The Roles of Cyclin-dependent Kinase 5 and Glycogen Synthase Kinase 3 in Tau Hyperphosphorylation

Florian Plattner1, Marco Angelo, and K. Peter Giese2

From the Wolfson Institute for Biomedical Research, University College London, London WC1E 6BT, United Kingdom

Hyperphosphorylation of the microtubule-associated protein tau is a characteristic feature of neurodegenerative tauopathies including Alzheimer disease. Over-activation of proline-directed kinases, such as cyclin-dependent kinase 5 (Cdk5) and glycogen synthase kinase 3 (GSK3), has been implicated in the aberrant phosphorylation of tau at proline-directed sites. In this study we tested the roles of Cdk5 and GSK3 in tau hyperphosphorylation in vivo using transgenic mice with p25-induced Cdk5 over-activation. We found that over-activation of Cdk5 in young transgenic animals does not induce tau hyperphosphorylation at sites recognized by the antibodies AT8, AT100, PHF-1, and TG3. In fact, we observed that Cdk5 over-activation leads to inhibition of GSK3. However, in old transgenic animals the inhibition of GSK3 is lost and results in increased GSK3 activity, which coincides with tau hyperphosphorylation at the AT8 and PHF-1 sites. Pharmacological inhibition of GSK3 in old transgenic mice by chronic treatment with lithium leads to a reduction of the age-dependent increase in tau hyperphosphorylation. Furthermore, we found that Cdk5, GSK3, and PP2A co-immunoprecipitate, suggesting a functional association of these molecules. Together, these results reveal the role of GSK3 as a key mediator of tau hyperphosphorylation, whereas Cdk5 acts as a modulator of tau hyperphosphorylation via the inhibitory regulation of GSK3. Furthermore, these findings suggest that disruption of regulation of GSK3 activity underlies tau hyperphosphorylation in neurodegenerative tauopathies. Hence, GSK3 may be a prime target for therapeutic intervention in tauopathies including Alzheimer disease.

Neurodegenerative tauopathies, including Alzheimer disease (AD),3 are characterized by abnormal hyperphosphorylation of the microtubule-associated protein tau at proline-directed serine/threonine phosphorylation sites (1). Amongst the main aberrantly hyperphosphorylated sites on tau are the pathological phospho-sites Ser-202/Thr-205 (AT8 site), Ser-214 and/or Ser-235 (AT100 site), Thr-231 and/or Ser-235 (TG3 site), and Ser-396/Ser-404 (PHF-1 site) (1–3). A well characterized function of tau is the assembly and stabilization of microtubules (1–3). Tau binds directly to microtubules and promotes microtubule polymerization (2, 3). Increasing phosphorylation of tau leads to its dissociation from microtubules and in turn to microtubule destabilization (4, 5). The interaction of tau and microtubules also plays an important role in the regulation of microtubule-dependent axonal transport (6, 7). A recent study proposed that deficient axonal transport may be important in the early stages of pathogenesis of AD (8). Precise regulation of phosphorylation of tau is probably important for its normal cellular functions; aberrant tau hyperphosphorylation is believed to disrupt cellular processes such as axonal transport. However, it is still not established what the physiological importance of the individual tau phosphorylation sites is.

The normal phosphorylation state of tau is balanced by antagonistic kinase and phosphatase activity. Thus, numerous protein kinases and protein phosphatases have been implicated in the abnormal hyperphosphorylation of tau (for review, see Ref. 3). The proline-directed serine/threonine kinases, cyclin-dependent kinase 5 (Cdk5) and glycogen synthase kinase 3 (GSK3), have been identified as prime candidates mediating aberrant tau phosphorylation at disease-associated sites (9–12). Cdk5 co-localizes with filamentous tau deposits and has increased activity in several tauopathies, including AD (Refs. 13–15; for review, see Ref. 16). GSK3 generates disease-associated phospho-epitopes on tau (17) and co-localizes with aggregates of hyperphosphorylated tau (18). Based on this, much research effort has been focused on the development of specific inhibitors for Cdk5 and GSK3 as potential therapeutic treatments in tauopathies (for review, see Refs. 19 and 20). However, the roles of Cdk5 and GSK3 in tau hyperphosphorylation are not fully established, and it still remains to determine the critical factors leading to abnormal tau hyperphosphorylation in tauopathies.

3 The abbreviations used are: AD, Alzheimer disease; APP, amyloid precursor protein; Cdk5, cyclin-dependent kinase 5; ERK, extracellular signal-regulated kinase; GSK-3, glycogen synthase kinase 3; IP, immunoprecipitation; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; TBS, Tris-buffered saline; TG, transgenic; WT, wild-type; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid.

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1 An Alzheimer’s Research Trust Research Fellow. Present address and to whom correspondence may be addressed: Dept. of Molecular Neuroscience, Institute of Neurology, University College London, Queen Square, London WC1N 3BG, UK. Tel.: 44-20-7676-2192; Fax: 44-20-7676-2157; E-mail: p.giese@ucl.ac.uk.

