Kidins220 accumulates with tau in human Alzheimer’s disease and related models: modulation of its calpain-processing by GSK3β/PP1 imbalance

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Failures in neurotrophic support and signalling play key roles in Alzheimer’s disease (AD) pathogenesis. We previously demonstrated that downregulation of the neurotrophin effector Kinase D interacting substrate (Kidins220) by excitotoxicity and cerebral ischaemia contributed to neuronal death. This downregulation, triggered through overactivation of N-methyl-D-aspartate receptors (NMDARs), involved proteolysis of Kidins220 by calpain and transcriptional inhibition. As excitotoxicity is at the basis of AD aetiology, we hypothesized that Kidins220 might also be downregulated in this disease. Unexpectedly, Kidins220 is augmented in necropsies from AD patients where it accumulates with hyperphosphorylated tau. This increase correlates with enhanced Kidins220 resistance to calpain processing but no higher gene transcription. Using AD brain necropsies, glycogen synthase kinase 3-β (GSK3β)-transgenic mice and cell models of AD-related neurodegeneration, we show that GSK3β phosphorylation decreases Kidins220 susceptibility to calpain proteolysis, while protein phosphatase 1 (PP1) action has the opposite effect. As altered activities of GSK3β and phosphatases are involved in tau aggregation and constitute hallmarks in AD, a GSK3β/PP1 imbalance may also contribute to Kidins220 decreased clearance, accumulation and hampered neurotrophin signalling from early stages of the disease pathogenesis. These results encourage searches for mutations in Kidins220 gene and their possible associations to dementias. Finally, our data support a model where the effects of excitotoxicity drastically differ when occurring in cerebral ischaemia versus progressively sustained toxicity along AD progression. The striking differences in Kidins220 stability resulting from chronic versus acute brain damage may also have important implications for the therapeutic intervention of neurodegenerative disorders.

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INTRODUCTION

Alzheimer’s disease (AD), the most prevalent age-related dementia, consists in progressive neurodegeneration and severe synaptic and neuronal loss. Neuropathological hallmarks of AD are extracellular senile plaques containing amyloid-β (Aβ) and intracellular neurofibrillary tangles (NFTs), highly enriched in hyperphosphorylated forms of the microtubule-associated protein (MAP) tau (1,2). Phosphorylation of tau is regulated by several kinases and phosphatases and, since expression levels and/or activity of some of them are modified in AD, hyperphosphorylation of this protein is likely due to an imbalance of phosphorylation–dephosphorylation systems. In fact, decreased activity of phosphatases has been proposed to be involved in the formation of NFTs (3) while, among protein kinases, increased activity of glycogen synthase kinase 3 (GSK3) has been largely correlated to the molecular mechanism/s underlying neuronal death in AD (4–8). Evenmore, GSK3 has been suggested to be the link between Aβ and tau hyperphosphorylation and aberrant accumulation in NFTs along AD pathogenesis (9–11). According to the GSK3 hypothesis of AD, overactivity of this kinase is a causal mediator of AD accounting for tau hyperphosphorylation, increased Aβ production and local plaque formation (9). In addition, Aβ activates GSK3 resulting in tau phosphorylation and aggravation of neuronal tauopathy (10,12).

Excitotoxicity, a type of neuronal death elicited by acute or chronic exposure of the glutamate N-methyl-D-aspartate receptors (NMDARs) to high glutamate concentrations, was proposed to be associated to AD and other neurodegenerative disorders (13,14). Indeed, treatment of AD patients with memantine, a non-competitive antagonist of NMDARs, results in functional improvement and reduction of care dependence (15). Recent publications indicate that tau is a critical effector by which NMDA and Aβ mediate their toxicity (16). First, it was shown that reduction of endogenous tau protects transgenic mice expressing human amyloid precursor protein against excitotoxic insults (17). In addition, it has been suggested that Aβ causes neuronal damage through tau by excitotoxic activation of dendritic post-synaptic NMDARs (18).

NMDARs are not only involved in neuronal death but also central to physiology, promoting neuronal survival and playing key functions in synaptic activity, plasticity, learning and memory (19,20). Function and signalling of NMDARs are intimately related to their interactions with other receptors such as the Trk family of neurotrophin receptors (21,22). Neurotrophins exert multiple functions in neuronal homeostasis, regulating neuronal development, survival and death (23). Notably, failures in neurotrophic support and signalling are involved in neurodegeneration (24–26).

A molecule associated to both neurotrophin receptors and NMDARs is Kinase D interacting substrate (Kidins220), also known as ankyrin repeat-rich membrane spanning or ‘ARMS’ (herein referred as Kidins220) (27–29). Kidins220 is an integral membrane protein that was initially identified as a substrate for protein kinase D, a kinase we have found to regulate its intracellular traffic in neurons (27,30,31). In addition, Kidins220 is a scaffold protein that has important implications in neurotrophin signalling (32–37), modulates the development and maturation of axons and dendrites (37,38) and interacts with and regulates the activity of components of the actin and microtubule cytoskeleton (35,38,39).

Among the latter, Kidins220 binds directly to MAP1 and MAP2 (38), members of the same family of microtubule regulatory proteins as tau, that are sequestered by hyperphosphorylated tau within NFTs in AD (40).

Our group identified the association of NMDARs with Kidins220 and the participation of this protein in neuronal survival and death pathways. We demonstrated that excitotoxicity sharply decreases Kidins220 expression levels in cortical neurons and transient cerebral ischaemia (29). This downregulation, that contributes to neuronal death, is triggered by high Ca2+ influx through NMDARs, and involves a dual mechanism: a massive and rapid cleavage by the Ca2+-dependent protease calpain and a long-term calpain-independent silencing of Kidins220 gene transcription. As excitotoxicity and calpain are active in AD (41,42), we hypothesized that Kidins220 could be downregulated in this disease. Unexpectedly, we found increased levels of Kidins220 in human temporal cortex necropsies from AD patients at different progression Braak stages. While we detect no changes in Kidins220 gene expression in these samples, we show evidence of higher resistance of Kidins220 to calpain cleavage in AD brain. Using different ex vivo and in vivo models of neurodegeneration related to AD, we have uncovered some of the major molecular mechanisms involved in Kidins220 proteolytic protection and accumulation with tau. Functional consequences and implications of these data for therapeutic intervention in neurodegeneration are also discussed.

