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Bioinformatic Prediction and Confirmation of β-Adducin as a Novel Substrate of Glycogen Synthase Kinase 3*[S]

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It is important to identify the true substrates of protein kinases because 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/T)P*XX*(S/T)P or (S/T)P*XXX*(S/T)P), 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 three 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)[2] 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 two 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 three 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 (Tyr-279 in GSK3α, Tyr-216 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 (Ser-21 in GSK3α, Ser-9 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 (containing PKA, PKG, and PKC families) family of kinases (*e.g.* Akt) and commonly occurs downstream of growth factor and PI3K signaling (7–9). Activation of the canonical Wnt signaling 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 disease (12–16). 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.

To delineate the mechanisms by which GSK3 regulates brain function, it is vital to identify its substrates because 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 (kinase substrate tracking and elucidation) 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 toward soluble abundant pro-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3 and Table 1.

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[2] The abbreviations used are: GSK3, glycogen synthase kinase 3; Cdk5, cyclin-dependent kinase 5; CRMP2, collapsin response mediator protein 2; PP1, protein phosphatase 1; AD, Alzheimer disease; pS, phosphoserine.

teins (CRMP2 constitutes 1% of total brain protein).[3] The 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, specialized 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 (supplemental Fig. 1). Advantages of this approach include the following. 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 specialized 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 focused 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′-GAATTCGCCACCATGGACTACAAGGACGACGATGACAAGAGCGAAGAGACGGTCC-3′ and 5′-GGCGAATTCTCAGGACTCCACTTTCTCC-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′-GGCGAATTCTCAGGTATCAACATCCGTGTCAGC-3′, ΔE610-5′-GGCGAATTCTCACTCTGCCTCCTTCGCTGG-3′, ΔA586-5′-GGCGAATTCTCAGGCAGTTTCTTTCTCT-CCATC-3′. The S697A/S613A/S600A triple mutant was generated using a QuikChange II site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. All constructs were verified by DNA sequencing. Phosphospecific antibodies were generated by injection of rabbits with the following peptides: pSer701-CGSPSKpSPSKKK, pSer693-CTSGPLpSPEGSP, pSer617-CKSPAVpSPSKTS, pSer613-CAGTKpSPAVS, pSer604-CASPVQpSPSKAG, and 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 dephosphopeptide to reduce nonspecific binding to dephosphorylated β-adducin. Total β-adducin goat polyclonal antibody was purchased from Santa Cruz Biotechnology (N-19, Santa Cruz, CA). Alexa Fluor 568-phalloidin was from Invitrogen (Mount Waverly, Australia), whereas anti-FLAG monoclonal antibody was from Sigma (Castle Hill, Australia). All fluorescent secondary antibodies were supplied by Jackson ImmunoResearch Laboratories (West Grove, PA). CT99021 was a kind gift from Dr. Rodolfo Marquez, University of Dundee, Scotland, UK. Rats were supplied by the Animal Resources Centre (Perth, Australia).

Prediction and Confirmation of Novel GSK3 Substrates—Bioinformatic screening for proteins containing a GSK3 phosphorylation consensus sequence ((S/T)P*XX*(S/T)P or (S/T)P*XX*(S/T)P*XX*(S/T)P) was performed using BlastP (blast.ncbi.nlm.nih.gov/Blast.cgi) and ScanSite. Conservation of potential phosphosites through evolution was determined by aligning sequences from different species using ClustalW. Proteins reported to be phosphorylated by large-scale phosphoproteomic studies were identified using PhosphoSite. Other criteria of particular interest (*e.g.* predominant expression in the brain, association with the cytoskeleton) were identified using UniProt and PubMed (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), and 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 milliunits; 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 radiolabeled Mg-[γ-^{32}P]ATP (30 °C, 0.5 h). Reactions were terminated by the addition of SDS loading buffer, subjected to SDS-PAGE, and stained with Coomassie Brilliant Blue (CBR-250). Radiolabeled bands were visualized by autoradiography and excised from gels, and the amount of ^{32}P incorporated into the candidate was determined by liquid scintillation counting.

