It is important to identify the true substrates of protein kinases, since this illuminates the primary function of any kinase. Here, we used bioinformatics and biochemical validation to identify novel brain substrates of the Ser/Thr kinase Glycogen Synthase Kinase 3 (GSK3). Briefly, sequence databases were searched for proteins containing a conserved GSK3 phosphorylation consensus sequence (S/TPXXS/TP or S/TPXXXS/TP), as well as other criteria of interest (e.g. brain proteins). Importantly, candidates were highlighted if they had previously been reported to be phosphorylated at these sites by large-scale phosphoproteomic studies. These criteria identified the brain-enriched cytoskeleton-associated protein β-adducin as a likely substrate of GSK3. To confirm this experimentally, it was cloned and subjected to a combination of cell culture and in vitro kinase assays that demonstrated direct phosphorylation by GSK3 in vitro and in cells. Phosphosites were mapped to 3 separate regions near the C-terminus and confirmed using phosphospecific antibodies. Prior priming phosphorylation by Cdk5 enhanced phosphorylation by GSK3. Expression of wild type, but not non-phosphorylatable (GSK3 insensitive) β-adducin increased axon and dendrite elongation in primary cortical neurons. Therefore, phosphorylation of β-adducin by GSK3 promotes efficient neurite outgrowth in neurons.

Glycogen synthase kinase 3 (GSK3) is a Ser/Thr protein kinase that is ubiquitously expressed in all mammalian tissues and subcellular organelles, but most highly in the brain. There are 2 isoforms encoded by separate genes (GSK3α and GSK3β) (1), plus a brain-specific isoform of GSK3β containing a 13 amino acid insert in the kinase domain generated by alternative splicing (GSK3β2) (2,3). GSK3 is critical for normal function of the central nervous system, where it regulates a variety of neuronal functions, including neurotransmission, neurite outgrowth, growth cone dynamics, cytoskeletal dynamics, synaptic plasticity, endocytosis, apoptosis and neurogenesis. Interestingly, it is one of the most unusual kinases in the human genome for 3 main reasons; 1) Most substrates require prior phosphorylation by another kinase before they can be efficiently phosphorylated at Ser/Thr residues by GSK3. This process is known as ‘priming’ and occurs 4 or 5 residues C-terminal to the GSK3 target site. 2) GSK3 is highly active in cells under basal conditions. This is partly due to constitutive phosphorylation of a conserved tyrosine residue on the activation loop of the kinase domain (Tyr279 in GSK3α, Tyr216 in GSK3β) that is absolutely required for kinase activity (4,5). 3) Phosphorylation of GSK3 at an N-terminal serine residue inhibits its kinase activity (Ser21 in GSK3α, Ser9 in GSK3β). This phosphoserine acts as a pseudo-substrate and binds to the phosphate-binding pocket on GSK3, preventing interaction with primed substrates (6). Phosphorylation at this site is mediated by members of the AGC family of kinases (e.g. Akt) and commonly occurs downstream of growth factor and PI3K signalling (7-9). Activation of the canonical Wnt signalling pathway also inhibits GSK3 activity, preventing phosphorylation of β-catenin, although this is not mediated by N-terminal phosphorylation, but by protein-protein interactions (10,11). Deregulated GSK3 activity has been implicated in the development of several psychiatric and neurodegenerative diseases, including Bipolar disorder, Schizophrenia and Alzheimer’s disease.
Therefore, it is important to identify downstream targets of GSK3 that maintain healthy brain function and to identify deregulated substrates in diseased brains that might become therapeutic targets.

In order to delineate the mechanisms by which GSK3 regulates brain function, it is vital to identify its substrates, since this is the key to illuminating the primary function of any protein kinase. So far, nearly 100 substrates for GSK3 have been identified, although only around half of these have been confirmed and it is likely that many more are yet to be discovered. Physiological substrates identified so far include several metabolic proteins, transcription factors and cytoskeleton-associated proteins. The challenge now is to complete the list of physiological targets of GSK3 and to assign functions for phosphorylation of each substrate. Previously, we used the KESTREL technique to identify a novel brain substrate of GSK3 called collapsin response mediator protein 2 (CRMP2) (17). However, no other substrates were identified in this screen. Like most other proteomic methods, the KESTREL screen was biased towards soluble abundant proteins (CRMP2 constitutes 1% of total brain protein; unpublished observation). High sensitivity of modern mass spectrometers has greatly improved detection of low abundance phosphorylated proteins, with many groups generating vast lists of phosphosites on endogenous proteins from various tissues. However, specialised mass spectrometers and computing power required for these phosphoproteomic studies are expensive and inaccessible to many researchers. Importantly, these databases do not yet contain information about the physiological kinases that target these sites. Therefore, we used an alternative approach that utilizes and extends the phosphoproteomic databases by assigning kinases to particular phosphorylated substrates. It uses bioinformatics to predict novel kinase substrates, followed by confirmation of candidates using a specific combination of cell culture and in vitro kinase assays (Suppl.Fig.1). Advantages of this approach include; 1) It is independent of abundance issues. 2) It can be targeted to particular classes of proteins of interest. 3) It does not require expensive specialised equipment. 4) If mammalian expression vectors are already available for predicted candidates, they can be experimentally confirmed within a few days. These attributes make it is accessible to all academic researchers conducting focussed research. Here, we used this approach to identify β-adducin as a novel substrate of GSK3 in the brain.