RESULTS

Increased levels and resistance to calpain proteolysis of Kidins220 in human AD necropsies

To study whether Kidins220 could be downregulated in AD, we compared control individuals and age-matched AD patients at progressive Braak stages (represented with a colour scale in Fig. 1A). We selected samples from temporal cortex necropsies with post-mortem times up to 5 h after confirming that spontaneous post-mortem calpain activation (43) decreased Kidins220 levels in human and mouse brain (Supplementary Material, Fig. S1). To perform an accurate comparative analysis, we ordered samples by increasing post-mortem delays (Fig. 1B). Paradoxically, Kidins220 content was increased in AD samples (3.5-fold increase; Fig. 1B and C) and higher levels tended to correlate with disease progression (Fig. 1C) while no differences could be attributed to gender (data not shown). Hyperphosphorylated tau (PHF1) was only detectable in late Braak stages (Fig. 1B).

This unexpected result led us to investigate the mechanism/s that could be at the basis of Kidins220 increase in AD. To explore a possible transcriptional control of Kidins220 gene we performed real-time quantitative PCR analysis of RNA samples obtained from the same brain necropsies used in Figure 1B. Kidins220 mRNA levels were almost identical between control and AD subjects, while protein amounts were statistically augmented in AD as before (Supplementary Material, Fig. S2).
Figure 1. Kidins220 presents increased levels and diminished sensitivity to calpain-processing in brain necropsies from AD patients, where it colocalizes with phospho-tau. (A) Age, gender and post-mortem interval of control (C) and AD patients used in this study. Braak stage progression is indicated (I–V) and represented in a colour scale. Numbers (1–6) correspond to different samples in each group. (B) Immunoblot analysis of the indicated proteins in temporal cortex necropsies. Processing of full-length brain spectrin (FL) into break-down products (BDPs) denoted the state of calpain activity. (C) Relative Kidins220 levels in control and AD samples. Results are expressed in arbitrary units. Each symbol represents an individual (C, n = 6; AD, n = 11). (D and E) Immunoblot and quantitation of Kidins220 levels in control or AD brain homogenates after 1 h of endogenous calpain activation by 5 mM CaCl₂ addition (C, n = 4; AD, n = 11). Only results corresponding to the digested samples are represented and are expressed relative to values from non-digested extracts, arbitrarily assigned a value of 100%. (F) Analysis of Kidins220 levels in brain homogenates untreated or incubated with CaCl₂ for 1 h in the presence or absence of calpain inhibitor III (CIII; 10 μM). (G) Confocal microscopy images showing Kidins220 (green) and phospho-tau (AT8) or Aβ (red) in hippocampal slices from AD Braak stage V/VI (n = 6). Nuclei were stained with TO-PRO-3-iodide (blue). Arrowheads indicate Kidins220 and phospho-tau stain overlapping. Scale bar: 30 μm.
Once demonstrated that levels of Kidins220 transcripts were unaffected by the disease, we hypothesized that increased accumulation of this protein in AD might correlate with a reduced sensitivity to calpain processing. To test this notion, we activated endogenous calpain in tissue homogenates by exposing them to CaCl$_2$ for 1 h. As shown in Figure 1D and E, Kidins220 downregulation was much larger in control extracts (85 ± 4%) than in AD samples (59 ± 7%, P < 0.01). This proteolytic process was calpain-dependent since it was blocked by addition of calpain inhibitor III (CiIII; Fig. 1F). These data suggested that Kidins220 accumulation in AD brain necropsies might be due to post-transductional modifications that conferred a partial calpain-resistant state to this protein.

Since phosphorylation modulates tau susceptibility to calpain cleavage favouring its accumulation in AD tissues (44,45), we anticipated Kidins220 presence in tau aggregates. Analysis of AD brain preparations (Braak stages V/VI) showed a partial overlapping of Kidins220 with phospho-tau (AT8), while there was not a clear co-localization with Aβ (Fig. 1G).

Neurodegeneration induced by okadaic acid increases Kidins220 levels and promotes its accumulation with tau and kinesin-1 in neuritic swellings

Tau resistance to calpain cleavage is acquired through deficient action of phosphatases (PPs) and excessive GSK3β activity (3,46–51). Therefore, we next examined the role of PPs on Kidins220 stability. Primary cultures of cortical neurons treated with the serine/threonine PPs inhibitor okadaic acid (OA) constitute a neurodegeneration model reproducing AD features such as intracellular accumulation of amyloid precursor protein (APP) and phospho-tau (52). Immunoblot of extracts from primary cortical neurons showed that OA doses able to inhibit PP1/PP2A activities induced a reduction in Kidins220 electrophoretic mobility, a feature frequently found in proteins after phosphorylation (Fig. 2A). Treatment with OA also provoked a significant increase in Kidins220 signal from very early time-points, and levels of this protein reached values of 224 ± 28% (P < 0.05) after 4 h of OA treatment compared with control cells (Fig. 2A and B). Importantly, this increase correlated with elevated APP levels and tau hyperphosphorylation (Fig. 2A). Additionally, Kidins220 sensitivity to in vitro calpain digestion in OA-treated neurons was notably and specifically diminished, while processing of spectrin did not render variations (Fig. 2C). These results indicate that Kidins220, as tau, is hyperphosphorylated in serines/threonines after inhibiting PPs activity with OA, and also demonstrate that this form of the protein has a higher endogenous stability and resistance to calpain cleavage. In addition, they suggest that inactivation of phosphatases in AD could contribute to Kidins220 accumulation.

