An unbiased approach to identifying tau kinases that phosphorylate tau at sites associated with Alzheimer’s disease

Annalisa Cavallini, Suzanne Brewerton, Amanda Bell, Samantha Sargent, Sarah Glover, Clare Hardy, Roger Moore, John Calley, Devaki Ramachandran, Michael Poidinger, Eric Karran, Peter Davies, Michael Hutton, Philip Szekeres & Suchira Bose

1Eli Lilly & Company Ltd., Erl Wood Manor, Sunninghill Road, Windlesham, Surrey, GU20 6PH, UK
2 Eli Lilly & Co Ltd., Corporate Centre, Indianapolis, USA
3Eli Lilly & Co Ltd., Lilly Singapore, Singapore Centre for Drug Discovery, Republic of Singapore
4Alzheimer’s Research UK, Cambridge, UK
5AECOM, The Einstein Institute for Medical Research, 350 Community Drive, Manhasset, NY 11030, USA

*Corresponding author: bose_suchira@lilly.com, +44 (1276) 483608

Keywords: Tau phosphorylation, kinases

Background: Abnormally hyperphosphorylated tau is present in neurofibrillary tangles in Alzheimer’s disease.

Results: Key kinases that phosphorylate tau at Alzheimer’s disease specific epitopes have been identified in a cell based screen of kinases.

Conclusions: GSK3α, GSK3β and MAPK13 were the most active tau kinases.

Significance: Findings identify novel tau kinases and novel pathways that may be relevant for Alzheimer’s disease and other tauopathies.

SUMMARY

Neurofibrillary tangles (NFT), one of the hallmarks of Alzheimer’s disease (AD), are composed of paired helical filaments (PHF) of abnormally hyperphosphorylated tau. The accumulation of these proteinaceous aggregates in AD correlates with synaptic loss and severity of dementia. Identifying the kinases involved in the pathological phosphorylation of tau may identify novel targets for AD. We have used an unbiased approach to study the effect of 352 human kinases on their ability to phosphorylate tau at epitopes associated with AD. The kinases were overexpressed together with the longest form of human tau in human neuroblastoma cells. Levels of total and phosphorylated tau (epitopes pS202, pT231, pS235 and pS396/404) were measured in cell lysates using AlphaScreen assays. GSK3α, GSK3β and MAPK13 were found to be the most active tau kinases, phosphorylating tau at all 4 epitopes. We further dissected the effects of GSK3α and GSK3β using pharmacological and genetic tools in hTau primary cortical neurons. Pathway analysis of the kinases identified in the screen suggested mechanisms for regulation of total tau levels and tau phosphorylation, for example kinases that affect total tau levels do so by inhibition or activation of translation. A network fishing approach with the kinase hits identified other key molecules putatively involved in tau phosphorylation pathways including the G-protein signaling through the Ras family of GTPases (MAPK family) pathway. The findings identify novel tau kinases and novel pathways that may be relevant for AD and other tauopathies.

Tauopathies, including AD, frontotemporal dementia with Parkinsonism linked to chromosome-17 (FTDP-17), progressive supranuclear palsy, Pick’s disease and corticobasal degeneration are all characterized by the progressive development of intracellular inclusions of the microtubule-stabilizing protein tau in affected brain regions (1,2). The accumulation of misfolded, hyperphosphorylated tau species in AD correlates...
with neuronal loss and cognitive impairment, unlike senile plaque burden (3). These findings have highlighted the need for identifying tau-based disease modifying therapies for AD and other tauopathies.

The primary function of tau is to facilitate assembly and maintenance of microtubules in neuronal axons, allowing transport of cellular cargo (4). Tau function is regulated by phosphorylation as well as by alternative splicing. There are 85 putative phosphorylation sites on the longest tau isoform and more than 20 Ser/Thr kinases have been shown to phosphorylate tau in vitro. However, there is still uncertainty about which of these kinases actually phosphorylate tau in vivo (for reviews, see 5,6). Mass spectrometric analysis of human brain tissue, combined with Edman sequencing and specific antibody reactivity, has been used to demonstrate numerous tau phosphorylation sites associated with tau dysfunction and neurodegeneration (6). Many of these are associated with the C-terminal repeat regions of tau, defined as microtubule binding domains (MTBD), as well as the flanking domains. Site-directed phosphorylation of tau in these two domains is essential for regulating tau function in microtubule assembly and stabilization. In AD brain, abnormal hyperphosphorylation of tau in these regions is thought to change the conformation of tau and decrease its affinity for microtubules resulting in microtubule instability and neurofibrillary tangle (NFT) formation (7,8). Loss of a functional microtubule cytoskeleton contributes to neuronal cell dysfunction and cell death.

Numerous tau phosphorylation sites are associated with tau dysfunction and neurodegeneration (5,6). Augustinack and colleagues used phosphorylation dependent tau antibodies and a panel of AD cases of varying severity to map epitopes that were associated with different stages of neurofibrillar tangle formation during disease progression (9, 10). Epitopes that were associated with pre-tangle, non-fibrillar tau included pT231, pS262 and pT153; epitopes associated with intraneuronal fibrillar structures include pS262/pS396, pS422 and pS214; finally, epitopes associated with intracellular and extracellular filamentous tau include pS199/pS202/pT205 and pS396/pS404. Phosphorylation of tau on pS262 and pS356 in adjacent microtubule binding repeats significantly reduces the affinity of tau for microtubules and renders tau less susceptible for degradation (11).

Phosphorylation of tau on pS214 and pT231 is also reported to reduce the ability of tau to bind microtubules (12). In p25 transgenic mice, significantly higher levels of pS235 positive tau relative to non-transgenic mice were present and therefore considered to be cdk5 specific epitope (13). Since cdk 5 is a well established tau kinase, we included this epitope in our screen. Identifying the kinases involved in phosphorylation of key residues associated with AD will increase our understanding of the mechanisms of tau dysfunction in AD and lead to identification of novel targets for therapeutic intervention. Here, we evaluated the effect of kinases to phosphorylate tau at epitopes critical for the progression of AD (pT231, pS202, pS235 and pS396/404).

In vitro, numerous kinases have been reported to phosphorylate tau on multiple epitopes (5), however, these may not reflect the situation in a cellular system. Here we report on an unbiased approach to study the effect of 352 human kinases on their ability to phosphorylate tau at epitopes associated with AD in human neuroblastoma cells. The kinases were co-transfected with the longest isoform of tau and their ability to phosphorylate tau at AD relevant epitopes (pS202, pT231, pS235 and pS396/404) was measured by AlphaScreen assays. Kinases that phosphorylated tau at one or more epitopes in a statistically significant manner were further validated in a second screen. Using the GeneGo MetaCore™ pathway analysis software we identified the mechanisms by which some kinases regulate total tau levels, through inhibition or activation of translation, and the phosphorylation of tau. A network fishing approach using the kinase ‘hits’ identified other molecules putatively involved in tau phosphorylation pathways and suggested the G-protein signalling through Ras family of GTPases (MAPK family) pathway to be a key regulator of tau phosphorylation. In addition, since GSK3α and GSK3β were found to be the most active tau kinases, phosphorylating tau at all 4 epitopes, we further dissected these effects using pharmacological and genetic tools in hTau primary cortical neurons. The data provides insights into novel targets and pathways for AD.