2 To whom correspondence may be addressed: Wolfson Institute for Biomedical Research, University College London, Gower St., London WC1E 6BT, UK. Tel.: 44-20-7679-6774; Fax: 44-20-7209-0470; E-mail: f.plattner@ucl.ac.uk.
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In the present study we have used a transgenic (TG) mouse line expressing low levels of the Cdk5 activator p25 (21) to analyze in vivo the impact of Cdk5 over-activation on tau hyperphosphorylation. We assessed the phosphorylation state of tau at sites characteristically hyperphosphorylated in tauopathies including the AT8, AT100, TG3, and PHF-1 sites. Surprisingly, we find that activation of Cdk5 does not affect tau hyperphosphorylation at these sites in young TG mice. However, p25-induced Cdk5 over-activation leads to inhibition of GSK3. This inhibitory cross-talk of Cdk5 and GSK3 is correlated with the finding that Cdk5 and GSK3 are associated in a complex. In old TG mice the inhibitory regulation of GSK3 is lost, and GSK3 activity is significantly increased. Furthermore, in old TG mice we observe increased phosphorylation at the AT8 and PHF-1 sites, which accords with the enhanced GSK3 activity. Consistently, we find that pharmacological inhibition of GSK3 with lithium leads to a reduction of the increased tau phosphorylation in old TG mice. Our study shows for the first time in vivo that Cdk5 can indirectly affect tau hyperphosphorylation via the regulation of GSK3 activity and establishes GSK3 as a key mediator of tau phosphorylation at disease-associated sites. Hence, these results suggest GSK3 as a prime target for therapeutic intervention in neurodegenerative tauopathies, whereas inhibition of Cdk5 may be inappropriate as a treatment for tauopathies.

**EXPERIMENTAL PROCEDURES**

**Animals**—Heterozygous p25 TG mice and wild-type (WT) control littersmates in the C57BL/6 genetic background were bred and genotyped by PCR analysis as reported (21). The mice were housed in groups of 2–5 and treated according to the Animals (Scientific Procedures) Act or 1986, UK.

**Antibodies and Reagents**—Primary antibodies used for immunoblotting are listed in Table 1. Horseradish peroxidase-conjugated secondary antibodies were from Perbio, and protease inhibitor Complete tablets EDTA-free were from Roche. All chemicals were from Sigma unless stated otherwise.

**Lysate Preparation**—Mice were euthanized using CO2, the brain was quickly removed, and the hippocampus was dissected in 4 °C lysate buffer. The hippocampi were Dounce-homogenized in 4 °C lysate buffer and stored at −80 °C. The lysate buffer (10 mM Tris, pH 7.4, 320 mM sucrose, 1% Triton X-100, 1% CHAPS and 0.025% NaN3) contained phosphatase and protease inhibitors (1 mM EDTA, 1 mM EGTA, 50 mM sodium fluoride, 2 mM sodium orthovanadate, 0.1 mM ammonium molybdate, 0.2 mM phenylarsine oxide, and Complete tablets). Protein concentrations were determined with the BCA assay (Perbio).

**Immunoblot Analysis**—Equal amounts of protein were separated on 4–16% polyacrylamide gels (Bio-Rad), transferred onto polyvinylidene difluoride membranes (Bio-Rad), and incubated in blocking buffer consisting of TBS (10 mM Tris, pH 7.6, and 150 mM NaCl) with either 3% milk or 3% bovine serum albumin. Blots were incubated overnight at 4 °C with primary antibodies (Table 1) in blocking buffer. After washing in several volumes of TBS with 0.05% Tween 20 (TBST), the blots were incubated with horseradish peroxidase-conjugated secondary antibodies in blocking buffer and washed again in TBST, and signals were visualized with the enhanced chemiluminescent system (Perbio). Band intensities from x-ray film (Amersham Biosciences) were quantified with Densitometer Quantity One (Bio-Rad) in the linear range. Blots were stripped with stripping buffer (Persbio) and reprobed with anti-β-actin and anti-synaptotagmin antibody to normalize for the amount of loaded protein.

**Immunoprecipitation (IP)**—Hippocampal lysate with a protein content of 1 mg was diluted in 1 ml of ice-cold IP buffer

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**Table 1**
Antibodies used for immunoblotting