**EXPERIMENTAL PROCEDURES**

**Materials** - The cDNA encoding full-length human β-adducin (SwissProt P35612) was amplified by PCR from Image clone #6142886 using the primers

5’-GAATTCGCCACCATGGACTACAAGGACGA CGATGACAAGAGGAAGACGGTCC-3’
5’-GGCGAATTCTCAGGACTCCACTTTTCTCC-3’,
including a 5’ (N-terminal) Flag tag. The PCR product was subcloned into pRK5 (CMV promoter) for mammalian expression. Truncation mutants were generated by PCR using the 5’ primer shown above and the following 3’ primers; ΔT679-5’-
GGCGAATTCTCAGGACTCCACTTTTCTCC-3’,
ΔE610-5’-
GGCGAATTCTCAGGACTCCACTTTTCTCC-3’,
ΔA586-5’-
GGCGAATTCTCAGGACTCCACTTTTCTCC-3’.

The S697/613/600A triple mutant was generated using a QuikChange II site-directed mutagenesis kit (Stratagene) according to the manufacturers instructions. All constructs were verified by DNA sequencing. Phosphospecific antibodies were generated by injection of rabbits with the following peptides; pSer701-CGSPSkPSKkKk, pSer693-CTSGPlpSPEGSP, pSer617-CKSPAvpSPSKTS, pSer613-CAGTKpSPAVS, pSer604-CASPvQpSPSKAG, pSer596-CGSPvKpSTPASP (where pS is phosphoserine). Peptides were conjugated to keyhole limpet hemocyanin. Antisera were affinity purified on a phosphopeptide antigen-agarose column. Immunoblotting and immunofluorescence analyses using purified phosphospecific antibodies were routinely performed in the presence of dephospho-peptide to reduce non-specific binding to dephosphorylated β-adducin. Total β-adducin goat polyclonal antibody was purchased from Santa Cruz.
Prediction and confirmation of novel GSK3 substrates - Bioinformatic screening for proteins containing a GSK3 phosphorylation consensus sequence (S/TPXS/TP or S/TPXXS/TPXXS/TP) was performed using BlastP (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and ScanSite (http://scansite.mit.edu/). Conservation of potential phosphosites through evolution was determined by aligning sequences from different species using ClustalW (http://www.genome.jp/tools/clustalw/). Proteins reported to be phosphorylated by large-scale phosphoproteomic studies were identified using PhosphoSite (http://www.phosphosite.org/homeAction.do). Other criteria of particular interest (e.g. predominant expression in the brain, association with the cytoskeleton) were identified using UniProt (http://www.uniprot.org/) and PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez). To confirm candidates experimentally, they were cloned into a mammalian expression vector (pRK5) tagged with a Flag peptide, expressed in HEK293 cells in the absence or presence of the highly specific GSK3 inhibitor CT99021 (2 µM, 12 h) (18), then isolated from 300 µg of transfected HEK293 cell lysate using 10 µl of anti-Flag agarose (Sigma). Following washing, purified substrates were separately subjected to in vitro kinase assays with recombinant GSK3β (50 mU; Millipore) in kinase buffer containing 50 mM Tris-HCl, pH 7.5, 0.03% (v/v) Brij-35, 0.1% (v/v) β-mercaptoethanol and radiolabelled Mg[γ-32P]-ATP (30°C, 0.5 h). Reactions were terminated by addition of SDS loading buffer, subjected to SDS-PAGE and stained with Coomassie Brilliant Blue (CBR-250). Radiolabelled bands were visualised by autoradiography, excised from gels and the amount of 32P incorporated into the candidate was determined by liquid scintillation counting.

Cell culture - HEK293 cells were maintained in DMEM media supplemented with 10% (v/v) heat-inactivated foetal bovine serum, 2 mM GlutaMax and penicillin/streptomycin at 37°C with 5% CO₂. HEK293 cells were transfected using Dharmafect transfection reagent according to the manufacturers instructions (Dharmacon, Lafayette, Colorado, USA). Cells were lysed in buffer containing 1% (v/v) Triton X-100, 50 mM Tris-HCl, pH 7.5, 0.27 M sucrose, 1 mM EDTA, 0.1 mM EGTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.1% (v/v) β-mercaptoethanol and Complete protease inhibitor tablets (Roche, Basel, Switzerland) (4°C). Following centrifugation to remove insoluble material, supernatants were collected and protein concentrations were determined using the Bradford method (Sigma; (19)). Primary cortical neurons were isolated from E17 Sprague-Dawley rats, plated onto glass coverslips coated with high molecular weight poly-D-lysine (Millipore) and incubated at 37°C with 5% CO₂ in Neurobasal media containing 2% (v/v) B27 serum replacement, 2 mM GlutaMax, Penicillin (50 units/ml) and Streptomycin (100 units/ml) (Invitrogen). Neurons were co-transfected with β-adducin and GFP constructs at 1 DIV using calcium phosphate precipitation and harvested at 3 DIV.