Cell Culture—HEK293 cells were maintained in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM GlutaMAX, and penicillin/streptomycin at 37 °C with 5% CO_2. HEK293 cells were transfected using DharmaFECT transfection reagent according to the manufacturer's instructions (Dharmacon, Lafayette, CO). 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 Applied Science, 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 embryonic day 17 Sprague-Dawley rats, plated onto glass coverslips coated with high molecular weight poly-D-lysine (Millipore), and incubated at 37 °C with 5% CO_2 in Neurobasal medium 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 day *in vitro* using calcium phosphate precipitation and harvested at 3 days *in vitro*.

[3] M. Soutar, A. R. Cole, and C. Sutherland, unpublished observation.

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 and 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 and then transferred to nitrocellulose membrane using the XCell II blot module (120 V-h; Invitrogen). Membranes were blocked in 5% (w/v) skim milk powder in PBS and 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, NE), washed again, and visualized using a LI-COR Odyssey infrared imaging system. Differential detergent fractionation of adult rat brain was performed using the Qproteome fractionation kit according the manufacturer's instructions (Qiagen).

Phosphatase Assays—Adult rat brains were homogenized 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 $MgCl_2$ (to reactivate endogenous phosphatases (20)). Reactions were terminated by the addition of SDS loading buffer, and proteins were visualized by Western blotting. For *in vitro* phosphatase assays, β-adducin and CRMP2 were isolated from 300 μg of transfected HEK293 cell lysate using 10 μl of anti-FLAG-agarose and phosphorylated using GSK3β or Cdk5 (2.5 milliunits/μl) in the presence of radiolabeled Mg-[γ-^{32}P]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 the addition of CT99021 (20 μM) or roscovitine (20 μM), respectively, followed by the addition of different amounts of recombinant protein phosphatase 1 (PP1; New England Biolabs; 30 °C, 30 min). Reactions were terminated by the addition of SDS loading buffer and then subjected to SDS-PAGE. Radiolabeled bands were visualized by autoradiography and excised from the gel, and the amount of ^{32}P 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 0.1 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 Axiocam mRm microscope (Zeiss, Germany) using 20× and 100× objectives. Image analysis was performed using the National Institutes of Health 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/T)P*XX*(S/T)P or (S/T)P*XXX*(S/T)P). 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 (supplemental 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 radiolabeled 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 radiolabeled phosphate into the protein isolated from inhibitor-treated cells as compared with 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) (supplemental 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 whether 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-[γ-^{32}P]ATP *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 *in vitro* and in cells.

Further examination of the amino acid sequence of the C-terminal region of β-adducin revealed three separate conserved GSK3 phosphorylation consensus sequences (Fig. 2*A*). 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 Ser-693–Ser-701; E610 mutant removes Ser-693–Ser-701 and Ser-613–Ser-617; A586 mutant removes Ser-693–Ser-701, Ser-613–Ser-617, and Ser-592–Ser-604). Phosphate incorporation was significantly increased in wild type, T679, and E610 truncation mutants isolated from cells treated with CT99021 as compared with untreated cells, but not for the A586 mutant, which incorporated essentially no phosphate (Fig. 2, *B* and *C*). These results show that GSK3 phosphorylates three separate regions in the C-terminal domain of β-adducin.

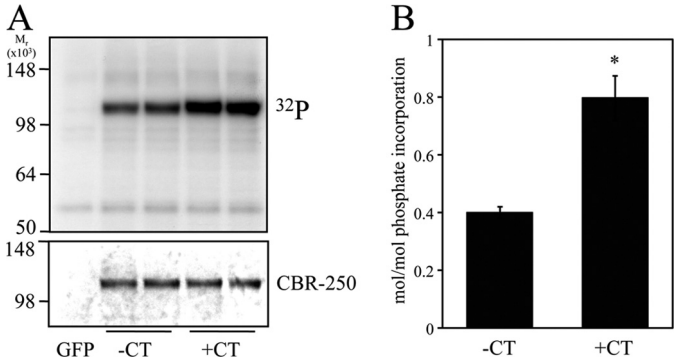

FIGURE 1. **Identification of β-adducin as a novel GSK3 substrate.** *A*, β-adducin was isolated from cells that were untreated or treated with CT99021 (2 μM, 12 h) and then subjected to *in vitro* kinase assays with GSK3β and Mg-[γ-^{32}P]ATP (30 °C, 30 min). The *upper panel* shows the amount of radiolabeled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