Neurodegeneration is accompanied by the formation of axonal and neuritic swellings (reminiscent of those found in AD-axonopathies) filled with tau, APP and transport proteins such as kinesin-1 complex (52). We examined these neuritic swellings in OA-treated neurons, analysing Kidins220 immunostaining and colocalization with those molecules (Fig. 2D). In untreated neurons, Kidins220 signal was more intense in some neurons and extensions and partially colocalized with tau while it showed little overlapping with kinesin heavy chain (KHC), a component of the kinesin-1 complex, or APP. However, OA-incubation enhanced Kidins220 staining in the neuritic network and increased Kidins220 accumulation with tau and KHC in the swellings, while it sparsely overlapped with APP aggregates.

Then, we analysed Kidins220 association with the kinesin-1 complex. KHC-immunoprecipitation assays confirmed that OA-treatment enhanced Kidins220 association with KHC (Fig. 2E, upper panel). After immunoprecipitating kinesin light chain (KLC) we also found a significant increase in Kidins220 binding to this component of the complex, eventhough KLC signal was lower in the immunoprecipitates from neurons incubated with OA (Fig. 2E, lower panel). In contrast to KHC and similar to Kidins220 and tau, the electrophoretic mobility of KLC, another known GSK3 substrate (53), was reduced in neurons treated with OA compared with untreated cells, suggesting a hyperphosphorylated state (Fig. 2E and F). These data show that hyperphosphorylation of Kidins220 by PPs inhibition enhances its binding to kinesin-1 complexes favouring its retention in neuritic swellings.

A similar approach showed an association of Kidins220 with tau (Fig. 2F). Again here, OA-treated neurons contained preferentially hyperphosphorylated tau (Fig. 2F). Equal signal of Kidins220 in tau immunoprecipitates indicates that these two molecules might also present a strong association when hyperphosphorylated.

PP1 inhibition in excitotoxicity attenuates Kidins220 calpain-processing in a GSK3-dependent manner

Kidins220 is rapidly cleaved by calpain during excitotoxicity (29), a form of neuronal death associated to PPs activation (54). Thus, we examined whether hyperphosphorylation was able to prevent Kidins220 processing induced by NMDAR-overactivation. Pre-treatment of cortical neurons with OA for 1 h highly attenuated Kidins220 downregulation induced by NMDA at all time-points studied, preserving 79 ± 5% of the protein after 4 h of excitotoxic insult compared with 27 ± 4% in OA-untreated cells (Fig. 3A and B). This effect was specific of PP1, and was not observed after inhibiting PP2A or PP2B (Supplementary Material, Fig. S3).

Since GSK3 activity is enhanced in AD, we next tested the role of this kinase in Kidins220 regulation during excitotoxicity. Cortical neurons were pre-treated with GSK3 inhibitors LiCl or SB415282 before adding OA and prior to NMDAR-overactivation. The inhibitors reversed the protective effect of OA and restored almost completely Kidins220 downregulation by excitotoxicity (Fig. 3C and D), while LiCl had no effect on Kidins220 basal levels (Fig. 3E). Altogether, these data show that Kidins220 stability and cleavage by calpain is under the control of GSK3 and PP1 activities.

GSK3β phosphorylates Kidins220 and increases its calpain resistance

Given the role played by GSK3 in the regulation of Kidins220 calpain-mediated proteolysis induced by excitotoxicity, we explored whether GSK3 could be exerting this regulation by
direct phosphorylation of Kidins220. We performed in silico analysis of human Kidins220 sequence and looked for species-conserved consensus GSK3 phosphorylation sites using four different protein motif scan tools (GPS2.1, NetPhosK, Phosida and Eukaryotic Linear Motif – ELM – Resource; see ‘Materials and Methods’ for details and references). Phosida
and ELM rendered very similar predictions and detected 30 common putative sites, 19 of them only found using these two software tools (Fig. 4A, black residues) while the 11 hits remaining were also predicted by GPS2.1 (Fig. 4A, red residues). Thirteen additional consensus sequences were recognized by GPS2.1 which corresponded to proline-directed sites or sequences found to be phosphorylated by GSK3 in other GSK3-substrates although they do not match the consensus (Fig. 4A, green). The most restrictive software, NetPhosK, identified 6 consensus sequences that were also hits for the other three tools. Altogether, Kidins220 contains a total of 43 putative GSK3-phosphorylatable residues absolutely