**EXPERIMENTAL PROCEDURES**

**Materials**-2N4R tau was obtained from Peter Davies, Albert Einstein College of Medicine, USA. Kinase clones used for screen were supplied dried in
96 well plates. The kinases were full-length cDNA clones containing native 5' and 3' untranslated region in pCMV vector (Origene #FKIB19604).

The total tau (TG5) and phosphospecific antibodies (MC6 (pS235), CP13 (pS202), CP17 (pT231), and PHF1 (pS396)) were obtained from Peter Davies, (Albert Einstein College of Medicine, USA). Antibodies to GSK3α, GSK3β and GAPDH were purchased from Abcam and Ambion, respectively.

shRNA lentiviral clones for GSK3α and GSK3β were purchased from Sigma. Five clones were tested for each target and the sequence that gave the highest knockdown with no off-target effects was used. The GSK3α lentiviral shRNA used in this study had the following sequence: GTGCTCCAGAACTCATCTTTG and the GSK3β lentiviral shRNA sequence was: CCGGATGAAAGTTAGCAGAGAT. Non-target (scrambled control) had the following sequence: CAACAAGATGAAGAGCACCAA.

**Cell culture**-SK-N-AS human neuroblastoma cells were maintained in Dulbecco’s Modified Eagle Medium with 1% non essential amino acids MEM, 1% PS (penicillin and streptomycin) and 10% foetal bovine serum.

Primary cultures were made from cortices of hTau foetal mice [embryonic day 18] (Taconic/Charles River Laboratories). These mice are C57/Blk6 mice expressing human MAPT (14) and were obtained from Peter Davies. Neurons were dissociated by incubating dissected cortices in trypsin-EDTA (Invitrogen) for 2 min at 37°C. Cells were resuspended in Neurobasal media supplemented with B27 supplement (Invitrogen) and glutamine, triturated and centrifuged at 200xg for 2 minutes. The resulting pellet was resuspended in neurobasal media and filtered through a 200µM mesh filter. Dissociated cortical neurons were cultured on poly-D-lysine coated plates at 1 x 10^6 cells/ml. Cells were maintained at 37°C in a humidified atmosphere of 5% CO2 for 6-7 days

**Reverse transfection**-SK-N-AS cells (3.5 x 10^5 cells/ml) were reverse co-transfected with human kinases (Origene) and 2N4R tau (1ng/well) using FuGene transfection reagent (Roche) at 6:1 ratio (FuGene:DNA). 48 hours post transfection, cells were lysed and AlphaScreen assays (Perkin Elmer) performed. GFP, cdk5/p25 and GSK3β cDNA were also co-transfected in each experiment to serve as controls.

**Cell lysis**-Cells were washed twice with ice-cold PBS followed by incubation in lysis buffer (Invitrogen, FNN0011 -10 mM Tris, pH 7.4,100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na_4P_2O_7, 2 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate with protease inhibitor tablet (Roche), 1mM PMSF (Sigma), benzonase nuclease (100 units/10ml buffer; Novagen and 1mM MgCl_2 (Sigma) added fresh) for 45 minutes on ice, with gentle agitation. Plates were sealed and stored at -80°C until further use.

**AlphaScreen assays**- The Alphascreen technology is a sandwich assay for detection of molecules of interest in serum, plasma, cell culture supernatants or cell lysates in a sensitive, quantitative, reproducible manner. In summary, a biotinylated anti-analyte antibody binds to streptavidin-Donor beads while another anti-analyte antibody is conjugated to Acceptor beads. In the presence of the analyte, the beads come into close proximity. The excitation of the Donor beads provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer in the Acceptor beads, resulting in light emission.

AlphaScreen assays (Perkin Elmer) were performed according to the manufacturer’s guidelines using tau specific antibodies. Optimised AlphaScreen assays were performed using biotinylated DA9 (bDA9) and acceptor (Ab-ACC) bead tau antibodies (TG5, total tau; PHF1, pS396/404; CP17, pT231; MC6, pS235 and CP13, pS202). 10µl / well of optimized antibody mix of Ab-ACC and bDA9 were added to 384 well assay plates (Greiner) together with 5µl/well of sample or standard diluted in Alphascreen assay buffer (0.1% casein in DPBS). Plates were incubated overnight in the dark at 4°C. After overnight incubation, 5µl/well of the streptavidin-coated donor beads diluted in Alphascreen buffer was added to each well and plates incubated in the dark with gentle agitation at room temperature for 4 hours. Plates were read at excitation 680nm and emission 520-620nm using an Envision plate reader (Perkin Elmer).

The corresponding standard curve for each phospho-epitope was used to convert the raw
AlphaScreen signal into % tau levels. Fold change relative to GFP was calculated by dividing the % tau level by the % tau level of the GFP control. Transient transfection of tau would probably result in variable expression levels. Thus the actual amount of total tau present was accounted for by dividing each fold change value by the total tau fold change for that well.

**Statistical analysis**-Each kinase was transfected in triplicate and then assayed on 3 separate days for each epitope. The fold change relative to total tau in each sample was calculated and ranked. Analyses were based on the assumption that most clones are inactive and should give fold-changes similar to GFP (negative control) of 1. To determine statistically significant hits from the first round of screening, the log of the fold change relative to total tau was determined for each run. Then for each run, the ‘mid inactives’ were selected and the mean of these were corrected to 1 and this correction was applied to all the kinases. The mean and standard deviation of the corrected log fold change values were calculated across the 3 runs. Actives were identified if the corrected fold change in phospho-tau epitope was >2 - 3 X the standard deviation (SD) above the corrected baseline, in two or more runs.

Statistically significant kinases were re-screened together with known literature tau kinases that were not present in the original set (46 kinases were re-screened). Following the second screen, statistically significant kinases were determined using one-way ANOVA test with Dunnet’s multiple comparison test on log fold change values relative to the negative control, GFP. This analysis was also applied to other results presented.

**qPCR for selection of shRNA lentiviral clone**-hTau mouse primary neuronal cultures (DIV6 or 7) were infected with lentivirus containing the appropriate shRNA clones (as described above) at MOI1 for 4 days. 4 days post-infection, RNA was isolated and reverse transcribed. The resulting cDNA was used for qPCR using SYBR Green master mix (Qiagen) and an ABI Prism PCR machine. The primers used were: GSK3a Forward 5’ AGT CCT GGT GAA CTG TCC; GSK3a Reverse 5’ GCT TGT GAG GAT GGG TTG T; GSK3b Forward 5’ GTC CGA GGA GAG CCC AAT G; GSK3b Reverse 5’ ACA ATT CAG CCA ACA CAC AGC. The relative levels of mRNA were normalised to housekeeping genes (β-actin). Expression levels were normalised to housekeeping genes and expressed as a percentage relative to the untreated cells (n=6 ±SEM).