| Antibody       | Epitope                      | Isotype   | Buffer   | Dilution | Source               |
|----------------|------------------------------|-----------|----------|----------|----------------------|
| Cdk5 (C-8)     | Cdk5 C terminus             | Rabbit IgG| BSA      | 1:3000   | Cell Signaling Technology |
| p35 (C-19)     | p35 and p25 C terminus      | Rabbit IgG| BSA      | 1:1000   | Cell Signaling Technology |
| 4G-1E          | Total GSK3α/β               | Mouse IgG | BSA      | 1:1000   | Cell Signaling Technology |
| 9332           | Total GSK3β                 | Rabbit IgG| BSA      | 1:1000   | Cell Signaling Technology |
| 9331           | Phospho-GSK3α/β (Ser-21/9)  | Rabbit IgG| BSA      | 1:1000   | Cell Signaling Technology |
| 5G-2F          | Phospho-GSK3α/β (Thr-279/276)| Mouse IgG| BSA      | 1:1000   | Cell Signaling Technology |
| AT-8           | Tau phospho-Ser-202/205     | Mouse IgG | BSA      | 1:1000   | Cell Signaling Technology |
| AT-100         | Tau phospho-Thr-212/S214    | Mouse IgG | BSA      | 1:1000   | Cell Signaling Technology |
| PHF1           | Tau phospho-Ser-396/404     | Mouse IgG | BSA      | 1:1000   | Cell Signaling Technology |
| TG3            | Tau phospho-Thr-231/S235, conformation-dependent | Mouse IgG | BSA      | 1:1000   | Cell Signaling Technology |
| BR134          | Pan-tau                     | Rabbit IgG| Milk     | 1:750    | M. Goedert           |
| TAU-5          | Pan-tau (residues 220–240)  | Mouse IgG | Milk     | 1:1000   | Chemicon             |
| SM131          | Phospho-neurofilament antibody cross-reacting with PHF-1 site on tau | Mouse IgG | Milk     | 1:1000   | Sternberger Monoclonals, Inc. |
| 9562           | β-Catenin                   | Rabbit IgG| BSA      | 1:3000   | Cell Signaling Technology |
| 06-182         | Total ERK1/2                | Mouse IgG | Milk     | 1:1000   | Upstate              |
| 4377           | Phospho-ERK1/2 (Thr-202/Tyr-204) | Monoclonal rabbit IgG | BSA | 1:1000   | Cell Signaling Technology |
| 9121           | Phospho-MEK1/2 (Ser-217/221)| Rabbit IgG| BSA      | 1:1000   | Cell Signaling Technology |
| 9251           | Phospho-SAPK/INK (Thr-183/Tyr-185) | Rabbit IgG| BSA      | 1:1000   | Cell Signaling Technology |
| PP2Ac          | Catalytic subunit of PP2A   | Mouse IgG | Milk     | 1:1000   | S. M. Dilworth       |
| PR65/A         | Scaffold/regulatory subunit of PP2A | Mouse IgG | Milk     | 1:1000   | S. M. Dilworth       |
| sc-7482        | Catalytic subunit of PP1 (total protein) | Mouse IgG | Milk     | 1:2000   | Sigma                |
| 2581           | Phospho-PP1 (Thr-320)       | Rabbit IgG| BSA      | 1:500    | Cell Signaling Technology |
| 2452           | APP (around unphosphorylated Thr-668) | Rabbit IgG| BSA      | 1:2000   | Cell Signaling Technology |
| 2451           | Phospho-APP (Thr-668)       | Rabbit IgG| BSA      | 1:1000   | Cell Signaling Technology |
| AS441          | β-AActin                    | Mouse IgG | Milk     | 1:50000  | Sigma                |
| S2177          | Synaptotagmin               | Rabbit IgG| BSA      | 1:40000  | Sigma                |

*SAPK, stress-activated protein kinase.*
(150 mM NaCl, 10 mM Tris-HCl, pH 7.6, 1% Triton X-100, 0.025% NaN₃) with protease and phosphatase inhibitors. The IP mix was precleared, and 5 µg of GSK3β antibody were added. The IP mix was incubated for 3 h on a rotator at 4 °C. One hundred microliters of IgG bead slurry were added and rotated for 3 h at 4 °C. The beads were washed 3 times with 1 ml of ice-cold IP buffer. Forty microliters of the kinase reaction mix (20 mM MgCl₂, 1 mM EDTA, 0.5 mM β-glycerol phosphate, 0.2 mM sodium orthovanadate) containing protease and phosphatase inhibitors and centrifuged, and supernatant was transferred to a new tube. One microgram of anti-Cdk5 antibody was added per tube, and the mix incubated for 1.5 h at 4 °C under constant shaking. Thirty microliters of protein A bead slurry were added and incubated for 1.5 h at 4 °C under constant shaking. After settling of the beads, the supernatant was discarded, and the beads were washed 3 times with 400 µl of IP buffer. Forty microliters of the kinase reaction mix (20 mM Tris, pH 7.6, 25 mM MgCl₂, 1 mM EDTA, 0.5 mM β-glycerol phosphate, 0.2 mM sodium orthovanadate) containing 20 µg of the Cdk5 substrate histone 1 (Upstate) and the inhibitor mixture (Upstate) with 5 µM protein kinase C inhibitor peptide, 0.5 µM cyclic AMP-dependent protein kinase A inhibitor peptide, and 0.5 µM Compound R24571 were added to the beads. The reaction was initiated by the addition of 10 µl of 100 µM ATP solution containing 5 µCi of radioactive-labeled [γ-³²P]ATP (GE Healthcare). The samples were incubated for 30 – 60 min at 30 °C. The reaction was stopped by spotting 25 µl of assay mix on p81 nitrocellulose paper (Upstate). The paper was washed 3 times in 0.75% phosphoric acid and then with acetone. The radioactivity was measured by Cherenkov counting using 25 ml of polyethylene scintillation vials filled with 12 ml of H₂O. Cdk5 activity was calculated as the difference between the activity with and without the presence of 10 µM roscovitine, a Cdk5 inhibitor.

