP processing/A β production, activation of NF- κ B and its proinflammatory targeted genes, and inhibition of multiple transcription factors involved in cell fate and survival (reviewed in Jope et al., 2007), and thus it is possible that A β exposure induces neurotoxicity through many different mechanisms, our in vitro and in vivo data are consistent with the possibility that GSK-3 β inhibition of the transcriptional program mediated by CREB plays a prominent role in the A β triggered neurodegenerative process. Moreover, our results implicate a soluble form of A β , likely oligomeric, as the responsible bioactive molecule that mediates the neuronal alterations that occur near plaques (Koffie et al., 2009). A second relevant implication of our results is that increased activity of GSK-3 β produces a phenocopy of these A β effects. Importantly, either immunodepletion of A β or pharmacological inhibition of GSK-3 β can inhibit these changes and lead to recovery of spine density and neurite structure. Moreover, our current data show that pharmacological reduction of aberrantly increased GSK-3 β activity not only blocks the morphological changes induced by A β on neuronal processes but also decreases brain oligomeric A β accrual in vivo, reinforcing the importance of this therapeutic strategy and the disease-relevance of these observations. However, our in vitro data also alert about potential adverse effects of excessive GSK-3 β inhibition on dendritic spines. Concordant with these data a recent study showed that administration of a novel ATP competitive GSK3 inhibitor (SB216763) to wt mice induced inflammation and behavioral deficits further indicating that the window to safely achieved beneficial GSK-3 inhibition needs to be carefully defined.

Finally, our results suggest that GSK-3 β and CREB mediated transcriptional program play a major role in neural sculpting. It has been established that GSK-3 β has a prominent role in memory and CREB-mediated gene expression plays a critical role in the best established form of synaptic plasticity related to learning, long-term potentiation (LTP), (Barco et al., 2002; Josselyn and Nguyen, 2005; Lonze and Ginty, 2002). However, a major role for GSK-3 β -CREB mediated transcriptional events in neurite remodeling in the adult brain or in disease conditions as we observed here has not been previously reported. Our data favor a model where the neurotoxic and structural damage effects of A β accrual may be mediated, at least in part, by aberrant activation of GSK-3 β and its consequent inhibition of CREB mediated transcriptional program providing a molecular mechanism of neurodegeneration in AD and further evidence that inhibition of GSK-3 β has the potential to be a disease-modifying strategy in this devastating disorder.

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Fig. 1.

(A, B) 24 hour exposure of neurons to Tg2576 CM resulted in a significant reduction of spine density when compared to neurons exposed to wt media. Exposure to oligomeric Aβ isolated on the SEC column from AD brains induced a similar reduction in the number of dendritic spines, and immunodepletion using 3D6 (=IDPTgCM) antibody completely rescued this phenotype. Data are derived from 20 neurons per condition. Calibration bars 10 μm. *** p<0.001.

Fig. 2.

(A, B). 24 hour exposure of neurons to Tg2576 CM significantly decreased the proportion of thin and mushroom spines. TDZD-8 treatment at 1 μ M increased the overall amount of dendritic spines by over 25%, restored the proportion of thin and mushroom spines to control levels, and increased the relative proportion of stubby spines. Transfection of neurons with a wt GSK-3 β isoform significantly decreased the amount of all three classes of spines, thin, mushroom and stubby in the absence of A β . Data are derived from 20 neurons per condition. *p<0.05 ** p<0.01 *** p<0.001.

Fig. 3.

(A, B). Total level of GSK-3β in nuclear extracts did not significantly change in primary cultured neurons after 24 hour exposure to Tg2576 CM compared to wt CM. GSK-3β/pSer9GSK-3β ratio was increased by 30% indicating that oligomeric Aβ exposure triggers a significant increase in GSK-3β activity. 6E10 immunodepletion completely prevented the elevation of GSK-3β activity. Treatment of cultured neurons with TDZD-8 at 1 μM significantly decreased GSK-3β/pSer9GSK-3β ratio indicating that the enzyme is inhibited by this treatment. Data are derived from 24 dishes per condition. *p<0.05 ** p<0.01.

Fig. 4. (A, B) Treatment of neurons with TDZD-8 at 1 μ M increased the overall amount of dendritic spines by over 25% in comparison to neurons exposed to wt CM. Concentrations of TDZD-8 higher than 1 μ M were toxic and resulted in significant reduction in the number of spines suggesting that excessive inhibition of GSK-3 β endogenous activity might not be safe. Data are derived from 20 neurons per condition. Calibration bars 10 μ m. * p <0.05 ** p <0.01.