β-Adducin Is Phosphorylated by GSK3 and Cdk5—Phosphospecific antibodies were generated that recognize two phosphorylation sites in each of the three 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. Fig. 3*A* shows that each antibody recognizes the wild type form of β-adducin, but not when the target site is mutated to a non-phosphorylatable alanine residue. In addition, although the pSer693 antibody recognizes wild type and S701A forms of β-adducin, it does not recognize the S697A or S693A mutants, indicating that phosphorylation of Ser-697 (but not Ser-701) acts as a priming site for subsequent phosphorylation of Ser-693 by GSK3. Similarly, the pSer613 antibody recognizes wild type, but not S617A or S613A mutants, indicating that pSer617 primes for GSK3 phosphorylation at Ser-613. Phosphorylation of wild type β-adducin at Ser-693 and Ser-613 was reduced in the presence of CT99021 (Fig. 3*B*), confirming that Ser-693 and Ser-613 are targeted by GSK3. Meanwhile, phosphorylation of the putative priming sites Ser-701, Ser-617, and Ser-604 was unaffected by CT99021. Note that data using the pSer596 antibody are not included in these figures because it was designed to recognize 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 Ser-693 and Ser-596 (Fig. 3*C*). 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

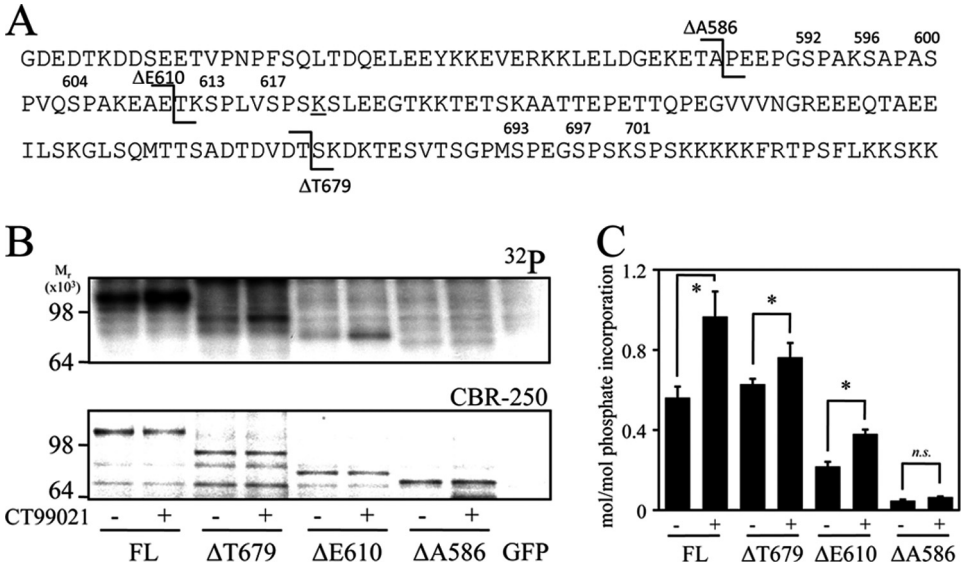

FIGURE 2. **Three separate regions in β-adducin are targeted by GSK3.** *A*, primary amino acid sequence of the C-terminal region of human β-adducin. *Numbered residues* are putative phosphorylation sites. *Lines* indicate the C termini of three truncation mutants. *B*, full-length (*FL*) and truncation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