Figure 3. GSK3β inhibition hampers the protective effect of OA on Kidins220 downregulation by excitotoxic NMDA. (A) Immunoblot of control (C) or treated neurons with 100 µM NMDA plus 10 µM glycine (from now on NMDA) for the indicated times, pre-incubated or not for 1 h with OA, as schematized. (B) Kidins220 levels after 4 h of NMDAR-overactivation expressed relative to values obtained in NMDA-untreated controls (+ OA). Results shown are the means ± s.e.m of six independent experiments. (C) Immunoblot of cortical neurons untreated or stimulated 3 h with 30 mM LiCl or 5 mM SB-415286 (SB) and further incubated 1 h with OA prior to overactivation of NMDAR for 4 h, as represented in the diagram. (D) and (E) Graphs showing the effects of LiCl and OA on Kidins220 stability during excitotoxicity. Kidins220 levels after NMDAR stimulation were determined in the presence of LiCl, either combined with OA (D) or alone (E). Data represented are the means ± s.e.m of five independent experiments and are expressed relative to values obtained in OA-incubated cells (D) or non-stimulated neurons (E), arbitrarily assigned a value of 100%.
Figure 4. GSK3β phosphorylation of Kidins220 diminishes its calpain-processing. (A) Evolutionary conserved consensus sites for GSK3 phosphorylation identified in Kidins220 amino acid sequence using Phosida, ELM, NetPhosK and GPS2.1 software. Only potential phosphosites conserved among Homo sapiens, Mus musculus, Rattus norvegicus, Gallus gallus, Xenopus tropicalis and Danio rerio are shown, except those in light blue boxes absent in the latter. Colour scale indicates the combination of prediction tools recognizing the corresponding position: green (GPS2.1), black (Phosida plus ELM), red (Phosida plus ELM and GPS2.1, being six of those sites also identified by NetPhosK). Note that Phosida, ELM and NetPhosK recognize only primed consensus GSK3-sequences (S/T-X-X-X-pS/pT), while GPS2.1 also identifies unprimed proline-directed sites (S/T-P) or sites that, without matching the consensus, had been found to be phosphorylated by GSK3 in other GSK3-substrates. (B) Kidins220 neuronal immunoprecipitates were phosphorylated \textit{in vitro} (IVK) using [γ-\textsuperscript{32}P]-ATP with or without purified GSK3β for 30 min. Autoradiography or immunoblots are shown. (C) Kidins220 immunoprecipitates were phosphorylated using non-radioactive ATP and then digested with calpain I (0 and 10 U/ml) for 30 min before detecting its signal by immunoblot. (D) Calpain I \textit{in vitro} digestion (0 and 10 U/ml, 30 min) of Kidins220 from HEK293T cells transfected with empty (control), myc-GSK3β wild-type (GSK3β wt) or kinase inactive (GSK3β ki) plasmids. Kidins220 and myc-GSK3β were detected by immunoblot. (E) Kidins220 sensitivity to calpain-processing after expression of GSK3β-con structs. Kidins220 signal was expressed relative to the corresponding signal in non-digested extract, arbitrarily assigned a 100% value. Data shown are the means ± s.e.m of three independent experiments. (F) Kidins220 immunoprecipitates from human temporal cortex from control or AD samples subjected to IVK using GSK3β as in (B).
conserved among human, rat, mouse, chicken and frog, being 34 of them also present in the zebra fish (Fig. 4A, light blue boxes show residues not conserved in zebra fish). Since human–fish comparisons provide a very useful evolutionary position for comparative sequence-based discovery, these criteria identified Kidins220 as a likely substrate for GSK3.

We confirmed that Kidins220 immunoprecipitated from neurons was a bona fide GSK3β substrate performing in vitro kinase assays with recombinant kinase (Fig. 4B). Additionally, we showed that phosphorylation of Kidins220 immunocomplexes by GSK3β decreased Kidins220 susceptibility to in vitro calpain-processing (Fig. 4C). To further support this finding, wild-type active and kinase inactive myc-GSK3β (GSK3β wt and GSK3β ki, respectively) were transfected into HEK293T cells. Of note are the considerable levels of endogenous Kidins220 found in lysates from this cell line (Supplementary Material, Fig. S4), that allowed to determine this protein susceptibility to calpain processing by performing in vitro digestion assays with purified calpain. While Kidins220 was efficiently proteolysed in extracts from cells transfected with empty vector or GSK3β ki, it was less sensitive to calpain cleavage after expression of GSK3β wt (percentage of decrease relative to non-digested extracts: control = 88 ± 4%; wt = 66 ± 11% and ki = 93 ± 2%; P < 0.05; Fig. 4D and E).

Next, we investigated the phosphorylation state of Kidins220 in human AD samples. We anticipated that if Kidins220 was already phosphorylated by GSK3β in vivo, the recombinant kinase would be less efficient incorporating radiolabelled phosphate in Kidins220-immunoprecipitates in an in vitro kinase assay. To test this hypothesis, we immunoprecipitated Kidins220 from control and AD brain temporal cortex extracts, and performed the in vitro kinase assay in the presence of recombinant GSK3β and 32P-γ-ATP (Fig. 4F). As expected, the protein obtained from AD samples was phosphorylated less efficiently than that precipitated from control extracts, eventhough higher amounts of Kidins220 were present in these immunocomplexes (Fig. 4F). Altogether, these data strongly suggest that Kidins220 is phosphorylated by GSK3β in AD brain. This modification could be protecting Kidins220 from calpain cleavage, contributing to a decreased clearance and accumulation of this protein in AD samples.

Kidins220 is more resistant to calpain-processing in brain from transgenic mice overexpressing GSK3β

To further support our previous findings, we used a mouse model where GSK3β is overexpressed in adult brain areas (mainly hippocampus and cortex) and animals developed increased apoptosis and atrophy of the dentate gyrus (DG) along ageing (55,56). First, we explored Kidins220 levels and distribution in cortex and DG and CA1 hippocampal regions from GSK3β transgenic mice. Brain sections were co-stained by immunohistochemistry for Kidins220 and β-galactosidase (a marker of the activity of the bidirectional promoter used by the GSK3β transgene) or the axonal marker SMI-31 (Fig. 5A). In addition, we analysed Kidins220 distribution in the somatodendritic compartment by staining adjacent serial sections with rabbit antibodies recognizing Kidins220 and the dendritic marker MAP2 (Fig. 5B). Confocal microscopy images showed that the degree of localization of Kidins220 with SMI-31 was low while Kidins220 and MAP2 presented a very similar staining pattern (Fig. 5). These data indicate that Kidins220 is mainly enriched in neuronal somatodendritic compartment in vivo. However, we found no significant alterations in Kidins220 immunostaining and localization in the brain of GSK3β mice compared with wild-type animals.

We next assayed Kidins220 sensitivity to calpain processing in extracts from brain frontal cortex (Fig. 6A) or hippocampus (Fig. 6B) from wild-type or GSK3β transgenic mice by incubating samples with CaCl₂ for 30 min in order to activate endogenous calpain. Kidins220 resulted to be significantly less vulnerable to Ca²⁺ action in the samples obtained from GSK3β transgenic animals compared with the wild-type mice in both cerebral regions (24 ± 5 and 56 ± 5% of protein left after digestion of extracts from cortex and hippocampus of GSK3β mice, respectively, versus 2 ± 0.4 and 7 ± 1% in wild-type animals). Importantly, spectrin proteolysis underwent no significant changes when comparing both genotypes, indicating that this effect was specific for Kidins220 and not due to calpain inhibition in GSK3β transgenic mice. This process was calpain-dependent since it was blocked by Cil3 but not the proteasome inhibitor lactacystin (Fig. 6C and D). The higher protection of Kidins220 to calpain action in hippocampus versus cortex (Fig. 6) was particularly significant since it correlated with the level of GSK3β overexpression found in these two brain regions of the transgenic mice. Expression of GSK3β increases a 40 or 17%, respectively, in the hippocampus and the cortex of the transgenic animals compared with wild-type mice (55). Notably, the functional effects (neuronal stress and death) described by Lucas et al. (55) are only detectable in the hippocampus of the GSK3β transgenic mice.