Western blotting - Whole brains or frontal cortex from rats and mice were homogenized in cold cell lysis buffer using a glass Dounce homogenizer. Cultured cells were rinsed once in cold PBS then scraped directly into cell lysis buffer. Insoluble material was removed by centrifugation and the protein concentration of the supernatant was determined using the Bradford assay. SDS loading buffer was added to cell lysates and subjected to SDS-PAGE, then transferred to nitrocellulose membrane using the XCell II blot module (120 Vh; Invitrogen). Membranes were blocked in 5% (w/v) skim milk powder in PBS then incubated with primary antibody overnight at 4°C (phosphospecific antibodies at 1 µg/ml in the presence of 1 µM dephosphopeptide; anti-Flag antibody 1 µg/ml). Following washing, membranes were incubated with fluorescent secondary antibodies (Li-Cor,
Lincoln, Nebraska, USA), washed again and visualised using a Li-Cor Odyssey infrared imaging system. Differential detergent fractionation of adult rat brain was performed using the Qproteome fractionation kit according to the manufacturer's instructions (Qiagen).

**Phosphatase assays** – Adult rat brains were homogenised using a 10 ml glass Dounce homogenizer in lysis buffer containing 1% (v/v) Triton X-100, 50 mM Tris-HCl, pH 7.5, 0.27 M sucrose, 1 mM sodium orthovanadate, 0.1% (v/v) β-mercaptoethanol and Complete protease inhibitor tablets (4°C). Following centrifugation to remove insoluble material, supernatants were collected and protein concentrations were determined using the Bradford method. Lysates, containing a mixture of endogenous phosphatases, were diluted to 1 mg/ml and incubated on ice or at 30°C for up to 4 h in the presence of 1 mM MgCl₂ (to re-activate endogenous phosphatases; (20)). Reactions were terminated by addition of SDS loading buffer and proteins were visualised by Western blotting. For in vitro phosphatase assays, β-adducin and CRMP2 were isolated from 300 µg of transfected HEK293 cell lysate using 10 ul anti-Flag-agarose and phosphorylated using GSK3β or Cdk5 (2.5 mU/ml) in the presence of radiolabelled Mg[γ³²P]-ATP in buffer containing 50 mM Tris-HCl, pH 7.5, 0.03% (v/v) Brij-35 and 0.1% (v/v) β-mercaptoethanol (30°C, 1 h). Kinases were inhibited by addition of CT99021 (20 µM) or roscovitine (20 µM), respectively, followed by addition of different amounts of recombinant Protein Phosphatase 1 (PP1; New England Biolabs; 30°C, 30 min). Reactions were terminated by addition of SDS loading buffer then subjected to SDS-PAGE. Radiolabelled bands were visualized by autoradiography, excised from the gel and the amount of ³²P released from CRMP2 was determined by liquid scintillation counting.

**Immunofluorescence Microscopy** - Neurons were fixed in 4% (w/v) paraformaldehyde, permeabilized with 0.5% (v/v) Triton X-100, blocked with 2% (w/v) BSA and incubated with primary antibodies in PBS for 2 h at room temperature (anti-pSer693 and anti-pSer604 at 0.02 µg/ml in the presence of 5 µM dephosphopeptide; anti-β-adducin N-19 at 2 µg/ml). Fluorescent secondary antibodies and Phalloidin were diluted 1:500 and incubated on neurons for 1 h at room temperature. Image acquisition was performed on a Zeiss Axioscam mRm microscope (Zeiss, Germany) using 20x and 100x objectives. Image analysis was performed using NIH Image J software. Statistical analyses were performed using paired Student’s t-test and results were considered significant when p<0.05.

**RESULTS**

Identification of novel GSK3 substrates. We performed a bioinformatic screen for proteins containing a GSK3 phosphorylation consensus sequence (S/TPXXS/TP or S/TPXXXS/TP). A similar survey of potential GSK3 substrates was recently published (21). Here, we have focused on brain-enriched proteins containing conserved GSK3 consensus sequences (at least in mammals), proline-directed serine/threonines and phosphosites that have previously been identified by large-scale phosphoproteomic studies. In these cases, the protein is phosphorylated in vivo within a GSK3 phosphorylation consensus sequence and is therefore more likely to be a physiological substrate of GSK3. Using these criteria, 219 potential substrates of GSK3 were identified (Suppl. Table 1).

To confirm that a candidate is indeed a target of GSK3 in cells, its cDNA sequence is cloned and placed into a mammalian expression vector (in this case, pRK5 under control of the CMV promoter and an N-terminal Flag tag added), transiently expressed in HEK293 cells that were untreated or treated with the highly-specific GSK3 inhibitor CT99021 (18), immunoprecipitated from both cell types via the N-terminal Flag tag and subjected to in vitro kinase assays with recombinant GSK3β and radiolabelled ATP. If the candidate is a physiological target of GSK3, transfection into HEK293 cells should result in phosphorylation by endogenous GSK3. This will be blocked by the GSK3 inhibitor, leaving GSK3 target sites vacant but any required priming events intact. In subsequent in vitro kinase assays, recombinant GSK3 should be able to incorporate more radiolabelled phosphate into the protein isolated from inhibitor-treated cells compared to untreated cells. If so, this would indicate that the protein is a good substrate for GSK3 both in vitro and in cells. This approach was validated using an
established substrate of GSK3 in the brain called CRMP2 (17,22-25) (Suppl. Fig.2). We suggest that this experimental validation is vital for confidence that proposed candidates are bona fide GSK3 substrates.