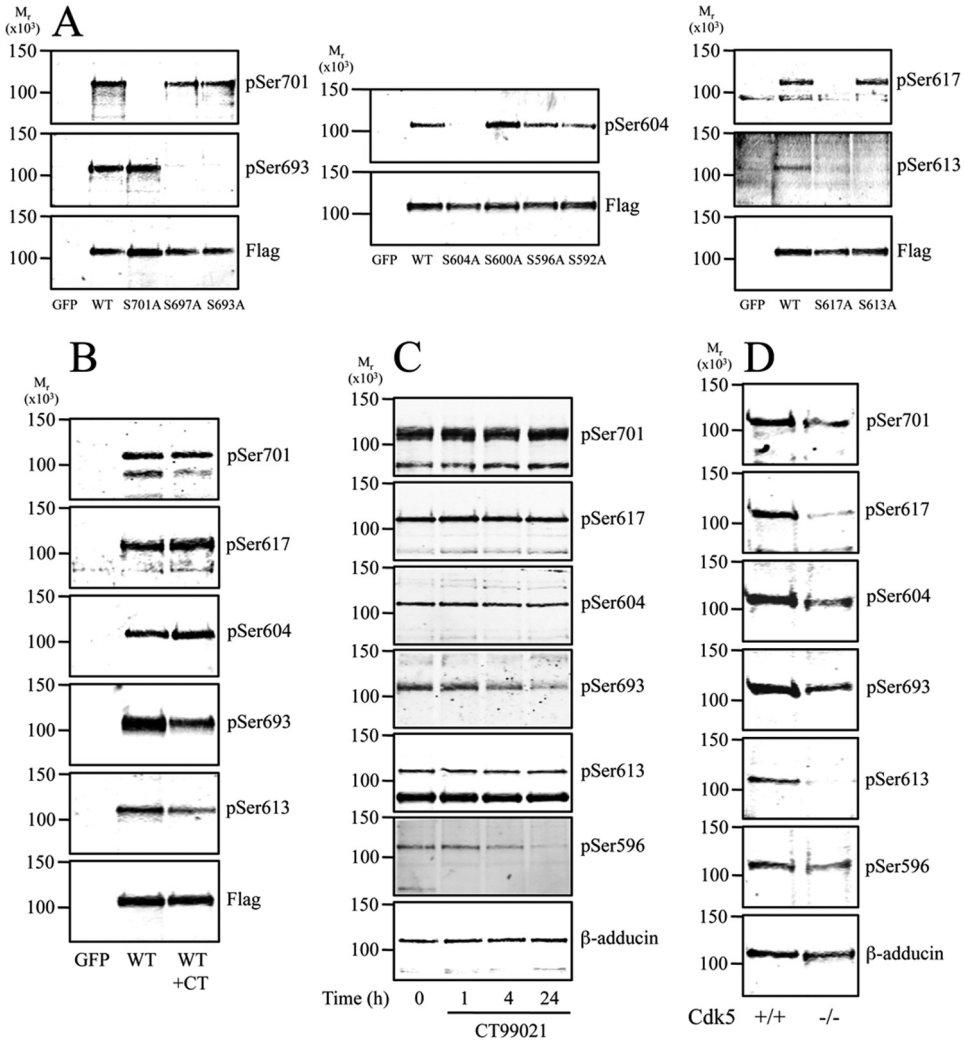

FIGURE 3. **Analysis of β-adducin phosphorylation using phosphospecific antibodies.** *A*, β-adducin wild type and various point mutants were transfected into HEK293 cells. Cell lysates were subjected to Western blot analysis using β-adducin phosphospecific antibodies and anti-FLAG as a loading control. *B*, wild type β-adducin was expressed in HEK293 cells in the absence or presence of CT99021 (*CT*, 2 μM). β-Adducin was isolated from lysates using anti-FLAG agarose and subjected to Western blot analysis using phosphospecific antibodies and 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 recognizes total β-adducin. *D*, cortex from wild type and Cdk5$^{-/-}$ mice (embryonic day 17) were homogenized and subjected to Western blot analysis using phosphospecific antibodies and a polyclonal antibody that recognizes total β-adducin.