Since these data supported that GSK3β phosphorylates Kidins220 in vivo, we assessed whether Kidins220 dephosphorylation facilitated its calpain-processing. For this purpose, cortical and hippocampal homogenates from GSK3β transgenic mice were incubated with lambda phosphatase (+λ) before triggering endogenous calpain activation for different time periods (Fig. 7A and B). As a control, phosphatase activity was confirmed by determining phospho-tau using the PHF1-antibody. Immunoblot and quantitative analysis showed that dephosphorylation sharply increased Kidins220 cleavage in cortex and hippocampus (Fig. 7A and B). These results suggest that the inaccessibility of potential calpain-digestion sites due to the phosphorylation of Kidins220 in GSK3β mice could contribute to its resistance to calpain cleavage, as it has been described for paired helical filaments isolated from adult human brain (44,45).

The lower susceptibility of Kidins220 to be proteolysed when phosphorylated by GSK3β could also lead to the accumulation of this protein over time in the GSK3β transgenic mice. In accordance with this hypothesis, cortices and hippocampi from aged 26-month-old transgenic mice contained higher amounts of Kidins220 than these same regions of wild-type animals of the same age (Fig. 8A and B). This result is particularly significant since we have shown that Kidins220 levels clearly decrease with ageing in rat brain (38). Altogether, these data suggest that in this transgenic model...
Kidins220 undergoes phosphorylation by GSK3β in vivo. This modification would be protecting Kidins220 from calpain proteolysis and leading to a decreased clearance and an accumulation along ageing. A similar molecular mechanism might be at the basis of the increased amounts of Kidins220 found in AD.

**Figure 5.** Distribution of Kidins220 in brain is not altered in GSK3β transgenic mice. Immunohistochemistry and confocal microscopy analysis of Kidins220 immunostaining in brain slices from 2-month-old wild-type or GSK3β transgenic mice. (A) Co-immunostainings of Kidins220 (green) and β-galactosidase (β-gal; marker of the activity of the bidirectional promoter used by the GSK3β transgene) or the axonal marker SMI-31 (red), in two regions from the hippocampus (DG, dentate gyrus, and CA1) and cortex (Cx). (B) Adjacent sections were immunostained for Kidins220 (green) or MAP2 (red) as a marker for the somatodendritic compartment. Images correspond to the same brain regions as in (A). Nuclei were stained with Dapi (blue). Merged confocal microscopy images are depicted. Scale bars: 10 μm.
DISCUSSION

Modification of Kidins220 could be at the basis of AD pathogenesis

This study is the first one to report that Kidins220 levels are increased in human brain necropsies from AD patients corresponding to different progression stages (Braak stages I–V), unveiling a very early alteration of this molecule along AD pathogenesis, which precedes the appearance of hyperphosphorylated tau. Notably, at late stages of the disease (Braak stages V/VI), Kidins220 localizes within hyperphosphorylated tau aggregates. We also find that
Kidins220 is a substrate for GSK3β and that this protein is likely phosphorylated by this kinase in AD brain, as it has been demonstrated for tau. Another common feature with tau is that Kidins220 contains a significant number of conserved sites for GSK3β phosphorylation, highly conserved through evolution. However, the rate of Kidins220 phosphorylation by recombinant GSK3β in vitro is low. A possible explanation could be that Kidins220 was already phosphorylated by GSK3β in vivo (in living cells or tissues), hindering further in vitro phosphorylation. Alternatively, GSK3β may need the priming by other kinase/s in order to further phosphorylate Kidins220, as has been described for most bona fide GSK3 substrates (57). Supporting this notion, we have observed a strong reduction in Kidins220 electrophoretic mobility provoked by inhibition of PP1 activity while specific GSK3 inhibitors were able to reverse this mobility upshift only partially (Fig. 3C). This result might be indicative of Kidins220 hyperphosphorylation by additional kinases. Together, our data suggest that although GSK3 is the key kinase protecting Kidins220 from calpain cleavage, as discussed further below, it might not be the major kinase phosphorylating this molecule in neurons.

An important issue to accomplish in the future will be to identify the specific residues and their temporal pattern of phosphorylation along AD progression and to establish a possible parallelism with tau phosphorylation. Equally important will be to search for mutations in Kidins220 gene that could lead to an aberrant phosphorylation and/or accumulation of this protein, as well as their possible associations to dementias, as it has been previously found for tau. Indeed, the identification of mutations in tau gene as the cause of inherited frontotemporal dementia with parkinsonism associated to chromosome 17 (FTDP-17) has confirmed that dysfunction of tau protein is sufficient to trigger neurodegeneration (58). Furthermore, Paudel’s group has demonstrated that missense tau mutations (G272V, P301L, V337M and R406W) found in FTDP-17 inhibit or promote tau dephosphorylation depending on the specific tau site, thus interfering with physiological phosphorylation/dephosphorylation of this protein (59).

Axonopathies are considered to be early events in the pathogenesis of AD, preceding the onset of known pathological changes such as formation of NFTs and senile plaques (60). Aberrant microtubule organization and axonal transport blockage lead to the formation of axonal swellings, precursors of some forms of dystrophic neurites with accumulated cytoskeletal proteins, such as neurofilaments, and the molecular motor complex kinesin-1 (60). Remarkably, sporadic AD, the major form of the disease with no precise underlying cause, might be initiated by age-dependent decline in axonal transport (60).