Fig. 5.

(A, B) TDZD-8 at 1 μM fully rescued spine loss triggered by exposure to oligomeric Aβ containing Tg2576 CM. 14 day transfection of wt GSK-3β into neurons led to a very robust spine loss recapitulating that observed after exposure to Tg2576 CM. The phenotype is even more dramatic after transfection of a constitutively active mutant form of GSK-3β (GSK-3β mut (S9A)) that leads to a complete loss of all dendritic spines in cultured neurons. Data are derived from 20 neurons per condition. Calibration bars 10 μm. *p<0.05 ** p<0.01 ***p<0.001.

Fig. 6. (A–D) GSK-3β levels in total homogenates (A, B) and nuclear fractions (C, D) prepared from the temporal cortex of AD samples were markedly decreased compared to those seen in control samples, as expected due to neuronal cell loss in AD. The ratio GSK-3β/pSer9GSK-3β was increased in the total homogenates from AD brains by 260%, and by 450% in the nuclear fraction when compared to controls indicating that GSK-3β is more active in AD brains (n=12 brains per group). *p<0.05 ** p<0.01.

Fig. 7.

(A) The total amount of Nab61 reactive oligomeric A β deposits was decreased in mice treated with NP12 in comparison to those treated with vehicle. (B) The regional exam further confirmed the treatment effect on Nab61 accumulation in the cingulate cortex and EC (n=6 animals per group). (C) Representative photomicrographs of sections immunostained with Nab61 through Cg, CA1 and EC from NP12 and vehicle treated animals. Calibration bars 200 μ m. EC = entorhinal cortex, Cg = cingulate. * p <0.05 ** p <0.01.

Fig. 8.

(A) Axons far (>50 μm) from plaques were significantly curvier in 15 month-old APP^{sw}-tau^{vlw} mice compared to non-Tg littermate controls. Treatment with NP12 restored the normal straight pattern of axons close to plaques (<50 μm) and far from them (n=90–135 axons analyzed per group). (B) Representative image of SMI312-positive neurites immunostaining (red) and senile plaque (Thioflavine S) (green). Curvature ratio was calculated by dividing the curvilinear length of the axon by the straight line length of the axon. (C) Neuritic dystrophies (size of dystrophies defined as areas of swelling >2.5 μm in diameter in neurites) can be seen in the brain of APP^{sw}-tau^{vlw} mice at 15 months of age in association with amyloid deposits. NP12 treatment significantly decreased the amount of dystrophies near plaques in the brain of APP^{sw}-tau^{vlw} compared to vehicle treated mice. (D) Representative image of SMI312-positive neurites immunostaining (red) and senile plaque (Thioflavine S) (green). Data are derived from 6 animals per group. Calibration bar 25 μm. **p<0.01 ***p<0.001.

Fig. 9.

(A–H) Increased activity of GSK-3 β triggered by exposure of neurons to Tg2576 CM media resulted in a significant increase in the inhibitory phosphorylation at serine 129 of CREB promoted by this enzyme and a significant decrease in the amount of BDNF. Treatment with TDZD-8 at 1 μ M concentration significantly decreased the inhibitory phosphorylation at Ser 129 of CREB mediated by GSK-3 β and restored the normal level of BDNF in neuronal cultures. Data are derived from 24 dishes per condition. *p<0.05 **p<0.01 ***p<0.001.

Fig. 10.

(A–H) A significant increase in GSK-3 β mediated inhibitory phosphorylation at serine 129 of CREB and concordant significant decreases in the amount of c-fos and ARC were observed in brain extracts from 15 month-old APP^{sw}-tau^{vlw} mice in comparison to similarly aged wt mice. These changes were fully rescued by pharmacological inhibition of GSK-3 β in APP^{sw}-tau^{vlw} mice. Data are derived from n=3 animals per group. *p<0.05 ** p<0.01 ***p<0.001.

Fig. 11.

(A–H) A significant increase in the inhibitory phosphorylation at serine 129 of CREB and a decrease in the levels of ARC and BDNF were observed in the temporal cortex of AD brains in comparison to cognitively normal controls. Data are derived from n=11–12 brains per group. * $p < 0.05$ *** $p < 0.001$.