A Positive Feedback Loop between Glycogen Synthase Kinase 3β and Protein Phosphatase 1 after Stimulation of NR2B NMDA Receptors in Forebrain Neurons

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Received for publication, March 11, 2005, and in revised form, August 19, 2005. Published, JBC Papers in Press, September 19, 2005, DOI 10.1074/jbc.M502699200

Glutamate is a major excitatory neurotransmitter in the central nervous system. In addition to its role in synaptic transmission, glutamate can also induce neuronal plasticity and promote neuronal survival during development. The latter effects are mediated by a subtype of glutamate ionotropic receptors, N-methyl-D-aspartate receptors (NMDARs) (1). On the other hand, excessive activation of NMDARs causes excitotoxic cell death that underlies neuron loss triggered by hypoxia, epilepsy, or neurotrauma (2).

It has been recently proposed that NMDARs may produce diverse outcomes depending on their subunit composition. Whereas the NR2A-containing NMDARs stimulate CRE-driven transcription and enhance neuronal survival, the NR2B NMDARs may inhibit CRE-mediated transcription and trigger excitotoxic cell death (3). Decreases in CRE-mediated transcription may play a role in neurodegeneration. For instance, Huntington disease (HD)-associated mutant forms of huntingtin inhibit transcription of CRE-regulated genes, which are required for proper neuronal function and long term neuronal survival (4–6).

Activation of CRE-driven transcription by NMDARs depends on phosphorylation of CREB at Ser133 (7, 8). Stimulation of NR2A NMDAR results in increased levels of phospho-Ser133 (pSer133) whereas stimulation of NR2B NMDAR reduces pSer133 (3). The latter effect may be because of activation of protein phosphatase 1 (PP1) that has been shown to target pSer133 in NMDAR-stimulated hippocampal or cerebellar neurons (9, 10).

PP1 activity is regulated by phosphorylation. For instance, PKA-mediated phosphorylation of a PP1 inhibitor 1 (I1) increases its potency to block PP1 (11, 12). Also, overexpression of I1 with a mutation that mimicked phosphorylation by PKA enhanced learning and learning-induced CRE transcription (13). On the other hand, phosphorylation of PP1 inhibitor 2 (I2) at threonine 72 (Thr72) reduces PP1 inhibition (11, 12). The kinases that phosphorylate Thr72 include GSKβ, ERK1/2, and CDKs (14–16).

Glycogen synthase kinase 3β (GSK3β) is a protein kinase that is up-regulated in the brains of Alzheimer disease (AD) patients where it phosphorylates Tau and may contribute to β-amyloid-induced neuronal death (17–20). Inhibition of GSK3β protects against cell death induced by overexpression of mutant huntingtin, suggesting a possible role in HD (21). In healthy neurons, the basal activity of GSK3β is limited by inhibition through phosphorylation at Ser9 (22, 23). Ser9 kinases include Akt, ERK1/2/p90RSK, p70S6 kinase, PKA, and protein kinase C (22, 23) whereas PP1 and protein phosphatase 2A (PP2A) are implicated in dephosphorylation of Ser9 (24–26). Interestingly, Zhang et al. (26) showed that in several cell lines, GSK3β engaged in a positive feedback loop with PP1 (26). This occurred through GSK-mediated increase of I2 phosphorylation, PP1 activation, PP1-mediated dephosphorylation of pSer9, and further activation of GSK3β (26).

As the mechanism of NR2B-mediated inhibition of CRE transcription is not fully understood, we investigated a possibility that CREB dephosphorylation following NR2B stimulation is regulated by GSK3β. We report the NR2B NMDAR-triggered activation of GSK3β that was mediated by PP1. We also show that GSK3β further amplified PP1 activity and enhanced CREB dephosphorylation by PP1.
EXPERIMENTAL PROCEDURES

Materials—Plasmids containing rat GSK3β wild-type and CRE-Luc (CRE-luciferase reporter) have been previously described (27, 28). The EF1α LacZ construct was from Invitrogen. The polyclonal anti-GSK3β antibody used for immunoprecipitation was from Santa Cruz Biotechnology. The rabbit antiphospho-ser9 GSK3β polyclonal antibody was from BIOSOURCE, International (Camarillo, CA); the mouse anti-GSK3α/monoclonal and rabbit anti-CREB monoclonal antibodies were from Upstate Biotechnology, Inc. The anti-Akt, anti-ERK1/2, anti-phospho-ser473 Akt, anti-CREB pSer133 antibodies were from Cell Signaling (Beverly, MA); the antiphospho-ERK1/2 antibody (anti-AC-TIVE™ MAPK pAb) was purchased from Promega; the antiphospho-ser202 Tau (clone AT8) was from Autogen Bioclear (Mile Elm, UK). The rabbit antiphospho-ser9 GSK3β polyclonal antibody was from Cell Signaling. The sheep anti-I2 antibody was a gift from Dr. David Brautigan, University of Virginia. The anti-NR2A, -NR2B, and -NR2C antibodies were provided by Dr. Anthone Dunah, Harvard Medical School. Okadaic acid (OA) and tautomycin were purchased from Calbiochem (San Diego, CA). FK506 (Tacrolimus) was from A.G. Scientific (San Diego, CA). SB216763 was from Tocris Bioscience (Ellisisville, MO). All other reagents were from Sigma.