neurons at Ser-613 was unaffected by treatment with CT99021 for up to 24 h, although longer time points show a small but significant decrease (data not shown). The putative priming sites Ser-701, Ser-617, and Ser-604 were unaffected by CT99021 treatment. The sequences surrounding the Ser-701, Ser-697, Ser-617, and Ser-604 conform to a Cdk5 phosphorylation consensus sequence ((S/T)P*X*(K/R)) (40). Cdk5 has been shown to acts as a priming kinase for other GSK3 substrates, including CRMP2 and Tau (22–24, 41). To determine whether Cdk5 targets these sites and primes for subsequent GSK3 phosphorylation at Ser-693, Ser-613, and Ser-596, respectively, the phosphorylation of endogenous β-adducin was measured in brain tissue from wild type and Cdk5$^{-/-}$ mice using Western blotting (Fig. 3*D*). 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 sites 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 whether 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 were measured using Western blotting. Fig. 4*A* shows that although there was rapid and complete dephosphorylation of Ser-596 in β-adducin and Thr-514/509 in CRMP2, there was

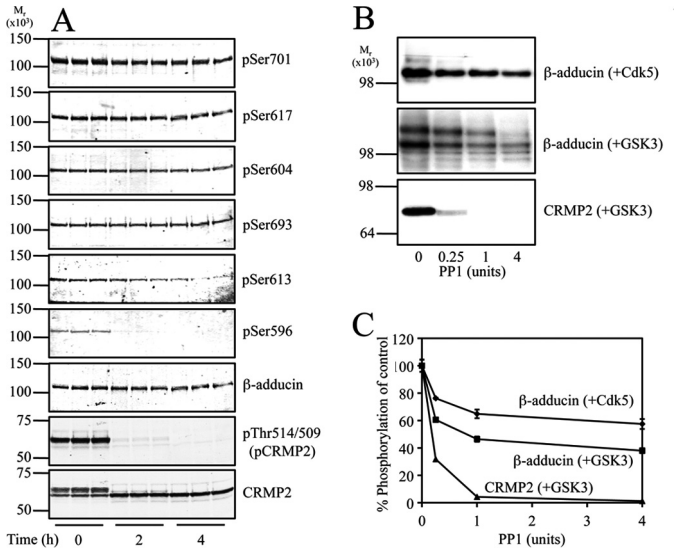

FIGURE 4. **β-Adducin phosphosites are relatively resistant to dephosphorylation by phosphatases.** *A*, adult rat brain lysate was incubated at 30 °C for up to 4 h. Dephosphorylation of endogenous β-adducin by endogenous phosphatases was determined by Western blot analysis using phosphospecific antibodies and a polyclonal antibody that recognizes total β-adducin, as well as antibodies that specifically recognize CRMP2 when phosphorylated at Thr-514/509 and total CRMP2 antibody. *B*, wild type β-adducin was phosphorylated using recombinant Cdk5 (*upper panel*) or GSK3β (*middle panel*), whereas wild type CRMP2 was phosphorylated using GSK3β in the presence of radiolabeled ATP (30 °C, 30 min). Following the addition of roscovitine or CT99021 to inhibit Cdk5 and GSK3β, respectively, different amounts of recombinant PP1 were added (30 °C, 30 min). Radiolabeled phosphate in β-adducin and CRMP2 was detected using autoradiography. *C*, the amount of radiolabeled phosphate removed from β-adducin and CRMP2 by PP1 was quantified by liquid scintillation counting and is presented as a graph ($n = 3$, average ± S.E.).