GSK3 Activity Assay—The GSK3 activity assay was performed similarly to the Cdk5 activity assay with the following changes; lysate containing 100 µg of protein was immunoprecipitated with 1 µg of anti-GSK3β antibody. The kinase reaction mix contained 5 µg of the GSK3 substrate phospho-glycogen synthase peptide-2 (Upstate), 0.2 µM okadaic acid (a protein phosphatase 1 and 2A (PP1; PP2A) inhibitor), and 10 µM roscovitine (a Cdk5 inhibitor). The kinase reaction mix was incubated for 1.5 h at 30 °C. GSK3 activity was calculated as the difference between the activity with and without the presence of 10 mM LiCl, a GSK3 inhibitor.

Chronic Lithium Administration—22–24-month-old TG mice (n = 4) and 18–19-month-old WT mice (n = 3) were chronically treated with lithium for 1 month. For the injection, a 0.1 M LiCl solution was prepared and filter-sterilized. The first 2 days the mice were injected intraperitoneally with 1.5 meq/kg LiCl (corresponding to 10.4 mg/kg). From days 3–7 a dose of 3 meq/kg was administered. Control aged p25 transgenic mice (n = 4) were injected with saline. Thereafter, the mice were fed with powdered chow containing 1.7 g of LiCl/kg of chow for 3 weeks. Control aged p25 transgenic mice were given powdered chow without LiCl. To prevent hyponatremia, water and 400 mM NaCl solution were available ad libitum to the mice.

Data Analysis—Statistical analysis was performed using one-way analysis of variance. Data are expressed as the mean ± S.E.

RESULTS

Age-dependent Increase in Tau Hyperphosphorylation in p25 TG Mice with Constant Cdk5 Over-activation—In this study we analyzed a TG mouse line expressing the Cdk5 activator p25 to investigate in vivo the roles of Cdk5 and GSK3 in tau hyperphosphorylation. Our transgenic mouse line, in which the p25 transgene is driven by the α-Ca²⁺/calmodulin-dependent kinase II promoter, displayed low level postnatal p25 expression restricted to forebrain, with the highest levels found in the hippocampus (21, 22). Hence, we focused on biochemical changes in the hippocampus, a brain area affected in many tauopathies, including AD (1, 23). We examined the impact of p25-induced Cdk5 over-activation on the phosphorylation level of tau at sites abnormally hyperphosphorylated in tauopathies in both young (3 months) and old (18 months) p25 TG mice. To investigate these disease-related phosphorylation sites on tau, we used phospho-specific antibodies commonly employed in neuropathological studies that recognize aberrantly hyperphosphorylated epitopes on tau including AT8 (Ser-202/Thr-205), AT100 (Thr-214 and/or Ser-212), TG3 (Thr-231/Ser-235), and PHF-1 (Ser-396/Ser-404). We observed an age-dependent increase in tau hyperphosphorylated at the AT-8 site and PHF-1 site in the hippocampus of TG mice (Fig. 1, A and B). In young TG mice no enhanced tau hyperphosphorylation was observed with these antibodies (Fig. 1, A and B; supplemental Fig. 1) even though Cdk5 activity was constantly increased by ~2-fold as demonstrated by a Cdk5 kinase activity assay (Fig. 2D). In contrast, old TG mice displayed significantly elevated levels of tau phosphorylation by ~90% using both AT8 and PHF-1 antibodies (Fig. 1, A and B). The results of the PHF-1 antibody were confirmed with the phosphorylation-dependent neurofilament antibody, SM131, known to cross-react with tau hyperphosphorylated at the PHF-1 site (24). The SM131 antibody showed no changes in young mice but revealed increased tau hyperphosphorylation in old TG mice (Fig. 1, A and B). The antibodies AT100 and TG3 did not detect significant changes in phosphorylation levels between TG and WT mice at an old age (Fig. 1A and supplemental Fig. 1).

To determine whether the age-dependent increase in tau hyperphosphorylation was related to changes in expression levels of p25, p35, and Cdk5 or the level of Cdk5 activity, we examined these parameters in young and old WT and TG mice. The p25 transgene was expressed constantly at low levels at around 33% of endogenous p35 expression (Fig. 2A and Ref. 21). No age-dependent changes were detected in the expression levels of p35 and Cdk5 (Fig. 2A). Consistent with these results, Cdk5 kinase activity was increased ~2-fold in both young and old TG mice as compared with WT controls (Fig. 2B). Thus, an age-de-
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A 3-month WT 18-month WT
AT8  
PHF-1  
SMI31  
AT100  
TG3  
BR134