Identification of β-adducin as a novel GSK3 substrate. Homology searches identified β-adducin as containing a conserved GSK3 phosphorylation consensus sequence that is very similar to other validated GSK3 substrates, such as CRMP2 and glycogen synthase. Furthermore, these sites have been detected as being phosphorylated in several large-scale phosphoproteomics studies (26-34). β-adducin is a cytoskeleton-associated protein that is predominantly expressed in the brain, where it is a constituent of synapses, dendritic spines and growth cones (35-37). It has been shown to regulate neuronal cytoarchitecture, long-term potentiation, motor coordination and learning (37-39). Therefore, we were interested to see if β-adducin is a novel brain substrate of GSK3. It was cloned and expressed in HEK293 cells in the absence or presence of CT99021. Purified Flag-tagged β-adducin was incubated with recombinant GSK3 and Mg\[^{32}\text{P}\]-ATP \textit{in vitro}. Significantly more phosphate was incorporated into β-adducin isolated from CT99021-treated cells than untreated cells (Fig.1), indicating that β-adducin is phosphorylated by GSK3 \textit{in vitro} and in cells.

Further examination of the amino acid sequence of the C-terminal region of β-adducin revealed 3 separate conserved GSK3 phosphorylation consensus sequences (Fig.2A). To determine which of these is targeted by GSK3, C-terminal truncation mutants of β-adducin were generated that sequentially removed one of the GSK3 consensus sequences at a time (T679 mutant removes Ser693-Ser701; E610 mutant removes Ser693-Ser701 and Ser613-Ser617; A586 mutant removes Ser693-Ser701, Ser613-Ser617 and Ser592-Ser604). Phosphate incorporation was significantly increased in wild type, T679 and E610 truncation mutants isolated from CT99021-treated cells compared to untreated cells, but not for the A586 mutant, which incorporated essentially no phosphate (Fig.2B, C). These results show that GSK3 phosphorylates 3 separate regions in the C-terminal domain of β-adducin.

β-adducin is phosphorylated by GSK3 and Cdk5. Phosphospecific antibodies were generated that recognise 2 phosphorylation sites in each of the 3 consensus sequences. The specificity of each antibody was confirmed by Western blotting of wild type β-adducin and non-phosphorylatable mutants expressed in HEK293 cells. Phosphospecific antibodies were raised against synthetic peptides containing pSer701, pSer693, pSer617, pSer613, pSer604 or pSer596. Figure 3A shows that each antibody recognises the wild type form of β-adducin, but not when the target site is mutated to a non-phosphorylatable alanine residue. In addition, while the pSer693 antibody recognises wild type and S701A forms of β-adducin, it does not recognise the S697A or S693A mutants, indicating that phosphorylation of S697 (but not S701) acts as a priming site for subsequent phosphorylation of S693 by GSK3. Similarly, the pSer613 antibody recognises wild type, but not S617A or S613A mutants, indicating that pSer617 primes for GSK3 phosphorylation at Ser613. Phosphorylation of wild type β-adducin at Ser693 and Ser613 was reduced in the presence of CT99021 (Fig.3B), confirming that Ser693 and Ser613 are targeted by GSK3. Meanwhile, phosphorylation of the putative priming sites Ser701, Ser617 and Ser604 were unaffected by CT99021. Note that data using the pSer596 antibody is not included in these figures because it was designed to recognise the rodent form of β-adducin, not the human form used in this experiment. Primary rat cortical neurons treated with CT99021 for up to 24 h displayed a reduction in phosphorylation of endogenous β-adducin at Ser693 and Ser596 (Fig.3C). Similar to transfected β-adducin in HEK293 cells, CT99021 treatment of cortical neurons reduced phosphorylation by around 50%. Surprisingly, phosphorylation of endogenous β-adducin in cortical neurons at Ser613 was unaffected by treatment with CT99021 for up to 24 h, although longer timepoints show a small but significant decrease (data not shown). The putative priming sites Ser701, Ser617 and Ser604 were unaffected by CT99021 treatment. The sequences surrounding the Ser701, Ser697, Ser617 and Ser604 conform to a Cdk5 phosphorylation consensus sequence (S/TPK/R) (40). Cdk5 has been shown to acts as a priming kinase for other
GSK3 substrates, including CRMP2 and Tau (22-24,41). In order to determine if Cdk5 targets these sites and primes for subsequent GSK3 phosphorylation at Ser693, Ser613 and Ser596, respectively, the phosphorylation of endogenous β-adducin was measured in brain tissue from wild type and Cdk5-/- mice using Western blotting (Fig.3D). Phosphorylation was reduced at all sites, supporting the hypothesis that Cdk5 is a priming kinase for subsequent GSK3 phosphorylation at each of the phosphorylation regions in the C-terminal domain of β-adducin.