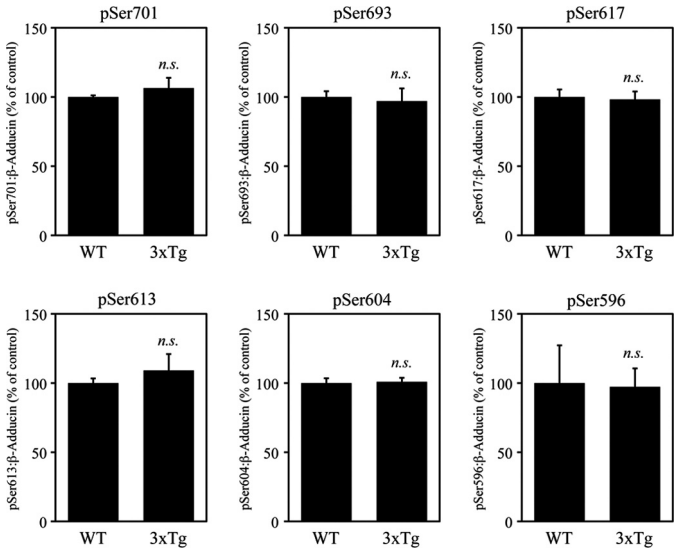

FIGURE 5. **Phosphorylation of β-adducin is not altered in a mouse model of Alzheimer disease.** Cortexes from 4-month-old female 3×Tg Alzheimer mice and wild type littermates were homogenized and subjected to Western blot analysis using β-adducin phosphospecific antibodies and a polyclonal antibody that recognizes total β-adducin. Following densitometric analysis, the ratio of phospho:total β-adducin was determined and is presented as graphs (wild type = 5, 3×Tg = 6; average ± S.E.; *n.s.*, not significant relative to control; Student's *t* test).

only partial dephosphorylation of pSer613 (~50%) but no change in phosphorylation at Ser-701, Ser-693, Ser-617, and Ser-604. In addition, β-adducin isolated from HEK293 cells was phosphorylated *in vitro* by recombinant Cdk5 or GSK3 in the presence of radiolabeled ATP, and following inhibition of the kinases, different amounts of recombinant PP1 were added to compare the efficiency of dephosphorylation (Fig. 4, *B* and *C*). Both the Cdk5 and the GSK3 phosphosites displayed relative resistance to dephosphorylation by PP1, whereas 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 disease (AD) patients for Tau and CRMP2 (42, 44). Because Cdk5 and GSK3 phosphosites in β-adducin display similar resistance to phosphatases, we investigated whether β-adducin was also hyperphosphorylated in a mouse model of Alzheimer disease. Phosphorylation levels were measured in two mouse models of AD, Triple-Tg (Fig. 5) and amyloid precursor protein/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 analyzed. Therefore, phosphorylation of β-adducin is normal in these mouse models of AD.

Phosphorylation of β-Adducin Regulates Its Subcellular Localization—The subcellular localization of β-adducin and its phosphorylated forms was investigated using immunofluorescence microscopy of cultured primary rat cortical neurons (Fig. 6*A*). β-Adducin was widely distributed throughout the cell body, nucleus, dendrites, axons, and growth cones of neurons. In particular, β-adducin was localized 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 although similar amounts of β-adducin were detected in all fractions, β-adducin phosphorylated at Ser-693 was highly enriched in the cytoplasm (Fig. 6, *B* and *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—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 (S697A/S613A/S600A) (supplemental Fig. 3) were transfected into cultured primary rat cortical neurons, and the morphology of transfected neurons after 3 days *in vitro* was analyzed using immunofluorescence microscopy (Fig. 7). Expression of wild type β-adducin increased axon elongation by ~25% in transfected neurons. In addition, the average length of branches from the axon and dendrite lengths were also increased by 25%, whereas the number of axonal branches and dendrites was 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