Cell Culture and Transfection—Cortical or hippocampal neurons were prepared from newborn Sprague-Dawley rats as described (29). Culture medium was Basal Medium Eagle (BME) supplemented with 10% heat-inactivated bovine calf serum (Hyclone, Logan, Ut), 35 mM glucose, 1 mM L-glutamine, 100 units/ml of penicillin, 0.1 mg/ml streptomycin. Cell-plating densities were 2 × 0.8 × 10⁶ per 35-mm plate for cortical or hippocampal neurons, respectively. Cytochrome arabinoside (2.5 μM) was added to cultures on the second day after seeding (DIV2) to inhibit the proliferation of non-neuronal cells. Additional glucose (4.5 mM) was added at DIV2 and then at DIV6. Cells were used for experiments at DIV6–8. Transient transfections were performed on DIV4 using the Lipofectamine 2000 reagent (Invitrogen) (30).

Drug Treatment—Dizocilpine maleate (MK-801), ifenprodil, Ro-25-6981, NBQX, CNQX, FK506, tautomycin, SB216763, and okadaic acid were dissolved in dimethyl sulfoxide (Me₂SO). The final concentration of Me₂SO in the medium was 0.2–0.4%. NMDA and glutamate were prepared from newborn Sprague-Dawley rats as described (29). Glucose, a physiological ligand of NMDAR, was added to the medium in amounts that would not affect the viability of the cells. The final concentration of glucose in the medium was 2% (w/v). The final concentration of NMDA in the medium was 0.2–0.4%. NMDA and glutamate were dissolved in culture medium. For experiments with tautomycin and SB216763, cells were dissolved in serum-free culture medium (BME supplemented with 35 mM glucose, 1 mM L-glutamine, 100 units/ml of penicillin, 0.1 mg/ml streptomycin, and 2.5 μM cytosine arabinoside). Tautomycin or SB216763 were added 1 or 2 h before NMDA, respectively.

Injections of Quinolinic Acid—Quinolinic acid (QA) was dissolved in 0.2 ml of 1.25% (v/v) 2-methyl-2-butanol (Sigma-Aldrich) in saline. These mice were stereotactically injected using a 10-μl Hamilton syringe with 1 μl of saline containing 0 or 30 nmol of quinolinic acid (cat. P63204, Sigma-Aldrich) at 4 coordinates (all from Bregma): for striatal injections 0.7 mm rostral, 1.9 mm lateral (left and right), 2.5 mm ventral, and for hippocampal injections 2.0 mm caudal, 1.5 mm lateral (left and right), 1.6 mm ventral. The injection of quinolinic acid was performed over 2 min, and the injection needle was retained in position for 2 min before and after injection. Mice were decapitated 1 h after injection and dissected on ice. The striata, hippocampi, and cerebellum were isolated, frozen on dry ice, and kept at −80 °C for future protein extraction.

GSK3β Kinase Assay—GSK3β activity was quantitated using an immune complex kinase assay as described previously (32).

Western Analysis and Immunostaining—Western blot analysis were performed as described (29, 33, 34). To detect the phosphorylation shift of I2 by Western blotting, 15% polyacrylamide gels were used. For all other epitopes, proteins were separated on 10% gels.

Promoter Assays—Promoter assays were performed as described (35). Briefly, 0.2 × 10⁶ cells were plated onto each well of a 24-well plate coated with poly-d-lysine. At DIV4–DIV5, neurons were co-transfected with the CRE-Luc (0.8 μg/wells) and EF1αLacZ DNA (0.55 μg/wells) with or without GSK3β (0.12–0.2 μg/wells) expression plasmid. Three days after transfection neurons were treated with NMDA for 20 h. The luciferase activity was determined using a luciferase assay kit (Promega) and normalized to β-galactosidase activity that was assayed using a kit from Promega.