B

WT TG

Normalized AT8 signal

Normalized PHF-1 signal

Normalized SMI31 signal

3 month 18 month 3 month 18 month 3 month 18 month

FIGURE 1. Age-dependent increase in tau hyperphosphorylation at the AT8 and PHF-1 sites in the hippocampus of p25-expressing TG mice. A, representative immunoblots of hippocampal lysates from 3- and 18-month-old WT and p25 TG mice probed with phospho-specific tau antibodies AT8 (Ser-202/Thr-205), AT100 (Thr-214 and/or Ser-212), TG3 (Thr-231/Ser-235), and PHF-1 (Ser-396/404) recognizing pathological phosphoepitopes on tau, pan-tau antibody BR134, and SMI31, a phospho-specific neurofilament antibody cross-reacting with the hyperphosphorylated PHF-1 epitope. B, quantification of immunoblots probed with AT8, PHF-1, and SMI31 (mean ± S.E.). At 18 months of age tau hyperphosphorylation at AT8 and PHF-1 sites was significantly increased in TG mice as compared with WT mice (n = 4; AT8, F1,6 = 55.4, ***, p < 0.001; PHF-1, F1,6 = 31.2, **, p < 0.01; SMI31, F1,6 = 27.1, **, p < 0.01).

A 3-month WT 18-month WT
p35  
p25  
Cdk5  
actin

B 3-month WT 18-month WT
Normalized cdk5 activity

Normalized p38 activity

Normalized actin activity

FIGURE 2. Constant Cdk5 over-activation induced by p25 expression in the hippocampus of TG mice. A, no age-dependent changes in expression of p25 and Cdk5 between genotypes were found. Immunoblots of hippocampal lysates from 3- and 18-month-old WT and TG mice were probed with C-19 (antibody detecting the Cdk5 activator proteins, p25 and p35) and Cdk5-specific antibodies. B, Cdk5 activity (mean ± S.E.) was increased by ~2-fold in both young and old TG mice as compared with WT mice (3-month, n = 4, F1,6 = 29.0, **, p < 0.01; 18-month, n = 4, F1,6 = 33.2, **, p < 0.01). C, increased phosphorylation levels of APP (pAPP) at the Cdk5-specific site Thr-668 (as per APP695 isoform) were detected in hippocampal lysates from 3- and 18-month-old TG mice as compared with WT mice. No differences in total protein levels of APP were detected between genotypes as tested with an antibody against an unphosphorylated epitope around Thr-668 (annotated as per APP695 isoform). This site is specifically phosphorylated by Cdk5 and, hence, displays enhanced levels of phosphorylation in p25-expressing TG mice with over-activation of Cdk5 (25, 26). Consistently, we found that the phosphorylation of APP at Thr-668 was significantly increased by ~60% in both young and aged TG mice as compared with control WT mice (Fig. 2, C and D). No changes in total APP protein levels were observed (Fig. 2C). This constant elevation of APP phosphorylation in the TG mice is in accordance with the results of the Cdk5 kinase activity assay. Together, these results confirm that Cdk5 activity is constantly increased in vivo in the p25 TG mice. Nevertheless, the p25-induced Cdk5 over-activation does not induce hyperphosphorylation at the assessed disease-associated sites on tau in young TG mice. However, in old TG mice the level of phosphorylation was significantly increased both at the AT8 and PHF-1 sites on tau.

Age-dependent Inhibitory Regulation of GSK3 Activity in p25 TG Mice—Because there were no age-related changes in the activity of Cdk5 to account for the increased tau phosphorylation in the TG mice, we studied the involvement of other proline-directed kinases that have been linked to aberrant tau hyperphosphorylation (1, 3). First, we tested the phosphorylation levels of the activating regulatory sites of extra-cellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and MAPK/ERK kinase 1/2 (MEK1/2). We observed no differences in the phosphorylation levels of the activating regulatory sites on JNK and MEK1/2 (Fig. 3A and supplemental Fig. 2). The phosphorylation level at the activation site of ERK was reduced in TG mice as compared with WT controls (Fig. 3, A and B). This is consistent with the observation that Cdk5 regulates the activity of ERK via the inhibition of MEK1 (27). Assessment of the phosphorylation level of the regulatory sites of GSK3 revealed a significant change between TG mice and WT controls. GSK3 is one of the major candidate mediators of tau hyperphosphorylation in tauopathies and has been shown to phosphorylate proline-directed serine/threonine sites similarly to Cdk5 (Ref. 7; for review, see Ref. 3). One particular feature of GSK3 is that it requires priming of some of its phosphorylation sites by other kinases such as cyclic AMP-dependent protein kinase A and Cdk5 (28, 29). It has been proposed that once the priming sites on tau are modified, GSK3 is able to sequentially phosphorylate adjacent sites and, hence, induce hyperphosphorylation (30). GSK3 activity is regulated by antagonistic serine and tyrosine phosphorylation. Phosphorylation at Ser-9 in the β-isofrom and at Ser-212 in the α-isofrom inhibits GSK3 (31), whereas phosphorylation of Tyr-216 in the β-isofrom and Tyr-279 in the α-isofrom is essential for GSK3 activation (32).
We observed that the activity of GSK3 was differentially regulated in TG mice. In young TG mice, phosphorylation of GSK3β at the inhibitory site was significantly increased by ~85% (Fig. 4, A and B). This resulted accordingly in reduced GSK3β activity as assayed in a kinase activity assay using the primed substrate phospho-glycogen synthase peptide-2 (Fig. 4C). No differences in phosphorylation levels at the activation site were detected between the genotypes at any age (supplemental Fig. 3, A and B). These findings indicate that there is inhibitory cross-talk between Cdk5 and GSK3 such that p25-induced Cdk5 over-activation negatively regulates the activity of GSK3. However, in old TG mice the phosphorylation level at the inhibitory site of GSK3 was no longer elevated (Fig. 4, A and B). Moreover, GSK3 activity was significantly increased by ~75% in old TG mice as compared with WT controls (Fig. 4C). Interestingly, the amount of GSK3β phosphorylated at the activation site was significantly increased at old age in both genotypes, whereas the level of phosphorylation at the activation site on GSK3α was unaffected by genotype and age (supplemental Fig. 3). Together, these results imply that the inhibitory regulation of GSK3 is altered in old TG mice leading to over-activation of GSK3.