Herein, we report a constitutive association of Kidins220 with tau and the two subunits of kinesin-1 complex. Furthermore, neurodegeneration induced by PPs inhibition provokes hyperphosphorylation of Kidins220, tau and KLC (all of them GSK3 substrates), and increases their accumulation in neuritic swellings. Given the scaffolding properties attributed to Kidins220 (61) and its ability to interact with MAP1 and MAP2 (38) and regulate axonal elongation (37,38), it is tempting to speculate that excessive amounts of Kidins220 could be
involved in the sequestration and aggregation of these microtubule-regulatory proteins, thereby helping to destabilize microtubules and contributing to axonal failure.

Fast axonal anterograde transport of tau and Kidins220 occurs via kinesin-1, both molecules interacting directly with KLCs (34,62). We show here that Kidins220 hyperphosphorylation potentiates its interaction with this motor protein as shown before for phospho-tau (63). Bracale et al. (34) reported that disruption of Kidins220/kinesin-1 complexes depletes Kidins220 from neuritic processes. In agreement with their work, we find that a tight interaction between these two proteins concentrates them in neuritic swellings. The robust association of Kidins220 with kinesin-1 complexes and their retention in axonal swellings indicates that Kidins220 anterograde trafficking might be compromised in AD-related neurodegeneration.

Schiavo’s group has also proposed that cellular responsiveness to neurotrophins depends on the correct delivery of Kidins220-positive carriers to the neurite tips (34). Thus, failures in Kidins220 anterograde transport will diminish the amount of molecules getting to the axonal terminals. Since Kidins220 is obligatory for retrograde transport of Trk receptors and neurotrophin signalling (32–37), this uncoupling might consequently mimic conditions of neurotrophin withdrawal. Notably, compelling evidence suggests that failures in neurotrophic support and signalling (24–26), and alterations in retrograde transport and other membrane trafficking pathways (64,65) play key roles in neuronal death and AD pathogenesis. Therefore, Kidins220 mislocalization may contribute to the impairment of neurotrophin retrograde signalling.

**Differential effect of chronic versus acute brain damage on Kidins220 stability: implications for therapeutic intervention**

Excitotoxicity is at the basis of the aetiology of AD and other neurodegenerative disorders, thus we hypothesized that Kidins220 might be downregulated in AD as seen before in NMDAR-overstimulated neurons and transient cerebral ischaemia (29). Contrary to our hypothesis, Kidins220 is significantly augmented in AD brains, this increase correlating with enhanced resistance to calpain-processing but not with higher transcription of the gene. Using AD brain necropsies, transgenic mice and cell models, we have established that Kidins220 is more resistant to calpain-processing when phosphorylated by GSK3β, or if PP1 activity is inhibited. Together, increased phosphorylation and lack of dephosphorylation may synergistically cooperate to render a highly phosphorylated stable form of Kidins220, of reduced susceptibility to calpain.

Our data strongly support that Kidins220 accumulation in AD patients is a consequence of its decreased clearance due to slow and progressive PPs inactivation and GSK3β activation occurring in this disease (3,6,66). The onset of a gradual imbalance in phosphorylation–dephosphorylation systems should occur at very early stages of AD, when excitotoxic neuronal death is still ‘OFF’ (Fig. 9). Consequently, Kidins220 phosphorylation will start rising progressively as disease evolves, as it has been described to happen with tau (67). At more advanced AD stages, when excitotoxicity is ‘ON’, calpain activation through NMDAR-overstimulation will not result in a rapid processing and downregulation of Kidins220 because the phosphorylated protein will escape from degradation and start to accumulate. This mechanism may be common to other calpain-substrates such as tau. Indeed, tau is cut by calpain under neurotoxic conditions but may be common to other calpain-substrates such as tau. Indeed, tau is cut by calpain under neurotoxic conditions but its processing is greatly impaired in AD and other tauopathies (44,45,68). Therefore, initial small and gradual non-reversible changes in Aβ, or other toxic ligands or stimuli and signalling pathways primarily implicated in the control of phosphorylation/dephosphorylation systems, may be at the basis of the accumulation of several GSK3β and calpain substrates and the aetiopathology of these dementias.

Our data provide novel evidence supporting a model where the effects of NMDAR-overstimulation differ drastically in ‘healthy’ versus ‘pathological’ backgrounds (Fig. 9). Excitotoxicity would readily downregulate Kidins220 by calpain-dependent mechanisms during acute brain damage (i.e. traumatic brain injury or an ischaemic episode), but fail to do so at initial/medium stages of AD. A critical point for such disparity might result from differences in PP1 activity. Acute NMDAR-overactivation induces a positive feedback loop between GSK3β and PP1 further activating this phosphatase (54), while PP1 activity is significantly decreased in AD (69), by mechanisms specifically induced by Aβ peptides and enhanced by Aβ aggregation (70). Initially, when exposed
shortly to a mild transient toxic environment, Kidins220 resistance to calpain in AD and other related pathologies may be a protective response to potentiate survival, giving neurons the opportunity to restore their physiological functions. This neuroprotective mechanism is supported by our previous results showing that Kidins220 silencing enhances neuronal death and makes neurons more vulnerable to excitotoxicity (29), and could be shared by other calpain-substrates, such as tau (71,72). However, the persistent activation of this pathway in AD-related pathologies by chronic, maintained or exacerbated toxic insults leading to aberrant accumulation of Kidins220 and other calpain-substrates may result detrimental to neurons in the long-term. Supporting the idea that an excess of Kidins220 might be deleterious for neural functions and survival, we have demonstrated that overexpression of this protein in primary hippocampal neurons hampers their development and renders rounded cells, with fragmented Golgi apparatus that detach and die (38). Altogether, our findings support a dual behaviour of Kidins220, where too low/unstable or too high/stable levels both contribute to neuronal death. Finally, these results may have important implications for the establishment of different therapeutic approaches to treat acute versus chronic neurodegeneration. Calpain inhibitors have emerged as drugs for the potential treatment of neurodegenerative diseases, although their precise contribution to neuroprotection remains unclear (73). Our data support that the use of calpain inhibitors as a neuroprotective strategy would be more successful to prevent extensive neuronal death in acute neurodegenerative pathologies such as ischaemia and traumatic brain injury. These drugs will be able to efficiently hamper the processing of molecules critical for neuronal survival, such as Kidins220. In contrast, calpain inhibitors will be less effective in treating AD-related neurodegeneration where some of these survival molecules have been post-translationally modified (i.e. hyperphosphorylated) and will be insensitive to calpain inhibition because they are already resistant to cleavage by this protease. Because AD is one of the earliest disorders linked to GSK3β dysfunction, clinical and experimental studies supporting the use of inhibitors of this kinase for therapies in AD and related dementias are already available (8). A therapeutic paradigm using a combination of GSK3β and calpain inhibitors at initial stages of AD (or at appropriate therapeutic–temporal windows), avoiding early hyperphosphorylation and accumulation of key proteins while preventing calpain-processing of their non-phosphorylated forms, might succeed in the treatment of this and other types of chronic neurodegenerative pathologies.