β-adducin phosphosites display relative resistance to phosphatases. Phosphorylation of β-adducin was reduced by treatment with CT99021 and in Cdk5-/- brain tissue, but not completely inhibited (Fig.3). This suggests that other kinases might also target these sites, or that other kinases can compensate for the loss of Cdk5 and GSK3 activity. Alternatively, removal of phosphate from these by phosphatases might be very inefficient, as has previously been shown for phosphosites in CRMP2 and Tau that are relatively resistant to dephosphorylation by phosphatases (42,43). To determine if any phosphosites in β-adducin display similar resistance to phosphatases, a rat brain lysate was incubated without phosphatase inhibitors at 30°C for up to 4 h. Relative rates of dephosphorylation at each site by endogenous phosphatases was measured using Western blotting. Figure 4A shows that while there was rapid and complete dephosphorylation of Ser596 in β-adducin and Thr514/509 in CRMP2, there was only partial dephosphorylation of pSer613 (approximately 50%) but no change in phosphorylation at Ser701, Ser693, Ser617 and Ser604. In addition, β-adducin isolated from HEK293 cells was phosphorylated in vitro by recombinant Cdk5 or GSK3 in the presence of radiolabelled ATP, and following inhibition of the kinases, different amounts of recombinant PP1 was added to compare the efficiency of dephosphorylation (Fig.4B, C). Both the Cdk5 and GSK3 phosphosites displayed relative resistance to dephosphorylation by PP1, while for comparison, the GSK3 phosphosites on CRMP2 were completely dephosphorylated by 1 Unit of PP1.

We have previously reported a correlation between relative resistance of Cdk5 target sites to phosphatases and hyperphosphorylation in the brains of Alzheimer’s disease (AD) patients for Tau and CRMP2 (42,44). Since Cdk5 and GSK3 phosphosites in β-adducin display similar resistance to phosphatases, we investigated if β-adducin was also hyperphosphorylated in a mouse model of Alzheimer’s disease. Phosphorylation levels were measured in 2 mouse models of AD, Triple-Tg (Fig.5) and APP/PS1 mice (data not shown) (45,46). Both types of mice display hyperphosphorylation of CRMP2 and Tau (44). Analysis of Western blot data showed no change in phosphorylation of β-adducin at any of the sites analysed. Therefore, phosphorylation of β-adducin is normal in these mouse models of AD.

Phosphorylation of β-adducin regulates its subcellular localisation. The subcellular localisation of β-adducin and its phosphorylated forms was investigated using immunofluorescence microscopy of cultured primary rat cortical neurons (Fig.6A). β-adducin was widely distributed throughout the cell body, nucleus, dendrites, axons and growth cones of neurons. In particular, β-adducin was localised to the periphery of the neuron cell body, consistent with its association with actin and spectrin cytoskeletal networks (35). Phosphorylated forms of β-adducin were also detected in all regions of the neuron, but were predominantly located in the cell body, not at its periphery, as determined using the pSer693 and pSer604 antibodies. This was supported by differential detergent fractionation of adult rat brain, which showed that while similar amounts of β-adducin were detected in all fractions, β-adducin phosphorylated at Ser693 was highly enriched in the cytoplasm (Fig.6B, C; other phosphosites displayed the same distribution, data not shown). Together, this suggests that phosphorylation of β-adducin regulates its subcellular localization, promoting movement away from the cell membrane to the cytoplasm.

Phosphorylation of β-adducin promotes neurite outgrowth. In order to determine the effect of β-adducin phosphorylation on neuronal function, wild type and a non-phosphorylatable mutant of β-adducin that blocks phosphorylation by GSK3 (S697/613/600A)(Suppl.Fig.3) were transfected into cultured primary rat cortical neurons and the morphology of transfected neurons after 3 days in vitro (DIV) was analysed.
using immunofluorescence microscopy (Fig. 7). Expression of wild type β-adducin increased axon elongation by approximately 25% in transfected neurons. In addition, the average length of branches from the axon and dendrite lengths were also increased by 25%, while the number of axonal branches and dendrites were unchanged. In contrast, expression of the non-phosphorylatable mutant did not increase axon or dendrite length, nor branch or dendrite number, although the average length of axonal branches was significantly increased, similar to wild type β-adducin. These observations show that β-adducin promotes neurite outgrowth in cortical neurons and that phosphorylation by GSK3 is required for this process.

DISCUSSION

In order to delineate the mechanisms by which GSK3 regulates healthy brain function, it is critical to identify the substrates that mediate its functions. We used bioinformatics to screen for candidates that contain a conserved GSK3 phosphorylation consensus sequence, followed by validation using a combination of cell culture and in vitro kinase assays. This approach is particularly well suited to substrates of GSK3, since GSK3 is ubiquitously expressed (including commonly used cell lines), is highly active under basal conditions and does not usually require additional adaptor proteins. Phosphorylation of most GSK3 substrates requires prior phosphorylation by a ‘priming’ kinase, although these are commonly widely expressed and highly active under basal conditions (e.g. casein kinase I/II, Cdk5, DYRK). Therefore, it is likely that many bona fide substrates of GSK3 will be phosphorylated in common cell lines, although some substrates requiring specific priming kinases or adaptor proteins may require the use of more specialised cell types.