Association of Cdk5, GSK3, and PP2A in a Functional Complex—We observed that p25-induced Cdk5 over-activation negatively regulated GSK3β activity. To mediate this inhibitory cross-talk, these two molecules should be localized in proximity to each other or even complexed together. We tested whether Cdk5 interacts with GSK3 using co-immunoprecipitation with anti-GSK3β antibody in hippocampal lysates of WT and TG mice. We detected GSK3β protein in the immune pellet showing that the antibody is suitable for immunoprecipitation experiments (Fig. 5A). GSK3β co-immunoprecipitated with Cdk5, the catalytic PP2A subunit PP2Ac, and the scaffolding/regulatory PP2A subunit, PR65/A (Fig. 5, A–D). These results show that GSK3, Cdk5, and PP2AA interact with each other. The protein levels of the PP2A subunits, PP2Ac and PR65/A, were not different between genotypes at young and old ages (data not shown). Further evidence for association of these molecules comes from GSK3 kinase activity assays using okadaic acid, a specific inhibitor of PP1 and PP2A. Without the addition of the phosphatase inhibitor, the GSK3β activity assay showed similar levels of kinase activity for TG and WT mice (Fig. 5E). The addition of okadaic acid had no significant effect on kinase activity levels in WT mice but produced a marked reduction in TG mice (Fig. 5E). These results demonstrate that the GSK3 activity in TG mice as compared with WT mice is differently regulated by phosphatase activity. In addition, these observations demonstrate the necessity of application of suitable kinase and phosphatase inhibitors in immunoprecipitation kinase activity assays. Because we found that Cdk5, GSK3, and PP2A co-immunoprecipitate and that GSK3 activity is inhibited by Cdk5, it is necessary to use both Cdk5 inhibitors and phosphatase inhibitors in GSK3 activity assays. Interestingly, a recent study suggests that PP1 is involved in the regulation of GSK3 via a Cdk5-mediated mechanism (33). Because PP1 is inhibited via...
phosphorylation of Thr-320 by Cdc2 kinase, a Cdk5 homologue, we examined whether p25-induced Cdk5 over-activation alters the phosphorylation level at this inhibitory site. We detected neither a difference in the phosphorylation level of PP1 at Thr-320 nor a difference in total protein levels of PP1 between genotypes (supplemental Fig. 4).

**Chronic Lithium Treatment Reduces Tau Hyperphosphorylation in Aged p25 TG Mice**—We tested whether the increased GSK3 activity is responsible for the hyperphosphorylation of tau in old TG mice. For this, old TG mice were treated chronically with lithium, a commonly used reversible inhibitor of GSK3 (20, 34, 35). Lithium specifically inhibits GSK3 but not other kinases (36, 37). Chronic lithium-treatment in 22–24-month-old TG mice resulted in a significant reduction in tau hyperphosphorylation at the AT-8 and PHF-1 sites as compared with untreated old TG mice (Fig. 6, A and B). The tau hyperphosphorylation was reduced to levels comparable with those detected in young mice. Chronic lithium treatment in 18–19-month-old WT mice induced a non-significant reduction of phosphorylation levels at the PHF-1 site but no changes at the AT8 site (supplemental Fig. 5, A and B).

To test whether the lithium treatment affects Cdk5 activity, we assessed the phosphorylation level of APP at Thr-668. No changes in APP phosphorylation at Thr-668 were observed in lithium-treated TG and WT mice as compared with untreated controls (Fig. 6C and supplemental Fig. 5C). The inhibitory effect of lithium on GSK3 was confirmed by assaying the protein levels of β-catenin. Phosphorylation of β-catenin by GSK3 targets the protein for degradation via the ubiquitin-proteasome pathway (38). The level of β-catenin was increased in lithium-treated TG and WT mice as compared with untreated controls (Fig. 6D and supplemental Fig. 5D), indicating reduced GSK3 activity in lithium-treated animals. Interestingly, we observed no differences in protein levels of β-catenin between untreated TG and WT mice, both young and old, despite the altered GSK3 β kinase activities (supplemental Fig. 6). Taken together, these findings indicate that the age-dependent increase in tau hyperphosphorylation in TG mice at the pathological phospho-epitopes AT8 and PHF-1 is mediated by GSK3 activity.