Statistical Analysis—Statistical analysis of the data was performed using one-way analysis of variance (ANOVA).

RESULTS

NMDAR Stimulation Activates GSK3β—To test the possibility that GSK3β participates in signaling activated by NMDARs, we evaluated the effects of NMDA on GSK3β activity. As others have shown that reduced levels of the inhibitory GSK3β phosphorylation at serine 9 correlate with its activation (22, 23), we measured the levels of phospho-ser9 (pSer9) in NMDA-stimulated neurons. Western blot analysis with an antibody specific for pSer9 revealed decreased levels of this phosphorylation in cultured neurons that were stimulated with NMDA (Fig. 1, A and B). A 60% decrease was evident in both hippocampal and cortical neurons 20 min after addition of 100 μM NMDA (Fig. 1, A and B). The reduced pSer9 levels were observed up to 6 h after initiation of the treatment (Fig. 1, A and B).

Dose response experiments showed that the decrease in pSer9 was triggered by NMDA concentrations as low as 10 μM (Fig. 1C). Also, glutamate, a physiological ligand of NMDAR, reduced the pSer9-dependent inhibition of GSK3β.

As it was reported that GSK3β may be activated through the phosphorylation at Tyr216 residue (36), we evaluated NMDA effects on pTyr216 levels. Phosphorylation of Tyr216 was not affected by NMDA in cortical or hippocampal neurons (Fig. 1E). This suggests that NMDAR signaling does not involve modulation of pTyr216.

To test whether NMDAR stimulation in the adult brain in vivo can also produce a decrease in GSK3β pSer9 levels, we injected a NMDAR agonist, quinolinic acid (QA) into neostriatum and hippocampus of adult mice. Intrastriatal injections of QA produce a pathology with a striking resemblance to HD (37). Intraplacement QA injections result in seizures and loss of pyramidal neurons (38). As a negative control we used cerebellum that is far from the forebrain injection sites. One hour after QA administration, there was a significant decline of pSer9 levels that reached 39 or 51% of control values in striata or hippocampi, respectively (Fig. 2). The cerebellar pSer9 levels in QA-treated mice were similar to those in control animals (Fig. 2). These data indicate that NMDAR stimulation in the adult mouse brain rapidly activates GSK3β by dephosphorylation of pSer9.

To verify that the NMDAR-triggered decrease of pSer9 was accompanied by an elevation of GSK3β activity, we performed a GSK3β immunoprecipitation kinase assay. In cultured hippocampal neurons, the kinase activity of GSK3β increased as early as 5 min after 100 μM NMDA treatment (159% increase, p < 0.01, Fig. 3A). The maximal
FIGURE 1. Stimulation of NMDAR decreases the inhibitory phosphorylation of GSK3β at Ser9. Cultured rat cortical or hippocampal neurons were isolated from P1 pups and treated as indicated, 6–8 days after plating (DIV6–7). The phosphorylation of GSK3β at Ser9 (pGSK3β) or Tyr216 (pTyr216/GSK3β) was analyzed by Western blotting and quantified after normalization against the total GSK3β content. A and B, NMDA at 100 μM decreased pSer9 in both hippocampal and cortical neurons. Data in the graph represent four independent experiments. Error bars are S.E. The decline in pSer9 was statistically significant (p < 0.001, cortical neurons; p < 0.001, hippocampal neurons, ANOVA). C, Ser9 dephosphorylation by NMDA was dose-dependent as measured at either 20 min (hippocampal) or 60 (cortical) minutes after treatment. D, glutamate-induced pSer9 dephosphorylation was mediated by NMDAR. Cortical neurons were exposed for 60 min to glutamate in the presence or absence of NMDAR blocker (MK, 10 μM MK801) or non-NMDA glutamate receptor blockers (CN, 10 μM CNQX; NB, 10 μM NBQX). E, phosphorylation of Tyr216 of GSK3β or Tyr279 of GSK3α was not affected by the NMDA treatment. The numbers under the blots in C, D, and E are relative pSer9 or pTyr216 levels. Results shown in C–E were replicated in three independent experiments.