**Knockdown of GSK3α and GSK3β in hTau primary cortical neurones**-hTau mouse primary neuronal cultures (DIV6 or 7) were infected with lentivirus containing the optimised shRNA clones (GSK3α: GTGCTCCAGAACCTCATCTTTG and GSK3β: CCGGCATGAAAAGTTAGCAGAGAT) at the indicated MOIs for 4 days. 4 days post infection, cells were lysed as described above. Levels of GSK3α and GSK3β were determined by qPCR (mRNA) and western blot (protein) and effect of knockdown of these kinases on total and phosphorylated tau levels were determined by AlphaScreen assays.

**Inhibition of GSK in hTau primary cortical neurones**-hTau mouse primary neuronal cultures (DIV6 or 7) were incubated with a selective ATP competitive GSK3 inhibitor, CT20026 (CHIRON patent, 15) at the indicated concentration for the indicated times. After incubation, cells were washed and lysed as above. Levels of total and phosphorylated tau were measured by Alphascreen assays.

**Pathway Analysis**-The kinases identified as having a significant effect on total tau levels or on tau phosphorylation in the second screen were subjected to pathway analysis using the MetaCore™ pathway analysis software (GeneGo, www.genego.com). Lists of kinases were compiled according to whether they were shown to significantly increase or decrease total tau levels or tau phosphorylation. The lists were uploaded to MetaCore™ as experimental datasets and used to identify GeneGO Pathway Maps significantly enriched for these kinases. The specific interactions between each of the kinases and the tau protein were explored using the ‘Build Network’ option to find the shortest path between objects using Dijkstra’s shortest paths algorithm (16). The ‘Trace pathways’ option was used to investigate the connections between the kinases and the other molecules on which the kinase pathways may converge.

**Domain Analysis**-Structural and functional domains were identified in the kinases that were shown to significantly increase or decrease total tau or tau phosphorylation using the Pfam protein families database (17).
RESULTS

We developed a high-throughput cell-based assay to identify kinases that are involved in phosphorylation of tau at AD relevant epitopes using phospho-epitope specific AlphaScreen assays. To assess the sensitivity and robustness of the assay before screening 352 kinases, we established the assay with two known tau kinases, CDK5 (13) and GSK3β (18) as well as GFP, the negative control. SK-N-AS human neuroblastoma cells were reverse co-transfected with known kinase or GFP and 2N4R tau in 96 well plates. 48 hours post transfection, cells were lysed and assayed for total tau and phosphorylated tau using epitope specific AlphaScreen assays. Transient transfection of tau would probably result in variable expression levels. Thus the amount of total tau present was corrected for by dividing each fold change value by the total tau fold change for that well. The effect of GFP, CDK5/p25 and GSK3β on the phosphorylation of pS202, pS235, pS396, pT231 and total tau levels is shown in figure 1A. GSK3β and CDK5/p25 both increased the phosphorylation levels of the tau epitopes tested relative to GFP control. CDK5/p25 was found to increase the phosphorylation levels of pT231, pS396 and pS202 to a much greater extent than GSK3β. Both kinases phosphorylate pS396 to a similar degree. Overexpression of CDK5 in SK-N-AS alone without the co-transfection of p25 did not increase tau phosphorylation at CDK5 sites, as shown for pS235 or pS396 (Figure 1B).

Screen of human kinome—Having established the conditions for the known tau kinases, we then performed a screen of 352 human kinases available from Origene, using the known kinases and the GFP as positive and negative controls, respectively. The 352 kinases were distributed in four 96 well plates, each plate containing 88 kinases. Each kinase was reverse co-transfected with human 2N4R tau into SK-N-AS neuroblastoma cells in triplicate. 48 hours post transfection, cells were lysed and AlphaScreen assays performed on each replicate on three separate days for each of the epitopes. CDK5/p25 and GSK3β were used as the positive controls and GFP as the negative control and screening plates were only accepted if the controls gave the expected response as shown in figure 1A. The repeats were averaged and statistical analysis performed to determine significant hits (Table 1) as described in the methods section. 41 kinases were identified following the first round of screening, 4 of which could phosphorylate all 4 phospho-epitopes tested (Table 2). The percentage of kinases able to phosphorylate each epitope in the initial screen were pS202 = 3.4%, pT231 = 2.8%, pS235 = 4.5%, and pS396 = 8.2%. Several known tau kinases such as GSK3β, CDK2, MAPK1, casein kinase 2, PKC and MARK as well as a number of novel kinases such as ERN1, ADRBK2, PLK3 and ADCK5 were identified in the first round of the screen.

We excluded false-positive hits by repeating the screen with the ‘hits’ identified from the first screen. We also included kinases from the literature that are reported to be tau kinases but were not in the original panel of kinases screened. 46 kinases (41 kinases from first round, Table 1, together with NIM1, SIK2, MAPK11, TTBK1, and TTBK2) were screened in the second round to confirm the effects of their overexpression on total and phospho-tau epitope levels and to rank the kinases in order of their effectiveness on tau phosphorylation. Again, CDK5/p25 and GSK3β and GFP were included as positive and negative controls respectively. Screens were accepted if the controls showed the expected pattern of phosphorylation as shown in figure 1. The effect and rank order of each of the kinases on each of the phospho-epitopes studied is shown in figure 2. Interestingly, several kinases were found to alter total tau levels (Table 3). Six kinases significantly decreased total tau levels (EIF2AK1, EIF2AK2, EIF2AK3, MARK2 MLKL and PLK3) and three kinases significantly increased total tau level (ACVR1, ADCK1 and MAPK1). Nine kinases (MARK2, EIF2AK2, GSK3α, GSK3β, EIF2AK3, MLKL, MAPK13, EIF2AK1 and PLK3) significantly increased pS396/404 levels (Figure 2A). However, six of these (MARK2, EIF2AK2, EIF2AK3, MLKL, EIF2AK1 and PLK3) significantly reduced total tau levels and therefore may not be true ‘hits’. In addition, overexpression of TTBK2, BCKDK, ADCK1, ACVR1 and MAPK1 all significantly reduced phosphorylation of tau at pS396. Three kinases (GSK3α, GSK3β, and MAPK13) significantly increased pS235 levels whilst EIF2AK1 and EIF2AK3 decreased phosphorylation at this epitope (Figure 2B). Eleven kinases (GSK3α, GSK3β, MAPK14, MAPK13, MAP2K3, PCTK2, TTBK1, CDK2, ADCK1, MAPK11, and ADRBK1) significantly increased pT231 levels and EIF2AK1 significantly reduced tau phosphorylation at this epitope (Figure 2C). Finally, nine kinases (GSK3α, EIF2AK2, GSK3β, MARK2, MAPK13, TTBK1, MAP2K3, MLKL,
and PLK3) significantly increased pS202 levels however, two of these (EIF2AK2 and MLKL) significantly reduced total tau levels and therefore may not be true 'hits' (Figure 2D). DDR2, BRSK2, BCKDK, ACVR1, PRKCD and ADCK1 significantly reduced phosphorylation at this epitope.