**DISCUSSION**

Hyperphosphorylation of tau is a common feature of neurodegenerative tauopathies including AD. Hence, much research has focused on the identification of the protein kinases and protein phosphatases that regulate tau phosphorylation. The proline-directed serine/threonine kinases Cdk5 and GSK3 have been identified as major candidates mediating tau hyperphosphorylation at sites characteristic for neurodegenerative tauopathies (9–12). The current belief is that Cdk5 directly induces hyperphosphorylation of tau at disease-associated sites such as AT8, AT100, TG3, and PHF-1 and that this may subsequently trigger neurofibrillary tangle formation in tauopathies (16, 26). However, our findings do not support a direct role for Cdk5 in tau hyperphosphorylation. Instead, we observe that Cdk5 affects tau hyperphosphorylation at AT8 and PHF-1 sites via the regulation of GSK3 activity. Despite constant over-activation of Cdk5, we detect no changes in tau hyperphosphorylation in young TG mice. Consistently, several other p25-expressing transgenic mouse lines do not display tau hyperphosphorylation (39–41). In our young TG mice p25-induced Cdk5 over-activation results in inhibition of GSK3. In old TG mice this inhibitory GSK3 regulation is lost. The disruption of the inhibitory regulation of GSK3 in old TG mice and subsequently elevated GSK3 activity correlates with the tau hyperphosphorylation at the AT8 and PHF-1 sites. Accordingly, chronic treatment with the GSK3 inhibitor lithium leads to a reduction of tau hyperphosphorylation in old TG mice. These results are consistent with the role of GSK3 as a key medi-
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These results suggest that in old TG mice the regulation of GSK3 is equivalent to old WT mice (Fig. 4 and supplemental Fig. 3). In old TG mice, however, we find increased GSK3 activity even though phosphorylation at the inhibitory site (Ser-9) is increased in young TG mice resulting in increased Ser-9 phosphorylation (42). This indicates that the presence of p25 leads to an altered regulation within the complex or even different complex composition. The molecular mechanisms by which Cdk5 regulates the protein phosphatases, PP1 and PP2A, are not yet resolved. Our findings indicate that Cdk5 does not regulate PP1 and PP2A by affecting their expression levels or by direct phosphorylation of the inhibitory site Thr-320 on PP1 (supplemental Fig. 4). In fact, control of protein phosphatase activity is commonly mediated by a diverse complement of regulatory partners. For example, for PP1 more than 50 regulatory subunits have been described (for review, see Ref. 47). Interestingly, the function of regulatory subunits of PP1 is often controlled via phosphorylation (e.g. regulation of DARPP-32 (dopamine and cyclic AMP-regulated phosphoprotein molecular mass 32 kDa) by cyclic AMP-dependent protein kinase A (48)). Thus, it is conceivable that Cdk5 mediates its effect on PP1 and ultimately on GSK3 indirectly via the phosphorylation of one of these regulatory subunits of PP1. However, further studies will be required to identify the intricate molecular mechanisms by which Cdk5 regulates phosphatase activity.

Taken together these results suggest that GSK3 activity is regulated by an intricate interplay of phosphatases, kinases, and possibly other associating proteins. We propose that the presence of p25 induces functional changes within the complex in young transgenic mice resulting in increased Ser-9 phosphorylation levels and in turn reduced GSK3β activity. Long-term expression of p25 expression leads to altered regulatory control of GSK3 in old TG mice. For example, it might be possible that the composition of the Cdk5-GSK3β-PP2A complex is altered in aged TG mice, leading to changes in GSK3 activity at an old age. Moreover, we speculate that this age-dependent disruption of the regulatory cross-talk between GSK3 and Cdk5 may underlie tau hyperphosphorylation in tauopathies.

We find that prolonged exposure to low levels of p25 expression induces tau hyperphosphorylation at the AT8 and PHF-1 sites (Fig. 1). Interestingly, a study analyzing two transgenic mouse lines expressing p25 at different levels at young age and GSK3β in a functional ~450-kDa complex containing tau (44). Furthermore, Cdk5 and GSK3 have been implicated in protein complex formation with PP1, inhibitor 2, and 14-3-3 (45, 46). Functional cross-talk has been observed for some of the proteins associated in these complexes. For example, PP1 activity is regulated by both Cdk5 and GSK3 via inhibitor 2 (45). We find that application of okadaic acid, a specific PP1 and PP2A inhibitor, alters the level of GSK3β activity in TG mice but not WT littermates (Fig. 5E). This observation is consistent with involvement of PP1 or PP2A in the regulation of GSK3. It further indicates that the protein complex involving GSK3, Cdk5, and PP2A is functionally different in TG mice as compared with WT mice. It is conceivable that the presence of p25 leads to an altered regulation within the complex or even different complex composition. The molecular mechanisms by which Cdk5 regulates the protein phosphatases, PP1 and PP2A, are not yet resolved. Our findings indicate that Cdk5 does not regulate PP1 and PP2A by affecting their expression levels or by direct phosphorylation of the inhibitory site Thr-320 on PP1 (supplemental Fig. 4). In fact, control of protein phosphatase activity is commonly mediated by a diverse complement of regulatory partners. For example, for PP1 more than 50 regulatory subunits have been described (for review, see Ref. 47). Interestingly, the function of regulatory subunits of PP1 is often controlled via phosphorylation (e.g. regulation of DARPP-32 (dopamine and cyclic AMP-regulated phosphoprotein molecular mass 32 kDa) by cyclic AMP-dependent protein kinase A (48)). Thus, it is conceivable that Cdk5 mediates its effect on PP1 and ultimately on GSK3 indirectly via the phosphorylation of one of these regulatory subunits of PP1. However, further studies will be required to identify the intricate molecular mechanisms by which Cdk5 regulates phosphatase activity.