FIGURE 2. The NMDAR agonist quinolinic acid reduces pSer9 levels in the adult mouse brain. 30 nmol of quinolinic acid (QA) or vehicle (saline, Veh) were injected into the striata and hippocampi of male FVB mice. Animals were sacrificed 60 min after the treatment. The phosphorylation of Ser9 of GSK3β (pGSK3β) was analyzed by Western blotting. A significant dephosphorylation of pSer9 was found in the striatum and the hippocampus (Hip). The pSer9 levels were unaffected in cerebellum (Cb), which was used as a negative control. Data on the graph represent the mean of 5–6 animals from two independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ANOVA.

increase of 172% was seen at 20 min after addition of 100 μM NMDA (p < 0.001, Fig. 3A). The elevated GSK activity was still present at 60 min (128%, p < 0.05, Fig. 3A) and declined to control values 3 h after treatment. The reduction of GSK3β activity was despite the persistent dephosphorylation of pSer9 (Fig. 1A). This discrepancy is likely because of decreased levels of the total GSK3β protein observed at 1 and 3 h after NMDA stimulation (Fig. 1A). The reduction of GSK3β levels may be caused by the degradation of this protein during excitotoxicity, which is induced by 100 μM NMDA.3

To determine if stimulation of NMDAR increased GSK3β activity in intact neurons, we evaluated the effects of NMDA on Tau phosphorylation at the Ser352 residue. This phosphorylation has been shown to be carried out by GSK3β (39). In cortical neurons, phospho-Tau levels increased by 42% at 5 min after treatment with 100 μM NMDA (p < 0.01) suggesting GSK3β activation. At later time points, phospho-Tau levels decreased (data not shown), which is consistent with reports that PP2B or PP1/PP2A dephosphorylate Tau after NMDAR stimulation (40, 41). Therefore, activation of Tau phosphatases may antagonize the effects of GSK3β on Ser352. Together, these data indicate that activation of NMDAR can increase the kinase activity of GSK3β.

GSK3β Activation Is a Specific Response to Stimulation of NR2B NMDARs—NMDARs are heterotetramers of two NR1 and two NR2 subunits (1, 42). There is one gene for NR1 subunit and four genes for NR2 subunits including NR2A, -B, -C, and -D. We found that NR2A, NR2B, and NR2C were expressed in cultured cortical or hippocampal neurons (Fig. 4A), whereas NR2D expression was undetectable (data not shown). This suggests that cultured neurons possess diverse NMDARs that may trigger distinct responses to NMDA. Therefore, we determined which NMDARs may mediate GSK3β activation by NMDA in neurons. The NMDA-induced decrease in GSK3β pSer9 levels was abolished by an NR2B-selective NMDAR blockers, ifenprodil or Ro-25-6981 (Fig. 4, B and C). The effect was present in both hippocampal and cortical neurons and was identical to that of APV, which is a non-

3 A. Habas, E. Szatmari, and M. Hetman, unpublished observation.
Selective NMDAR antagonists (Fig. 4, B and C). This suggests that pSer9 dephosphorylation in NMDA-treated neurons is mediated by NMDARs that contain the NR2B subunit.

NMDARs act as ion channels, which upon activation increase intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) (42). Because the rising [Ca\(^{2+}\)]\(_i\), is critical for mobilization of NMDAR-activated signaling pathways (42), we tested whether Ca\(^{2+}\) influx by routes other than NMDAR can reduce pSer9 levels. Treatment with 55 mM KCl in the presence of a NMDAR selective antagonist (Fig. 4, B) did not significantly affect pSer9 levels (Fig. 4A). To quantify the pSer9 levels, β-actin was used for normalization. Data in the graph represent the mean of eight independent experiments ± S.E.; **, p < 0.01, ANOVA.

PP1 Mediates NMDA-induced GSK Activation—The NMDA-triggered decrease in pSer9 levels may be caused either by inhibition of Ser9 kinases or by stimulation of Ser9 phosphatases. As a first step to differentiate between these two possibilities, we examined the activity of Akt, the main GSK3β Ser9 kinase in cultured neurons (30). Because in some cell types ERK1/2 signaling may increase GSK3β Ser9 phosphorylation (25, 44–46), we also studied the status of ERK1/2 after NMDA treatment. The activities of Akt and ERK1/2 were monitored by Western blotting with antibodies specifically recognizing the phosphorylated, activated forms of these kinases. NMDA stimulation did not affect Akt whereas it activated ERK1/2 at 5, 20, or 60 min after treatment (Fig. 5A). However, pSer9 levels were significantly reduced at these time points (Fig. 5A). Therefore, inhibition of Ser9 kinases including Akt or ERK1/2-activated p90RSK is not a likely explanation for GSK3β activation by NMDA.