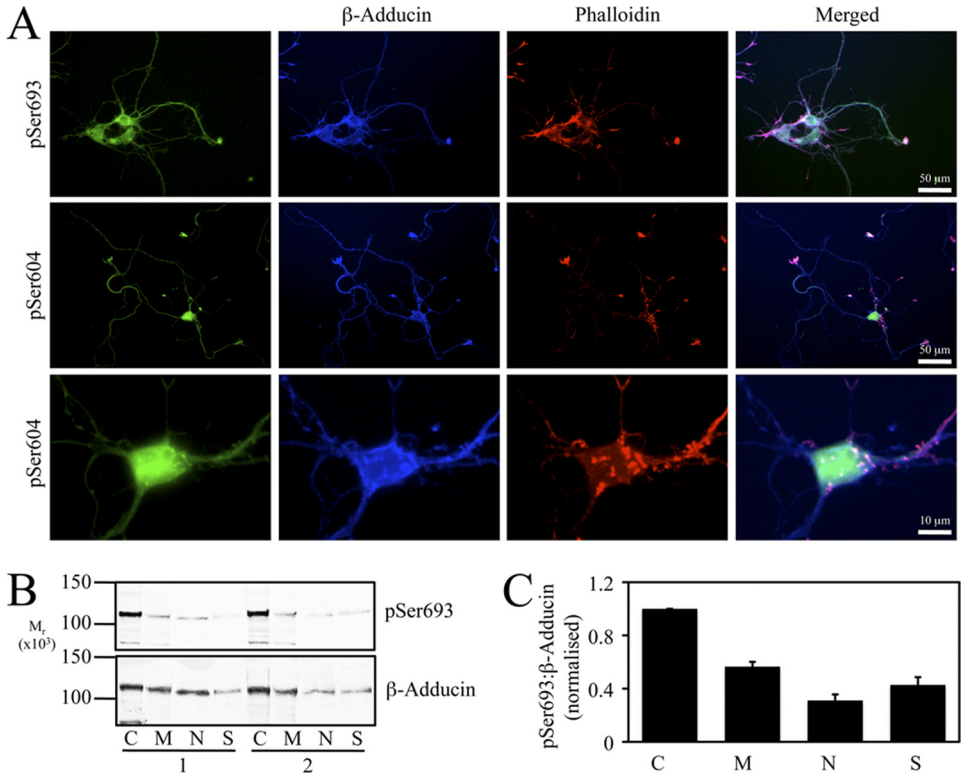

FIGURE 6. **Localization of β-adducin in neurons.** *A*, cortical neurons (3 days *in vitro*) were fixed and incubated with antibodies that recognize β-adducin phosphorylated at Ser-693 or Ser-604 (*green*), as well an antibody that recognizes total β-adducin (*blue*) and phalloidin (*red*). *B*, adult 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 recognizes β-adducin when phosphorylated at Ser-693 (*upper panel*) and an antibody that recognizes 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 ± S.E.).

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

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 because 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 (dual-specificity tyrosine phosphorylation-regulated kinase)). 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 specialized 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 three regions can be phosphorylated simultaneously on the same molecule (data not shown). This would result in phosphorylation of up to nine sites in the C-terminal domain of β-adducin, which would presumably induce signifi-