Here, we identified β-adducin as a novel target of GSK3 in the brain. β-adducin is a cytoskeleton-associated protein that is exclusively expressed in the central nervous system and erythrocytes (35,36). In the brain, its expression is particularly high in regions associated with high levels of plasticity, such as the hippocampus (39). It forms heterodimers or heterotetramers with α- (but not γ) adducin subunits (47), providing links between actin and spectrin cytoskeletal networks at the fast growing ends of actin filaments (48). It also binds to Rabphilin-3A, which is a synaptic vesicle protein that regulates neurotransmitter release (49). β-adducin knock-out mice display impairments in long term potentiation induction in hippocampal neurons and impaired performance in fear conditioning and water maze tasks, which are measures of learning (38,39). These observations support the idea that β-adducin is involved in neuroplasticity underlying learning and memory. This might involve changes in neuronal morphology, including neurite outgrowth and synapse formation. Consistent with this, the spectrin network is important for maintaining the structural integrity of axons and dendrites (50).

Three separate regions in the C-terminal domain of β-adducin are targeted by GSK3. Efficient phosphorylation at each of these sites required prior phosphorylation by Cdk5. This priming mechanism is characteristic of GSK3 substrates. The C-terminal domain is predicted to be relatively unstructured (random coil), which is also typical of GSK3 target sites and proline-directed Ser/Thr phosphosites in general (30). Preliminary pulldown experiments using phosphospecific antibodies suggests that all 3 regions can be phosphorylated simultaneously on the same molecule (data not shown). This would result in phosphorylation of up to 9 sites in the C-terminal domain of β-adducin, which would presumably induce significant structural changes in this domain. It is likely that this would affect interactions with other proteins, including actin and spectrin networks. Efforts are continuing in our lab to identify β-adducin-binding proteins that are affected by phosphorylation of the C-terminal domain.

Several phosphosites were found to be relatively resistant to dephosphorylation by phosphatases. β-adducin is the third substrate of Cdk5 demonstrated to display relative resistance to phosphatases (others being CRMP2 and Tau; (42,43)), but this is the first time a GSK3 phosphosite has been demonstrated to display relative resistance. We previously showed that resistance to phosphatases was in part due to the presence of a basic residue at the +3 position, which stabilizes the negative charge on the
phosphorylated serine (42). All 3 Cdk5 phosphosites in β-adducin contain a basic residue at the +3 position, consistent with this pattern. In contrast, the GSK3 target site Ser693 does not, although it is located close to the MARCKS domain at the C-terminus, which contains many lysine residues. It is tempting to speculate that phosphorylation of up to 9 residues by GSK3 and Cdk5 induces a dramatic conformational change that protects at least some of the phosphosites from phosphatases, although this remains to be proven.

β-adducin is distributed throughout the cytoplasm, plasma membrane, nucleus and cytoskeleton, whereas GSK3/Cdk5-phosphorylated β-adducin is predominantly cytoplasmic. This contrasts with a previous study showing phosphorylation of α-adducin at Ser716 and Ser726 by PKC (these sites are conserved and phosphorylated by PKC in β-adducin) localizes with spectrin at the cell membrane of MDCK cells and hippocampal neurons, in particular in dendritic spines (37). Expression of a non-PKC-phosphorylatable mutant of α-adducin (S716/726A) exhibited a punctate distribution of α-adducin in the cytoplasm, as well as increased cytoplasmic spectrin. This suggests that two opposing signalling pathways regulate adducin localization with spectrin at the cell membrane. PKC is amongst a number of AGC kinases that are able to phosphorylate GSK3 and inhibit its activity (51). Therefore, it is possible that activation of PKC activity could simultaneously invoke increased phosphorylation at the PKC sites and decreased phosphorylation at the GSK3 phosphosites, promoting localization of β-adducin with actin/spectrin at the cell membrane.

Cdk5 and its essential co-factors p35 and p39 are localised to the membrane via myristolation at the N-terminus of p35 and p39 (52). It is possible that Cdk5 phosphorylates β-adducin at the cell membrane, releasing it into the cytoplasm. In AD, p35 is cleaved by the protease calpain near the N-terminus, releasing its daughter product p25 into the cytoplasm (53), which could affect β-adducin phosphorylation levels. We did not observe any changes in β-adducin phosphorylation in 2 mouse models of AD. However, these mice do not generate p25 (44) or develop neurodegeneration. Therefore, it will be interesting to measure β-adducin phosphorylation levels in post-mortem brain tissue from AD versus control patients.

Transfection of wild type β-adducin, but not a non-phosphorylatable mutant, into primary cortical neurons increased the rate of elongation of both axons, axon branches and dendrites, suggesting that phosphorylation of β-adducin modulates this key neuronal process. Similar results were observed for CRMP2, another brain enriched cytoskeleton-associated protein that is phosphorylated by GSK3 (17). Pharmacological inhibitors and shRNA knockdown of GSK3 also reduce neurite outgrowth (54,55). Cellular GSK3 activity is regulated by various environmental signals, including growth factors, neurotransmitters, semaphorins and Wnt, all of which influence neurite outgrowth. Therefore, GSK3 might be a central node linking environmental cues to neuronal morphology via coordinating the activity of specific substrates. β-adducin and CRMP2 are 2 substrates demonstrated to mediate the effects of GSK3 on neurite outgrowth, presumably via regulation of actin/spectrin and microtubule dynamics, respectively. However, other targets of GSK3 remain to be discovered, for which the approach described here is an ideal tool.