**MATERIALS AND METHODS**

**Materials and chemicals**

N-Methyl-D-aspartate (NMDA), glycine, cytosine β-D-arabino furanoside (AraC), poly-L-lysine, L-laminin, lithium chloride, SB-415286 and Protein A/G-Sepharose were from Sigma Co. (St Louis, MO, USA). Calpain I (µ-calpain), clasto-Lactacystin β-Lactone (Lactacystin) and carbobenzoxy-valinil-phenilalanine
(CIII) were from Calbiochem-Merck Bioscience (Darmstadt, Germany). Okadaic acid (OA) was from Tocris Bioscience (Bristol, UK). Cyclosporine A (CsA) and FK-506 were from Sandoz (Vienna, Austria) and LC Laboratories (Wobum, MA, USA). Lambda protein phosphatase was from New England BioLabs (Ipswich, MA, USA). [γ-32P]-ATP (370 MBq/ml) was from PerkinElmer, Inc. (Boston, MA, USA).

Antibodies

Rabbit polyclonal were: Kidins220, prepared as before (27) or obtained from Abcam (Cambridge, UK); NSE (ICN Biomedicals; Costa Mesa, CA, USA); mouse and human C-terminal tau (DakoCytomation; Glostrup, Denmark); APP C-terminal (Sigma Co.; St Louis, MO, USA); high molecular weight MAP2 (74). Mouse monoclonals were: Myc and GSK3β (Cell Signaling Technology; Beverly, MA, USA); Spectrin (DakoCytomation; Glostrup, Denmark); SMI-31 (Abcam; Cambridge, UK); NSE (ICN Biomedicals; Costa Mesa, CA, USA); mouse and human C-terminal tau (DakoCytomation; Glostrup, Denmark); APP C-terminal (Sigma Co.; St Louis, MO, USA); β-galactosidase (Promega Corporation; Madison, WI, USA); Kinesin Heavy Chain (KHC) and Kinesin Light Chain (KLC) (Millipore Corporation; Billerica, MA, USA); phospho-tau-Ser202/Ser205/Ser396 (Millipore Corporation; Billerica, MA, USA) and phospho-tau -Ser396/Ser404 (PHF1) (a gift from Dr Davies; Albert Einstein College, NY, USA); APP N-terminal (Millipore Corporation; Billerica, MA, USA); Aβ clone 6F/3D (DakoCytomation; Glostrup, Denmark); SMI-31 (Abcam; Cambridge, UK). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were from General Electric (Fairfield, CT, USA) and Alexa-Fluor-488, 555 and 647 conjugated antibodies were from Invitrogen-Life Technologies (Carlsbad, CA, USA).

Cell culture and treatment of primary cortical neurons

Neuronal cultures were prepared from cerebral cortex of 19-day-old Wistar rat embryos as we have previously described (29). Rats were obtained from the animal care facility at the Instituto de Investigaciones Biomedicas ‘Alberto Sols’ (CSIC-UAM, Madrid, Spain). Animal procedures were approved by the Ethical Committee from the CSIC and performed in compliance with European Directive 2010/63/EU. Neurons were used after 14 days in vitro (DIV). Excitotoxicity was induced with treatment with the NMDAR co-agonists NMDA and glycine. Neurons were pre-treated or treated for different times as indicated with the following concentrations of reactives: 100 μM NMDA, 10 μM glycine, 10 μM CiIII, 30 mM LiCl, 5 μM SB-415286, 15 μM Lactacystin, 200 ng/ml FK-506, 100 ng/ml CsA and 1 mM or 500 mM OA.

Plasmids and transfection of HEK293T cells

Wild-type myc-GSK3β and its kinase inactive mutant (R85A) cloned in the expression vector pcDNA3.1 (Invitrogen-Life Technologies, Carlsbad, CA, USA) have been previously described (75). Human embryonic kidney 293T cells (HEK293T) were transfected with Lipofectamine 2000® (Invitrogen-Life Tech; Carlsbad, CA, USA) and collected for processing 48 h later.

GSK3β transgenic mice

Mice overexpressing GSK3β were generated as previously described (55). Transgenic animals as well as wild-type mice (C57BL/6) were bred at the animal care facility of the Centro de Biologia Molecular ‘Severo Ochoa’ (CSIC-UAM, Madrid, Spain). As discussed earlier, mice were handled in strict accordance with good animal practice as defined by the national animal welfare bodies and institutional guidelines. For biochemical studies, animals were sacrificed by cervical dislocation at 2 and 26 months of age and frontal cortex and hippocampus were immediately dissected and stored at -80°C until use. When aimed for histological purposes, 2-month-old mice were completely anaesthetized with an intraperitoneal pentobarbital injection and then perfused with saline followed by 4% paraformaldehyde in phosphate buffer. The brains of the animals were removed and post-fixed overnight in the same fixative. Sagittal sections (35 μm thick) from both hemispheres were obtained on a Leica VT1000S vibratome and series of sections were generated, comprising every 24th section.