Table 3 summarises the outcome of the second screen with respect to which kinases increase or decrease total or phosphorylated tau at each epitope. 12 out of the 24 kinases identified as hits in the second screen are known tau kinases (Figure 3; 5,6). GSK3α, GSK3β and MAPK13 were identified as key kinases as they phosphorylated all 4 epitopes in both screens. The remaining 12 kinases were identified as novel kinases that modulated either tau phosphorylation or tau expression levels (Figure 3).

Of the various kinases implicated in tau phosphorylation associated with AD, considerable data support GSK3β as being a well validated target (reviewed in 2). Our data suggest that both isoforms of GSK (α and β) are able to phosphorylate tau at AD relevant epitopes (Figure 2). Indeed, GSK3α exhibited the greater magnitude of phosphorylation at all these sites.

**Effect of GSK3 inhibition or knockdown on tau phosphorylation:** In order to further dissect the role of GSK3 α and β on tau phosphorylation, inhibitors to GSK3 or shRNA to GSK3 α and β were evaluated in hTau primary cortical culture. shRNA lentivirus (as shown in Methods) to GSK3 α and β were optimised by qPCR (data not shown). The optimised shRNA lentiviruses were GSK3α: GTGCTCCAGAACTCATCTTTT and GSK3β: CCGGCATGAAAGTTAGCAGAGAT, and were chosen based on their ability to selectively knockdown the target genes (expression of cdk5 and Erk1/2 or housekeeping gene, actin, were not affected; data not shown). These were used to knockdown the expression of GSK3 α and/or β in hTau primary cortical neurones at the indicated MOIs for 4 days. The levels of GSK3 α and/or β were evaluated by qPCR (data not shown) and western blotting (Figure 4). The empty vector control at MOI 10 depressed levels of all proteins (target and non-target), suggesting that this is non-specific. In contrast, the non-target control at MOI 10 did not affect protein levels and therefore effects observed with target shRNA at MOI 10 were perceived to be specific. Dose dependent decrease in GSK3 α or β were obtained when the target-specific shRNA lentivirus clone was used (Figure 4B). GSK3α shRNA lentivirus at the highest titre tested, MOI of 10 achieved ~55% knockdown of GSK3α protein level and GSK3β shRNA lentivirus at MOI of 2 achieved ~70% knockdown of GSK3β protein level. Increasing the virus titre resulted in non-specific knockdown of non-target proteins (data not shown). Knockdown of both GSK3 α and β together showed similar knockdown pattern to that observed with GSK3β knockdown alone. The effect of GSK3 α and/or β knockdown on total and phosphorylated tau levels in hTau primary cortical neurones were evaluated by AlphaScreen assays (Figure 5). Knockdown of either GSK3α or GSK3β expression levels of 55% and 70% respectively had no effect on total tau levels. The highest reduction was observed in levels of pT231 and pS235. A dose dependent reduction in pT231 and pS235 was achieved by knockdown of either GSK3α or GSK3β and the double knockdown predominantly resembled the effect of GSK3β knockdown alone. At the highest MOI, GSK3α knockdown resulted in 40% reduction in pT231 and 60% reduction in pS235 levels; the latter being significant. GSK3β knockdown resulted in significant reduction in pT231 (65%) and reduction in pS235 (80%) levels. GSK3α knockdown had modest effects on pS202 and pS396/404 levels (~20% reduction at highest MOI). Knockdown of GSK3β (highest MOI) resulted in 40% reduction in pS202 and 45% reduction in pS396/404 levels. The literature would suggest pS396/404 to be a key epitope for GSK3 phosphorylation of tau (19). These genetic knockdown data seem to suggest tau phosphorylation at pT231 and pS235 are greatly reduced by knockdown of GSK3 protein.

To explore the above observations further, we evaluated the effect of a selective GSK3 inhibitor, CT20026 (Figure 6A), on total and phosphorylated tau levels in hTau primary cortical neurones. The potency of CT20026 was tested in GSKS enzyme activity assays and the compound exhibited IC50 = 11.9nM for GSK3β and IC50 of 5.53nM for GSK3α (data not shown). The compound was tested in a panel of 100 kinases (Invitrogen), all with IC50 > 1μM (data not shown). Primary cortical neurones prepared from hTau mice were treated with CT20026 at the indicated concentrations for 2 hours and cells were harvested and total and phosphorylated tau levels measured by AlphaScreen assays (Figure 6B). Acute inhibition of GSK3 activity did not alter total tau levels but resulted in
dose dependent decrease in levels of pS396/404 > pS202 > pT231 = pS235. Thus, selective inhibition of GSK3 activity resulted in greatest reduction (~85% at 5 and 10µM) of pS396/404 levels, an epitope that is known to be affected by GSK3 activity. This discrepancy between knockdown of GSK3 protein levels and inhibition of GSK3 activity could be due to incomplete knockdown of protein expression levels or due to the differences in treatment paradigms. The knockdown experiments required a sub-chronic (4-day) treatment of cells with shRNA whilst the inhibitor was tested acutely for 2 hours. To test this further, we evaluated the effect of increased duration of GSK3 inhibition on tau phosphorylation. Primary cortical neurones prepared from hTau mice were treated with 1µM CT20026 for 2, 24, 48 and 96 hours. For the longer time points, the inhibitor was added to fresh media, daily. Cells were harvested at the appropriate time and analysed for total and phosphorylated tau levels by AlphaScreen assays (Figure 7). Inhibition of GSK3 activity with CT20026 did not alter total tau levels. We observed a time dependent decrease in the levels of pT231 and pS235 with increased duration of GSK3 activity inhibition with CT20026. pT231 levels were decreased by ~75% at 2 hours and greater than 90% after 96 hours whilst pS235 levels were decreased by ~50% at 2 hours and greater than 90% after 96 hours. In contrast, the levels of pS396/404 and pS202 were decreased ~50-60% and remained constant despite enhanced duration of inhibition of GSK3 activity. The 96 hour inhibition of GSK3 activity data suggest that pT231 and pS235 are greatly reduced by prolonged GSK3 inhibition and resemble the data obtained with knockdown of GSK3 expression levels.