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**FOOTNOTES**

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The abbreviations used are: Cdk5, cyclin-dependent kinase 5; GSK3, glycogen synthase kinase 3; CRMP2, collapsin response mediator protein 2; PP1, protein phosphatase 1; AD, Alzheimer’s disease.
**FIGURE LEGENDS**

**Fig.1.** Identification of β-adducin as a novel GSK3 substrate. *A*, β-adducin was isolated from cells that were untreated or treated with CT99021 (2 µM, 12 h), then subjected to *in vitro* kinase assays with GSK3β and [γ]32P-MgATP (30°C, 30 min). The upper panel shows the amount of radiolabelled phosphate incorporated into β-adducin, while the lower panel shows CBR-250-stained β-adducin as a loading control (GFP control, lane 1; no CT99021, lanes 2-3; +CT99021, lanes 4-5). *B*, The stoichiometry of radiolabelled phosphate incorporated into β-adducin in *A* is presented as a graph (average ± SEM; *p*<0.05 relative to control; Student’s t-test).

**Fig.2.** Three separate regions in β-adducin are targeted by GSK3. *A*, Primary amino acid sequence of the C-terminal region of human β-adducin. Numbered residues are putative phosphorylation sites. Lines indicate the C-termini of 3 truncation mutants. *B*, Full length and truncation mutants of β-adducin were isolated from cells that were untreated or treated with CT99021 (2 µM, 12 h), then subjected to *in vitro* kinase assays with GSK3β and [γ]32P-MgATP (30°C, 30 min). The upper panel shows the amount of radiolabelled phosphate incorporated into β-adducin, while the lower panel shows CBR-250-stained β-adducin as a loading control. *C*, The stoichiometry of radiolabelled phosphate incorporated into full length and truncated β-adducin -/+ CT99021 in *B* is presented as a graph (average ± SEM; *p*<0.05 relative to control; n.s. = not significant; Student’s t-test).

**Fig.3.** Analysis of β-adducin phosphorylation using phosphospecific antibodies. *A*, β-adducin wild type and various point mutants were transfected into HEK293 cells. Cell lysates were subjected to Western blot analysis using β-adducin phosphospecific antibodies and anti-Flag as a loading control. *B*, Wild type β-adducin was expressed in HEK293 cells in the absence or presence of CT99021 (2 µM). β-adducin was isolated from lysates using anti-Flag agarose and subjected to Western blot analysis using phosphospecific antibodies and an anti-Flag as a loading control. *C*, Cultured rat cortical neurons were treated with CT99021 for the times indicated. Neurons were harvested in cell lysis buffer and lysates were subjected to Western blot analysis using phosphospecific antibodies and a polyclonal antibody that recognises total β-adducin. *D*, Cortex from wild type and Cdk5-/- mice (E17) were homogenised and subjected to Western blot analysis using phosphospecific antibodies and a polyclonal antibody that recognises total β-adducin.

**Fig.4.** β-adducin phosphosites are relatively resistant to dephosphorylation by phosphatases. *A*, Adult rat brain lysate was incubated at 30°C for up to 4 h. Dephosphorylation of endogenous β-adducin by endogenous phosphatases was determined by Western blot analysis using phosphospecific antibodies and a polyclonal antibody that recognises total β-adducin, as well as antibodies that specifically recognise CRMP2 when phosphorylated at Thr514/509 and total CRMP2 antibody. *B*, Wild type β-adducin was phosphorylated using recombinant Cdk5 (upper panel) or GSK3β (middle panel), while wild type CRMP2 was phosphorylated using GSK3β in the presence of radiolabelled ATP (30°C, 30 min). Following addition of roscovitine or CT99021 to inhibit Cdk5 and GSK3β, respectively, different amounts of recombinant PP1 were added (30°C, 30 min). Radiolabelled phosphate in β-adducin and CRMP2 was detected using autoradiography. *C*, The amount of radiolabelled phosphate removed from β-adducin and CRMP2 by PP1 was quantified by liquid scintillation counting and is presented as a graph (n=3, average +/- SEM).

**Fig.5.** Phosphorylation of β-adducin is not altered in a mouse model of Alzheimer’s disease. Cortex from 4 month old female 3xTg Alzheimer’s mice and wild type litter mates were homogenised and subjected to Western blot analysis using β-adducin phosphospecific antibodies and a polyclonal antibody that
recognises total β-adducin. Following densitometric analysis, the ratio of phospho:total β-adducin was determined and is presented as graphs (wild type=5, 3xTg=6; average +/- SEM; n.s., not significant relative to control; Student’s t-test).

**Fig.6.** Localisation of β-adducin in neurons. *A*, Cortical neurons (3 DIV) were fixed and incubated with antibodies that recognise β-adducin phosphorylated at Ser693 or Ser604 (green), as well an antibody that recognises total β-adducin (blue) and phalloidin (red). *B*, Adult rat brain was subjected to differential detergent fractionation and separated into cytoplasm (C), membrane (M), nucleus (N) and cytoskeletal (S) fractions, which were subjected to Western blot analysis using an antibody that recognises β-adducin when phosphorylated at Ser693 (upper panel) and an antibody that recognises total β-adducin (lower panel). *C*, Following densitometric analysis, the ratio of phospho:total β-adducin in each fraction was determined and is presented as a graph (average +/- SEM).