Human brain samples

Brain tissue was obtained from the Institute of Neuropathology Brain Bank following Spanish legislation and local Ethics Committee guidelines and processed as described (76). Briefly, one hemisphere was immediately dissected and selected areas of the encephalon were stored at −80°C until use for biochemical studies. The other hemisphere was fixed by immersion in 4% buffered formalin for 3 weeks for morphological studies. Small vessel disease was a common feature in these aged groups but cases with lacunae and infarcts were excluded.

Preparation of protein extracts

Protein lysates from mouse brains, cortical neurons and HEK293T cells were obtained as previously described (29). Extracts from human brain cortex were prepared in 1% SDS in PBS, 5 mM EDTA and 5 mM EGTA, containing protease and phosphatase inhibitors. Homogenization of the samples was performed with a POLYTRON® System PT 1200 E (Kinematica AG, Lucerne, Switzerland) and lysates were then centrifuged at 21 000g for 30 min at 4°C.

Immunoprecipitation and immunoblot analysis

Immunoprecipitation assays were performed as previously described (29). Equal amounts of total lysates or equivalent volumes of immunocomplexes were analysed by immunoblot. Membranes were incubated with different primary and secondary antibodies, and immunoreactive bands were detected by ECL (General Electric; Fairfield, CT, USA). Visualization of tau immunoprecipitates required the use of an specific secondary antibody recognizing only those rabbit immunoglobulins present in their native form (ImmunoCruz™ IP/WB Optima F System; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).
Calpain I in vitro proteolysis

Cellular extracts were digested in vitro with purified calpain I as reported (29). Endogenous calpain activation was induced by adding 2.5 mM CaCl₂ to mouse brain lysates diluted with 5 mM DTT. Digestions proceeded at 37°C for 7, 15 and 30 min. Dephosphorylation by lambda-phosphatase was performed when indicated prior to CaCl₂ incubation. In order to prevent endogenous calpain inhibition by high concentrations of SDS, human samples were first homogenized in 50 mM Tris–HCl, pH 7.4, incubated with 5 mM CaCl₂ for 1 h, and then lysed in 1% SDS lysis buffer. Calpain activation was followed detecting full-length brain spectrin (FL) and its breakdown products (BDPs) by immunoblot.

In vitro kinase assay

Kidins220 phosphorylation by active recombinant kinase GSK3β (Invitrogen-Life Technologies, Carlsbad, CA, USA) was determined performing in vitro kinase assays in Kidins220 immunoprecipitates as described (31). Reaction proceeded for 30 min and samples were analysed by SDS–PAGE followed by autoradiography and immunoblot.

Quantitative real-time PCR

Total RNA was prepared using TRI® Reagent (Sigma Co; St Louis, MO, USA) and RNeasy® Mini Kit (Qiagen; Benelux, The Netherlands) spin columns. Two micrograms of total RNA were treated with RQ1 RNase-free DNase (Promega Corporation; Madison, WI, USA), purified within Microcon-100 columns (Millipore Corporation; Billerica, MA, USA) and analysed using an Agilent 2100 Bioanalyzer to determine their integrity level. Only RNAs whose RNA integrity number (RIN) was above 6 were considered adequate as templates for the reverse transcription reaction. RNA was retrotranscribed by oligo-dT extension with Superscript II. PCR reaction was performed as previously reported (29). Kidins220 forward primer spanned nucleotides 5389–5408 of its cDNA (‘5′-CGCCCATCACCTTTCTACAT-3′) and the reverse primer spanned nucleotides 5461–5480 (‘5′-TGCTCCCTTCGTCACTAG-3′), giving a PCR product of 92 pb. The amplification of Kidins220 transcripts was normalized against the human gene encoding glyceraldehyde 3-phosphate dehydrogenase (GAPDH). In this case, forward primer spanned nucleotides 454–473 (‘5′-TGCACCACCAACTGCTTAGC-3′) and reverse primers spanned nucleotides 520–540 (‘5′-GGCATGGACTGTGGTCATGAG-3′) of GAPDH cDNA. These primers amplified a fragment of 87 pb.

Immunofluorescence and confocal microscopy

Immunofluorescence of neuronal cultures and human and murine brain sections was performed as before (29,76,77). Images are single sections of a z-series acquiring each channel in a sequential mode using an inverted Zeiss LSM710 confocal microscope with a 63X/1.4 Plan-Apochromatic objective. Pictures were processed with ZEN 2009 light Edition (Carl Zeiss MicroImaging) and Adobe CS3 Extended (Adobe Systems Inc.) software.

In silico analysis of GSK3β putative phosphorylation sites in Kidins220

Putative GSK3 phosphorylation sites in human Kidins220 sequence (NCBI Accession NP_065789.1) were predicted using the protein motif scan tools: GPS2.1 (78), NetPhosK (79), Phosida (80) and the Eukaryotic Linear Motif Resource (ELM) (81). Amino acids with a score higher than 0.5 (NetPhosK 2.0) as well as those identified in GPS2.1, Phosida and ELM prediction using a high threshold were selected. Similar scans were applied to Kidins220 sequences from Rattus norvegicus (NP_446247.1), Mus musculus (NP_001074847.1), Gallus gallus (XP_419939.2), Xenopus tropicalis (NP_001120159.1) and Danio rerio (AAH61450.1). Only species-conserved consensus sites were finally selected.

Quantitative and statistical analysis

Immunoblot signals were quantified by densitometric analysis (NIH Image), normalized using NSE (or β-actin in the case of the HEK293T transfected cells) and expressed relative to values obtained in their respective controls. Results are shown as mean ± standard error of the mean (s.e.m) of 3–8 independent experiments. Statistical significance was determined by Student’s t-test. A P-value <0.05 was considered statistically significant: *P < 0.05, **P < 0.01, ***P < 0.001.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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