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FIGURE 7. Proposed mechanism of the inhibitory cross-talk of Cdk5 and GSK3. Association of p25 with Cdk5 leads to activation of Cdk5. Activated Cdk5 can inhibit GSK3 by indirect regulation of the inhibitory serine phosphorylation of GSK3. Because several protein kinases and phosphatases have been implicated in the direct regulation of the inhibitory phosphorylation of GSK3 (35), it is conceivable that Cdk5 acts via these protein kinases and phosphatases. Consistently, our results and data from others (33) indicate that Cdk5 acts via protein phosphatases such as PP1 and PP2A, possibly by affecting regulatory subunits. GSK3 phosphorylates tau at several disease-associated epitopes. Thus, disruption of inhibitory GSK3 regulation, for example by altered Cdk5 function, may lead to tau hyperphosphorylation. However, pharmacological GSK3 inhibitors, such as lithium, can diminish levels of tau hyperphosphorylation and, hence, may be relevant to the treatment of tauopathies.

observed tau hyperphosphorylation at the AT8 and PHF-1 sites in the high level p25-expressing line but not in the low level p25 line (26). The fact that tau hyperphosphorylation is equivalently affected by a short period of high level p25 expression and a long period of low level p25 expression suggests that comparable processes are induced in both cases independently of aging factors. Concluding from this, “lifetime exposure” to p25 could account for changes in GSK3 activity with age in our p25-expressing mice independently of aging effects. Furthermore, this may have implication in AD where p25 has been proposed to accumulate (15).

Next to the inhibitory regulation of GSK3β, another mechanism by which Cdk5 can affect tau hyperphosphorylation is the priming of phosphorylation sites on tau (28, 50). Cdk5 has been implicated in the priming of tau at the distinct sites Ser-202, Ser-235, and Ser-404 for GSK3β (44, 50). Priming of these sites by Cdk5 and subsequent GSK3β-catalyzed phosphorylation at the adjacent sites results in the generation of the disease-associated phospho-epitopes, AT8, TG3, and PHF-1. Hence, it is conceivable that in old p25 TG mice, where both Cdk5 and GSK3 are simultaneously over-activated, these two kinases synergize to induce tau hyperphosphorylation at these sites. Accordingly, we observe hyperphosphorylation of tau at the AT8 and PHF-1 sites. In this context, the inhibitory regulation of GSK3 by Cdk5 might represent an important mechanism to regulate the level of tau phosphorylation and prevent excessive phosphorylation at primed sites. The phosphorylation of Ser-214 on tau is thought to be regulated by cyclic AMP-dependent protein kinase A (PKA), which can also prime tau at Ser-202 and Ser-404 for GSK3β (51). We do not observe tau hyperphosphorylation at the AT100 site (Ser-214 and/or Ser-212) in old TG mice, suggesting that this site is specifically phosphorylated by PKA and not by Cdk5 or GSK3 in vivo. In conjunction, these results reveal that different kinases may mediate phosphorylation at distinct sites on tau to serve specific physiological functions.

The inhibitory control of GSK3 by Cdk5 might constitute an important mechanism underlying the regulation of physiological functions such as the control of tau phosphorylation and regulation of axonal transport. An important implication of the inhibitory cross-talk of Cdk5 and GSK3 in regard to the development of therapeutic treatments is that the application of pharmacological Cdk5 inhibitors may not be an appropriate treatment for tau hyperphosphorylation. Our results suggest that inhibition of Cdk5 might render GSK3 more active, resulting in enhanced tau phosphorylation. Furthermore, Cdk5 inhibition might impact on cognitive processes, as Cdk5 activity has been implicated in synaptic plasticity (Ref. 22; for review, see Ref. 52). Because our findings indicate that GSK3 is a key mediator of tau phosphorylation, we suggest that specific inhibitors of GSK3 may be a useful tool for therapeutic intervention in neurodegenerative tauopathies, including AD. Moreover, GSK3 regulates abnormal amyloid-β peptide production (49), another characteristic feature of AD (23). In conjunction, these data imply a central role for GSK3 signaling in molecular changes underlying AD, which suggests GSK3 as a prime therapeutic target.

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