**Fig.7.** β-adducin regulates neurite outgrowth in cortical neurons. *A*, Cortical neurons were co-transfected with GFP and either empty vector (upper panels), β-adducin wild type (middle panels) or β-adducin S600/613/697A mutant (lower panels). Neurons were fixed and visualized using fluorescence microscopy. The morphology of transfected neurons was quantitated using Image J software and comparisons of average axon length (*B*), number of neurites (*C*), dendrite length (*D*), number of branches from axons (*E*) and branch length (*F*) are presented as graphs (GFP control n=109, β-adducin wild type n=112, β-adducin S-Triple-A n=110; average ± SEM; *p*<0.05 relative to control; n.s., not significant; Student’s t-test).
Fig. 2

A

GDEDTKDDSEEETVPNPFSQLTDDQEELLEYKKEVERKKLELDEGEKETAPAPEGSPAKSAPAS
PVQSPAKEAETKSPLVSPSKEEGT KTETSKAATTEPETTQPEGVVVNGREEEQTAEE
ILSKGLSQMTTSADTDVDTSKDKTESVTSGPMSPEGSPSKESSKKFRTPSFLKKSKK

B

C

ΔT679

ΔE610

ΔA586

ΔT679

ΔE610

ΔA586

GFP

mol/mol phosphate incorporation

- + - + - + CT99021

FL ΔT679 ΔE610 ΔA586

32P

Mₐ (x10³)

98

FL ΔT679 ΔE610 ΔA586 GFP

CBR-250

- + - + - +

0.4

0.8

1.2

n.s.
Fig. 3

**B**

| M_r (x10^3) | pSer701 | pSer617 | pSer604 | pSer693 | pSer613 | Flag |
|-------------|---------|---------|---------|---------|---------|------|
| 150         |         |         |         |         |         |      |
| 100         |         |         |         |         |         |      |
| 150         |         |         |         |         |         |      |
| 100         |         |         |         |         |         |      |
| 150         |         |         |         |         |         |      |
| 100         |         |         |         |         |         |      |
| 150         |         |         |         |         |         |      |
| 100         |         |         |         |         |         |      |

**C**

| M_r (x10^3) | pSer701 | pSer617 | pSer604 | pSer693 | pSer613 | pSer596 | β-adducin |
|-------------|---------|---------|---------|---------|---------|---------|-----------|
| 150         |         |         |         |         |         |         |           |
| 100         |         |         |         |         |         |         |           |
| 150         |         |         |         |         |         |         |           |
| 100         |         |         |         |         |         |         |           |
| 150         |         |         |         |         |         |         |           |
| 100         |         |         |         |         |         |         |           |
| 150         |         |         |         |         |         |         |           |
| 100         |         |         |         |         |         |         |           |

**D**

| M_r (x10^3) | pSer701 | pSer617 | pSer604 | pSer693 | pSer613 | pSer596 | β-adducin |
|-------------|---------|---------|---------|---------|---------|---------|-----------|
| 150         |         |         |         |         |         |         |           |
| 100         |         |         |         |         |         |         |           |
| 150         |         |         |         |         |         |         |           |
| 100         |         |         |         |         |         |         |           |
| 150         |         |         |         |         |         |         |           |
| 100         |         |         |         |         |         |         |           |
| 150         |         |         |         |         |         |         |           |
| 100         |         |         |         |         |         |         |           |

Time (h) 0 1 4 24

CT99021

**Cdk5**

+/-  +/-
Fig. 5

**pSer701**

- WT: 100%
- 3xTg: n.s.

**pSer693**

- WT: 100%
- 3xTg: n.s.

**pSer617**

- WT: 100%
- 3xTg: n.s.

**pSer613**

- WT: 100%
- 3xTg: n.s.

**pSer604**

- WT: 100%
- 3xTg: n.s.

**pSer596**

- WT: 100%
- 3xTg: n.s.
**Fig. 6**

**A**

- **pSer693**
- **pSer604**
- **pSer604**

**B**

- Western blot analysis of **pSer693** and **β-Adducin** with different treatments (C, M, N, S).

**C**

- Bar graph showing the normalized levels of **pSer693:β-Adducin** across different treatments (C, M, N, S).
Fig. 7

(A) GFP, β-Adducin WT, β-Adducin S600/613/697A

Scale bar: 50 μm
Fig. 7

B. Axon length

C. Number of neurites

D. Dendrite length

E. Branches from axon

F. Branch length

|               | GFP  | β-Add WT | β-Add S-Triple-A |
|---------------|------|----------|------------------|
| Axon length   | 300  | 250      | 200              |
| Number of neurites | 6    | 4        | 4                |
| Dendrite length | 30   | 30       | 30               |
| Branches from axon | 3    | 3        | 3                |
| Branch length  | 40   | 40       | 40               |

* n.s.
Bioinformatic prediction and confirmation of β-adducin as a novel substrate of glycogen synthase kinase 3

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