Bioinformatic analysis of screen data—Pathway analysis using the GeneGo MetaCore™ pathway analysis software was performed using the kinase hits from the second screen as input. Kinases were separated into four categories 1) kinases that increase total tau, 2) kinases that decrease total tau, 3) kinases that increase phospho-tau (with no effect on total tau), 4) kinases that decrease phospho-tau (with no effect on total tau). GeneGo Pathway Maps significantly enriched for each category were identified. Nearly all of the pathways identified were focused on a single kinase or kinase group (e.g. p38) and did not reveal any particular association between the putative tau kinases in a single pathway. The exception to this is shown in Figure 8. Several of the kinases identified from the screen are involved in the Regulation of EIF2 Activity GeneGo Pathway Map. In particular EIF2AK1, EIF2AK2 (PKR) and EIF2AK3 all significantly decreased the total tau levels in the screen and MAPK1 significantly increased the total tau in the screen. The pathway in Figure 8 shows that EIF2AK1, 2 and 3 phosphorylate eIF2 subunit 1 and inhibit initiation of translation. Conversely MAPK1 is responsible for phosphorylating the protein phosphatase 1 (PP1) catalytic subunit which allows PP1 to dephosphorylate and reactivate eIF2S1 and initiation of translation. These observations lead to the hypothesis that the changes in total tau levels observed in the screen are, at least in part, due to inhibition or activation of initiation of translation. A number of the kinases identified to significantly increase or decrease phospho-tau have been previously annotated in the literature to phosphorylate tau directly. MetaCore™ was used to explore how some of the kinases identified may indirectly alter the phosphorylation state of tau. The interactions shown in figure 9 were identified using the build network, shortest paths option in the software. EIF2AK2 (PKR) for example was shown to increase phosphorylation of tau at epitopes pS396/pS404 and pS202, but also to significantly decrease total tau levels. It was therefore postulated that this kinase may be a false positive. The interactions presented in figure 9 show that EIF2AK2 activates AKT (PKB) and p38 MAPK which would in turn result in increased phosphorylation of tau, indicating that this may be a true hit after all. A similar situation is seen for PLK3, shown in figure 9 to activate Chk2, which in turn phosphorylates tau. Interestingly, there is also a documented interaction of PLK3 with the AP-1 transcription factor which could be responsible for the observed decrease in total tau. Both CDK2 and ADRBK1 inhibit kinases (Chk1 and PKC respectively) which phosphorylate tau. Although these kinases are seen to increase phosphorylation of tau at pT231 it may be that they decrease phosphorylation at other epitopes.

Figure 10 shows the network generated using the build network, trace pathways option in the MetaCore™ software. The network building algorithm was set to search for pathways that go ‘from’ or ‘through’ any of the kinases identified in the screen as having an effect on phospho-tau levels and ‘to’ the tau protein. The number of steps was set to 2, such that the molecules identified were within two interaction steps of at least one of the
kinases or the tau protein. This ‘network fishing’ approach is a method for identification of molecules, up and downstream of the experimentally determined kinases, that are putatively involved in the tau phosphorylation pathways. The table below the network diagram in figure 10 shows the list of molecules that are making >4 interactions with other molecules in the network. While it is expected that the input kinases and tau form the most significant hubs in the network it is interesting to see which of the kinases are forming the most interactions and which other molecules are highly connected in the network.

The domain composition of each of the kinases identified by the screen as having an effect on phospho-tau levels was identified from the Pfam database of protein families (data not shown). The known tau kinases GSK3β, GSK3α, MAPK1, 11, 13, 14, MAPK2K3 and CDK2 consist of a short sequence and a single kinase domain. Many of the novel tau kinases identified by this screen have more complex domain structures, consisting of longer sequences and other domains as well as the kinase domain. The kinases which have a more complex domain structure include a variety of domains such as regulator of G-protein signaling (RGS), pleckstrin homology (PH), ATPase (HATPase_c), C1 and discoidin domains.

DISCUSSION

Attenuation of tau hyperphosphorylation through inhibition of key tau kinases is an attractive therapeutic approach for the treatment of AD and other tauopathies. Identification of key and novel tau kinases would therefore be of benefit. Here, we report on an unbiased approach to study the effect of 352 human kinases on their ability to phosphorylate tau at epitopes associated with AD in an attempt to identify novel kinases, and generate hypothesis regarding both direct and indirect phosphorylation of tau. We developed a high-throughput reverse co-transfection cell based assay to identify kinases that are involved in phosphorylation of tau at AD relevant epitopes using phospho-epitope specific AlphaScreen assays. Prior to initiating the screen the robustness of the protocol was examined using two well characterized tau kinases CDK5 (13) and GSK3β (18) as well as GFP, the negative control. Several cell lines were examined including CHO cells stably overexpressing 2N4R tau (data not shown) and SK- N-AS. SK-N-AS human neuroblastoma cells were chosen for the screen as the effects of selective inhibitors and over-expression of kinases mirrored each other on the epitopes studied (data not shown) thus suggesting SK-N-AS cells represent a robust and reproducible host cell line for the screen. In selecting a single cell line to perform the screen, false negatives were expected due to specific activators not being present in the SK-N-AS cell line e.g. CDK5 did not appear as a hit unless co-expressed with p25 (Figure 1B). There is also the possibility of detecting false positives or false negatives due to differential expression levels of each kinase; expression levels were not confirmed by RT-PCR or western blotting. Conversely, kinases previously not identified as tau kinases in vitro, may be identified as modulators of tau phosphorylation via activation of pathways upon expression of a certain kinase e.g. pathway analysis suggests PLK3, shown in figure 9 to activate Chk2, which in turn phosphorylates tau. In addition, since 2N4R tau was overexpressed in the assay to increase the signal window, there is the possibility that increased cytoplasmic pool of tau unbound to microtubules may result in activation of pathways that do not normally regulate tau. However, this may also mimic pathways that are activated during the disease process where cytosolic pool of hyperphosphorylated tau has been observed in AD brain to sequester normal tau and disrupt the microtubule network (20). Our screen has identified 12 known and 12 novel tau kinases; the true function of these kinases in AD and other tauopathies will have to be further evaluated in neuronal cell lines and animal models of AD and other tauopathies using genetic and pharmacological tools for each kinase. In addition, the expression levels and activation state of these kinases in AD tissue relative to control patients, in particular the novel kinases, will have to be further evaluated.

A number of candidate kinases have been proposed to phosphorylate tau, however it is still unclear which kinases are key for disease progression. Our screen has identified three well characterized known tau kinases, GSK3α, GSK3β and MAPK13 (SAPK4) to phosphorylate all four AD relevant tau epitopes (Table 3) and indeed these kinases were also shown to have multiple interactions in the network fishing pathway analysis (Figure 10). GSK3 is a proline directed serine threonine kinase that acts as a multifunctional downstream switch that determines the output of
numerous signaling pathways. Two mammalian GSK3 isoforms encoded by distinct genes, GSK3α and GSK3β, are structurally similar, and have common and non-overlapping cellular functions. There is considerable literature evidence that support GSK3β as being a well validated tau kinase target. Dysregulated GSK3β has been implicated in the pathogenesis of Alzheimer’s disease (AD), and reducing its activity may have therapeutic efficacy (2). Our screen data suggested that not only is GSK3β a good candidate target, but that GSK3α is also a strong candidate tau kinase as GSK3α exhibited the greater magnitude of phosphorylation at all the AD associated epitopes tested. Moreover, recent data exploring the specific contributions of each of the GSK3 α and β isoforms in AD disease progression has been described using selective viral and gene silencing techniques in transgenic mouse models of AD (21). Their data indicate that GSK3α contributes to both senile plaque and NFT pathogenesis whilst GSK3β only affected NFT formation, thus supporting the importance of GSK3α as a therapeutic target for AD.