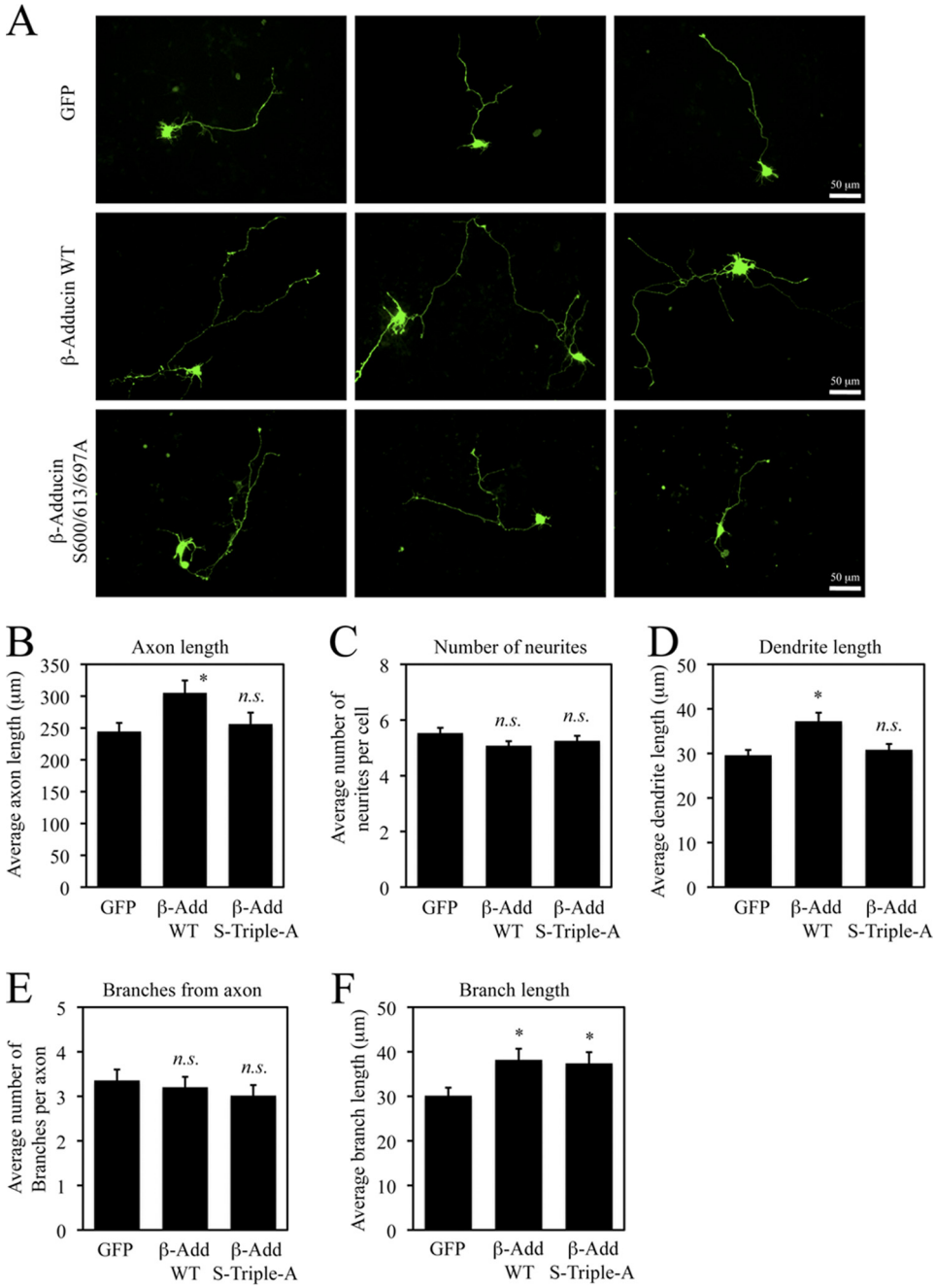

FIGURE 7. ***β*-Adducin regulates neurite outgrowth in cortical neurons.** *A*, cortical neurons were co-transfected with GFP and empty vector (*upper panels*), *β*-adducin wild type (*WT*) (*middle panels*), or *β*-adducin S697A/S613A/S600A (*S-Triple-A*) mutant (*lower panels*). Neurons were fixed and visualized using fluorescence microscopy. The morphology of transfected neurons was quantitated using the 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 (*β-Add*) wild type $n = 112$, *β*-adducin S-Triple-A $n = 110$; average ± S.E.; *, $p < 0.05$ relative to control; *n.s.*, not significant; Student's *t* test).

cant 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 laboratory 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 three Cdk5 phosphosites in *β*-adducin contain a basic residue at the +3 position, consistent with this pattern. In contrast, the GSK3 target site Ser-693 does not, although it is located close to the myristoylated alanine-rich protein kinase C substrate (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 pro-

tects 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 that phosphorylation of α-adducin at Ser-716 and Ser-726 by PKC (these sites are conserved and phosphorylated by PKC in β-adducin) localizes with spectrin at the cell membrane of Madin-Darby canine kidney cells and hippocampal neurons, in particular in dendritic spines (37). Expression of a non-PKC-phosphorylatable mutant of α-adducin (S716A/S726A) exhibited a punctate distribution of α-adducin in the cytoplasm, as well as increased cytoplasmic spectrin. This suggests that two opposing signaling pathways regulate adducin localization with spectrin at the cell membrane. PKC is among 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 localized to the membrane via myristoylation 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 two 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 postmortem 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 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 two 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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