We therefore further dissected the roles of the 2 isoforms of GSK3 on tau phosphorylation in primary neuronal cultures prepared from hTau mice. In knockdown studies the data were limited by the variable knockdown efficiencies of GSK3α and GSK3β. We were able to achieve ~55% and 70% knockdown of protein levels of GSK3α and GSK3β, respectively, therefore the relative contribution of GSK3α could not be validated. Under these conditions, tau phosphorylation was decreased with a rank order of pS235>pT231>pS396/404>pS202. Knockdown of GSK3α only reduced phosphorylation at pS396/404 by ~20% whilst GSK3β had a more robust effect, suggesting that phosphorylation of tau at these sites is predominantly mediated by GSK3β. Knockdown of GSK3β expression had a greater effect than GSK3α knockdown on tau phosphorylation levels, but this may have been due to the greater reduction in GSK3β levels achieved in the knockdown studies. Interestingly, the knockdown experiments suggested that pT231 and pS235 are the epitopes that are largely reduced after reduction of GSK3 expression levels. These epitopes are normally associated with CDK5 phosphorylation of Tau (22) but cdk5, p35 and p25 protein expression levels were not affected under these knockdown conditions (data not shown). We therefore compared these knockdown data to pharmacological inhibition of GSK3 activity using a selective GSK3 inhibitor, CT20026. We observed differences in inhibition of tau phosphorylation epitopes upon acute (2 hours) inhibition versus longer inhibition (24, 48 and 96 hours) of GSK3 activity. Acute inhibition of GSK3 activity did not alter total tau levels but resulted in dose dependent decrease in levels of pS396/404 > pS202 > pT231 = pS235. However, prolonged inhibition of GSK3 activity resulted in decreased levels of pS235 and pT231 (~90%) whilst levels of pS396/404 and pS202 remained constant at ~40-45% reduction. These data are consistent with the data obtained with knockdown of GSK3 expression levels where reduction of pT231 and pS235 were the key epitopes affected. This suggests that prolonged inhibition of GSK3 activity results in modulation of other signalling pathways that may be involved in tau phosphorylation. Indeed, the network shown in Figure 10 suggests that GSK3α/β interacts with 19 other proteins associated with tau phosphorylation. Taken together, these data have important implications when considering the clinical utility of GSK3 inhibitors for the treatment of disease. In a cellular context, prolonged kinase inhibition may result in modulation of multiple signaling pathways that could represent new points of therapeutic intervention in tau phosphorylation pathways.

Our screen also identified MAPK13 (SAPK4/p38δ) to be key in phosphorylating the four AD relevant tau epitopes. Indeed, five of the 24 hit kinases were from the MAPK pathways and a network fishing approach with the kinase hits identified other key molecules putatively involved in tau phosphorylation pathways including the G-protein signaling through the Ras family of GTPases (MAPK family) pathway. In addition, 4 of the 5 MAPK phosphorylated tau at pT231, an epitope that is associated with early AD (9). There is also literature evidence that links the MAPK pathway, including the stress activated protein kinases (SAP), to tau phosphorylation and neurodegenerative diseases (23; 24, for review see 25). In human disease including AD, tau inclusions colocalize with activated MAPK family members, in particular SAPKs (26; 27). Similarly, activated SAPKs, JNK and p38 were found to colocalize with hyperphosphorylated tau in the human P301S tau transgenic model (28). These data suggest that targeting the MAPK pathway may be of therapeutic benefit for the treatment of AD and other tauopathies. In particular, the p38 pathway is
attractive as it has been linked to inflammatory diseases and this pathway plays a key role in the activation and production of key proinflammatory cytokines. Moreover, neuroinflammation is increasingly linked to pathogenesis of Alzheimer’s disease (29, 30) thus inhibition of the p38 pathway may slow AD progression through anti-inflammatory mechanisms.

Several of the kinases identified from the screen are involved in the activation or inhibition of initiation of translation (Figure 8). In particular, EIF2AK1, EIF2AK2 (PKR) and EIF2AK3 all significantly decreased the total tau levels in the screen (Table 3). Interestingly, Azorsa et al., (31) recently performed a high-content siRNA screen of the kinome looking at the phospho-epitope pS262/pS356 (12E8 epitope) on tau. They also identified EIF2AK2 as potentially affecting tau expression levels. EIF2AK1, EIF2AK2 (PKR) and EIF2AK3 indentified here, are activated by different stressors and are all involved in phosphorylation and inhibition of the translation initiation factor, eukaryotic translation initiation factor 2 subunit 1 (eIF2S1). Phosphorylation of eIF2S1 results in shut down of protein synthesis. In addition, polymorphisms within EIF2AK2 have been genetically associated with AD (32) and EIF2AK2 has been shown to be activated in AD brain (33). Reductions in tau levels have also been shown to prevent Aβ from causing neuronal deficits in cell culture and hAPP transgenic mice (34). The authors also suggest that in mice, 50% reduction of endogenous tau is well tolerated, increases resistance to chemically induced seizures, and markedly reduces Aβ and ApoE4 fragment-induced neuronal and cognitive impairments in vivo (34). These data suggest that reducing overall tau levels may be of therapeutic value and down-regulation of the EIF2 pathway may be one approach to reducing tau levels.

The variety of domain structures, in particular for the novel kinases identified in this study (data not shown), demonstrate the complex functionality of these kinases. It is possible that the cell-based approach to the screen has enabled the identification of these kinases because they are not acting in isolation, but in the context of the cell, require the other factors available to function. It is also noteworthy that EIF2AK3, BRSK2, ACVR1 and DDR2 are transmembrane proteins and ADRBK1 (GRK2) and PKC (PRCKD in this case) are membrane associated proteins. There is some evidence that the phosphorylation state of tau directly impacts its localization and that tau is trafficked between the cytosol and the neuronal membrane depending on the phosphorylation state (35). BCKDK is localized to the mitochondria and although very little is known about ADCK1, the ABC1 protein in yeast, from which the ABC1 domain was identified, is imported into the mitochondria. It has been suggested that hyperphosphorylation of tau may contribute to axonal degeneration by disrupting mitochondrial transport in AD (36).

The network shown in figure 10 identifies many molecules, other than kinases, that are likely to be affected or involved in the pathways around tau phosphorylation. For example, it has been demonstrated that tau can bind to the N-terminal SH3 domain of Grb2. Hyperphosphorylated tau from AD brain did not bind SH3 domain proteins but the binding was restored by phosphatase treatment (37). It may be that signaling through these molecules is disrupted in AD by hyperphosphorylation. Histone acetyltransferase p300 also makes 5 interactions in the network. Min et al. (38), recently showed p300 is involved in tau acetylation and that inhibiting p300 promoted deacetylation of tau and eliminated tau phosphorylation associated with tauopathy in primary neurons. Calpain 2 is also identified by the network fishing approach, as having a role to play in tau phosphorylation pathways. It has recently been shown that genetic deficiency of calpastatin (CS), a calpain-specific inhibitor protein, augments tau phosphorylation and the Aβ-triggered pathological cascade, and increased mortality in APP-Tg mice (39). These molecules and some of the others identified by the protein interaction network approach may represent new points of therapeutic intervention in tau phosphorylation pathways.

A therapeutic strategy for AD and other tauopathies based on inhibition of tau phosphorylation is appealing. However, kinases are a difficult class of drug targets as they modulate multiple biological pathways and therefore designing selective molecules that avoid on-target toxicity is a challenge. An alternative approach would be to investigate non-selective kinase inhibitors as reducing the overall level of tau phosphorylation may be beneficial. In this respect, a moderate reduction in multiple kinase activities
might lead to a reduction in the on- and off-target side effects that are observed when a specific kinase is completely suppressed. Indeed, Le Corre et al., (40) used an analogue of K252a (SRN-003-556), a molecule that inhibits Gsk3, Erk2, cdk1, PKA and protein kinase C with equal efficacies, on JNPL3 tau transgenic mice and showed decreased hyperphosphorylated tau and improved motor impairments.

The data from this screen together with the pathway analysis have identified novel tau kinases and pathways which may be involved in generating phosphorylation of tau associated with pathological forms of the protein observed in AD and other tauopathies. The exact role of these kinases on tau phosphorylation will need to be evaluated in further studies.
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Figure Legends

Figure 1: Effect of known tau kinases on tau phosphorylation

A. The effect of CDK5/p25, GSK3β and GFP on the phosphorylation of pS202, pS235, pS396, pT231 and total tau levels was measured using phospho-epitope specific AlphaScreen assays. B. The effect of CDK5, CDK5/p25, GSK3β and GFP on the phosphorylation of pS235, pS396 is shown. The phospho tau levels were normalised to total tau levels. Data shown are average of 3 or 4 separate experiments, n=3 per experiment. Statistical analysis: One-way ANOVA test with Dunnets multiple comparison test on log fold change values (*, **, *** = p<0.05, 0.01, 0.001 vs GFP).

Figure 2 Summary of screening data at each epitope (A) pS396, (B) pS235, (C) pT231 and (D) pS202

41 kinases from first round, shown in Table 1, together with NIM1, SIK2, MAPK11, TTBK1, and TTBK2 were re-screened. The effect of these kinases and GFP on the phosphorylation of pS202, pS235, pS396, pT231 levels was measured using phospho-epitope specific AlphaScreen assays. The phospho tau levels were normalised to total tau levels. Average of three separate transfections shown. Statistical analysis: One-way ANOVA test with Dunnets multiple comparison test on log fold change values (*, **, *** = p<0.05, 0.01, 0.001 vs GFP). Red arrows represent kinases that significantly reduced total tau levels.

Figure 3 Hit kinases separated into kinase families.

The diagram shows an adaptation of the phylogenetic tree where each branch represents a kinase in the human kinome as described in Manning et al. (41, Illustration reproduced courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com)). The labeled kinases are those that were associated with changes in phosphorylated tau or total tau in the kinome screen. Red text = Known tau kinases, Black text = Novel tau kinases.

Figure 4 GSK3 α and β expression levels in hTau primary corticals following knockdown with shRNA.

Representative western blots (A) showing levels of GSK3 α and β, in duplicates, following knockdown of GSK3 α and/or β with shRNA lentivirus at various MOIs. Quantitative analysis of GSK3 α and β levels (B) of 3 independent knockdown experiments at the indicated MOI's, n=3 per experiment. (EVC: empty vector control, NTC: non-targeting shRNA control, UNTR: uninfected cells). Statistical analysis: One-way ANOVA test with Dunnets multiple comparison test on relative expression (*, **, *** = p<0.05, 0.01, 0.001).

Figure 5 Effect of GSK3 α and/or β knockdown on total and phosphorylated tau levels in hTau primary cortical neurones.

AlphaScreen assays were performed to quantitate the levels of total and phosphorylated tau (pT231, pS202, pS235 and pS396/404) in hTau primary cortical neurones following knockdown of GSK3 α and/or β with shRNA lentivirus at various MOIs; average of 2 separate experiments shown, n=3 per experiment. Reduction of GSK3 α and /or β levels had the greatest effect on pT231 and pS235 levels. Statistical analysis: One-way ANOVA test with Dunnets multiple comparison test on phosphorylation levels (*, **, *** = p<0.05, 0.01, 0.001)

Figure 6 Effect of selective GSK3 inhibitor on tau phosphorylation levels in hTau primary cortical neurones.

Structure of selective GSK3 inhibitor (A), Chiron CT20026. (B) Acute inhibition of GSK3 activity (2 hours) in hTau primary cortical neurones resulted in dose dependent decrease in pS396>pS202>pT231>pS235 (representative data shown from 2 separate experiments, n=3 per experiment). Statistical analysis: One-way ANOVA test with Dunnets multiple comparison test on phosphorylation levels (*, **, *** = p<0.05, 0.01, 0.001).
Figure 7 Comparison of short-term and long-term inhibition of GSK3 activity on tau phosphorylation levels.

hTau primary cortical neurones were treated with 1µM CT20026 for the indicated times. Total and phosphorylated tau levels were analysed by AlphaScreen assays. Long-term inhibition of GSK3 activity enhanced inhibition of pT231 and pS235 to greater than 90% while pS396/404 and pS202 inhibition remained constant at ~ 50-60% (representative data shown from 2 separate experiments; n=3 per experiment). Statistical analysis: One-way ANOVA test with Dunnets multiple comparison test on phosphorylation levels (*,**,***= p<0.05, 0.01, 0.001).

Figure 8 GeneGo Pathway Map Translation Regulation of EIF2 Activity

Part of the GeneGo Pathway Map for Translation Regulation of EIF2 Activity is represented in the diagram. Molecules are represented by node icons. Different shaped node icons represent different families of proteins. Edges between nodes represent an inhibitory interaction (red), and activating interaction (green) or an unknown interaction (grey). The flow of the pathway is represented by the directionality of the arrows. The shapes in the centre of the arrows describe the nature of the interaction (e.g. phosphorylation, binding etc.). This pathway was significantly enriched for the kinases identified to significantly decrease (-) total tau (pValue = 1.494e-6). The table below the pathway diagram describes whether total or phospho-tau are increased (+) or decreased (-) in the kinome screen. EIF2AK1, EIF2AK3 and EIF2AK2 (PKR) are all involved in phosphorylation of, and inhibition of eukaryotic translation initiation factor 2 subunit 1 (eIF2S1). MAPK1, as part of the ERK complex is involved in regulating the dephosphorylation of eIF2S1 via protein phosphatase 1 (PP1). GSK3 alpha and beta are involved in phosphorylation of, and inhibition of eIF2B5.

Figure 9 Evidence for Indirect Influence of Kinases on Tau Phosphorylation

The diagrams shown represent known interactions, direct and indirect, between kinases (green circles) and tau (red circles). Molecules are represented by node icons. Different shaped node icons represent different families of proteins. Edges between nodes represent an inhibitory interaction (red), and activating interaction (green) or an unknown interaction (grey). The kinases shown (green circles) were identified as having a putative indirect effect on tau (red circles) phosphorylation by activation or inhibition of an intermediary protein. Interactions were identified using the GeneGo MetaCore™ pathway analysis software shortest paths algorithm.

Figure 10 Network Fishing for Molecules Involved Tau Phosphorylation Pathways

The network shown was generated using the GeneGo MetaCore™ pathway analysis software to trace the putative pathways ‘from’ and ‘through’ the kinases identified in the kinase screen and ‘to’ the tau protein. Red circles behind the icons show nodes used as input to the network building algorithm. For ease of representation transcription factors were removed from the network, the nodes were organised by protein families and the edges were greyed out. The table below the network shows some of the hubs in the network and the number of interactions being made by each molecule.
Table 1 Summary of statistically significant kinases identified at each epitope after the first screen.

Statistically significant kinase activity was identified if the log fold change in phospho-tau epitope was >2 (orange) or 3 (red) X the standard deviation (SD) above the baseline, in two or more runs. Kinases were considered ‘hits’ if they were red or orange in 2 or more runs for each epitope. White represents not statistically significant and yellow represents 1 x the standard deviation; these were not considered as hits. The percentage of kinases able to phosphorylate each epitope were: pS202 = 3.4%, pS235 = 4.5%, pT231 = 2.8%, pS396 = 8.2%
| Kinase  | Number of epitopes phosphorylated | Epitopes phosphorylated |
|---------|----------------------------------|--------------------------|
| MAP2K3  | 4                                | pS396;pT231;pS235;pS202  |
| GSK3α   | 4                                | pS396;pT231;pS235;pS202  |
| MAPK13  | 4                                | pS396;pT231;pS235;pS202  |
| GSK3β   | 4                                | pS396;pT231;pS235;pS202  |
| DDR2    | 3                                | pT231;pS235;pS202        |
| MAPK1   | 3                                | pT231;pS235;pS202        |
| MAPKAPK3| 3                                | pS396;pT231;pS235;pS202  |
| PRKCD   | 3                                | pS396;pT231;pS235        |
| FLT3    | 3                                | pS396;pT231;pS202        |
| CSNK2A1 | 2                                | pS235;pS202             |
| CDK2    | 2                                | pT231;pS235             |
| MAPK14  | 2                                | pT231;pS235             |
| MARK2   | 2                                | pS396;pS202             |

**Table 2 Kinases that phosphorylate more than one epitope**

Kinases that phosphorylated more than one epitope in the first round of the screen are shown.

| Kinase | Tau | pS396 | pS202 | pT231 | pS235 |
|--------|-----|-------|-------|-------|-------|
| EIF2AK1| -   | +     | -     | -     | -     |
| EIF2AK2| -   | +     | +     |       |       |
| EIF2AK3| -   | +     |       |       |       |
| MARK2  | -   | +     |       |       |       |
| MLKL   | -   | +     | +     |       |       |
| PLK3   | -   | +     | +     |       |       |
| ACVR1  | +   | -     | -     |       |       |
| ADCK1  | +   | -     | -     |       |       |
| MAPK1  | +   | -     |       |       |       |
| BCKDK  |     |       |       |       |       |
| TTBK2  |     |       |       |       |       |
| BRSK2  |     |       |       |       |       |
| DDR2   |     |       |       |       |       |
| PRKCD  |     |       |       |       |       |
| ADRBK1 |     |       |       |       |       |
| CDK2   |     |       |       |       |       |
| MAP2K3 |     |       |       |       |       |
| MAPK11 |     |       |       |       |       |
| MAPK14 |     |       |       |       |       |
| PCTK2  |     |       |       |       |       |
| TTBK1  |     |       |       |       |       |
| GSK3A  |     |       |       |       |       |
| GSK3B  |     |       |       |       |       |
| MAPK13 |     |       |       |       |       |
Table 3 Summary of statistically significant kinases identified that either increase (+) or decrease (-) total or phospho-tau levels.

41 kinases from first round, shown in Table 1, together with NIM1, SIK2, MAPK11, TTBK1, and TTBK2 were re-screened. The kinases validated in the second round of screening are shown.
Figure 1
Figure 2

Fold change relative to total tau

GSK3A
GSK3B
MAPK7
MAPK11
TTPK1
MAP2K3
MLKL
PLK3
HSPB8
PCTK2
MAPK14
EIF2AK3
NIM1
CSNK2A1 (T2)
CSNK2A1 (T1)
MAPK11
PKC2
SGK1
MAP4K1
ADRBK2
EPHB3
S6K1
ADRBK1
MAPK4
MAPK1
ADCK5
FLT1
CDK2 (T2)
CDK2 (T1)
FLT4
DYRK3
ERN1
CDK2
IGF1R
TTBK2
PDGFR
MAPK12
BMP2
CDK5
ADCK1
PRKCD
ACVR1
BCKD1
BSK2
DOR2
GFP
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9
| Network Object         | Interactions | Network Object         | Interactions | Network Object         | Interactions |
|------------------------|--------------|------------------------|--------------|------------------------|--------------|
| ERK1/2                 | 29           | FGF1                   | 5            | CDC25                  | 4            |
| PKC                    | 27           | GRB2                   | 5            | Calpain 2(m)           | 4            |
| CDK2                   | 24           | MAP2                   | 5            | DDR2                   | 4            |
| p38 MAPK               | 21           | MARK2                  | 5            | FAK1                   | 4            |
| GSK3 alpha/beta        | 19           | NAD(P)H + O2 Reaction  | 5            | H+ + O2 Reaction       | 4            |
| PKR (EIF2AK2)          | 12           | Shc                    | 5            | MEKK1 (MAP3K1)         | 4            |
| Tau (MAPT)             | 12           | c-Src                  | 5            | NCOA3 (pCIP/ SRC3)     | 4            |
| FRS2                   | 6            | p21                    | 5            | PLK3 (CNK)             | 4            |
| MEK3 (MAPK7)           | 6            | p300                   | 5            | PP1                    | 4            |
| Caspase-3              | 5            | GRK2 (ARDBK1)          | 5            | ZFP36 (Tristetraprolin)| 4            |

Figure 10
An unbiased approach to identifying tau kinases that phosphorylate tau at sites associated with Alzheimer's disease
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*J. Biol. Chem. published online June 24, 2013*

Access the most updated version of this article at doi: 10.1074/jbc.M113.463984

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