GSK3β and PP1 in NMDA Receptor Signaling

NMDARs activate several protein kinases including PP1, PP2A, and PP2B (47, 48). Therefore, we determined the effects of protein phosphatase inhibitors OA, tautomycin, and FK506 on the NMDA-triggered pSer9 decline. If applied to cultured cells, 1 μM OA inhibits PP2A and PP1, whereas at the lower concentrations it acts selectively on PP2A (49). Indeed, OA at concentrations ≥10 nM significantly increased phosphorylation of a recognized PP2A target, ERK1/2 (Ref. 50 and Fig. 5B). Similarly, OA applied at the concentrations ≥10 nM increased basal pSer9 levels (Fig. 5, C and D). The increase was concentration-dependent reaching maximal values at 1 μM (10.9- or 5.7-fold of control levels in cortical or hippocampal neurons, respectively; Fig. 5, C and D). These data suggest that under basal conditions, Ser9 dephosphorylation may be carried out by both PP2A and PP1. On the other hand, the NMDA-induced reduction in pSer9 levels was abolished by 1 μM OA but not by lower OA concentrations (Fig. 5, C and D). In addition, a PP1-specific inhibitor, tautomycin (51) did not affect the basal pSer9 or pERK1/2 levels (Fig. 5E). In contrast, tautomycin prevented pSer9 decline in NMDA-treated neurons (Fig. 5E). PP2B inhibition by FK506 did not affect pSer9 levels in basal conditions or after NMDAR activation (Fig. 5F). These data suggest that a PP1-like phosphatase activity mediates the NMDA-induced dephosphorylation of pSer9 whereas a PP2A-like phosphatase dephosphorylates pSer9 under basal conditions.

GSK3β Enhances PP1 Activation in NMDAR-stimulated Neurons—GSK3β can stimulate PP1 activity by phosphorylation of I2 (11). Therefore, we tested if NMDAR-mediated GSK3β activation by PP1 can lead to a further increase of PP1 activity. To address this issue, we inhibited GSK3β with LiCl or a specific pharmacological inhibitor, SB216763 (52–54). In basal conditions and after NMDA treatment, 20 mM LiCl effectively reduced Tau phosphorylation at Ser202, indicating GSK3β inhibition (Fig. 6A). Similar effects were observed if GSK3β was inhibited with 5 μM SB216763 (Fig. 6B). Interestingly, LiCl or SB216763 reduced the NMDA-induced decline of pSer9 suggesting that GSK contributes to the NMDA-triggered increase of PP1 activity that targets GSK3β itself (Fig. 6, C and D). GSK3β activates PP1 by inhibitory phosphorylation of I2 at the Thr72 (55). This phosphorylation can be detected by a mobility shift of phospho-I2 visualized by SDS/PAGE and Western blotting (16). Therefore, we used this technique to determine if GSK may phosphorylate I2 in primary neurons. We found that in cortical neurons, inhibition of PP1 with OA produced accumulation of phospho-I2 (Fig. 6E). This indicates that in neurons, I2 phosphorylation is transient because of rapid dephosphorylation and reactivation by PP1. Indeed, we did not detect pI2 in either NMDA-stimulated or unstimulated neurons if OA was not included in the media (data not shown). OA-induced accumulation of pI2 was blocked by LiCl, suggesting that GSK3β phosphorylates I2 in intact neurons (Fig. 6E). Together, these data indicate that in NMDA-treated neurons, PP1 activates GSK3β which further stimulates PP1. That effect may be mediated at least in part by the transient phosphorylation of I2.

GSK3β Contributes to the PP1-mediated Inactivation of CREB—In mature neurons, activation of CREB by NMDAR is mediated through the transient increase in phosphorylation of Ser133 (3, 9). Also in our hands, a 5-min treatment of cortical neurons with 100 μM NMDA increased pSer133 levels 6.3-fold above controls (Fig. 7A). The increase was followed by a decline reaching 0.32- or 0.29-fold of control levels 20 or 60 min after treatment, respectively (Fig. 7A). This suggests that NMDA triggers phosphorylation and then dephosphorylation of CREB Ser133. The reduction of pSer133 levels at 60 min was blocked by OA at 1 μM but not at the lower concentrations that affected PP2A (Figs. 7B and 5B). Also a PP1-specific inhibitor, tautomycin abolished pSer133 phosphorylation (Fig. 7C).
dephosphorylation after 60 min of NMDA exposure (Fig. 7C). These results indicate a PP1 role in CREB dephosphorylation after NMDA treatment.

Because NR2B NMDARs appear to mediate both GSK3β-dependent amplification of PP1 activity (Fig. 6) and pSer133 dephosphorylation (3), we tested if GSK3β may contribute to CREB dephosphorylation at Ser133. First, we determined if GSK3β inhibition may affect the increase in pSer133 that was present at 5 min of NMDAR stimulation (Fig. 7, A and D) and that reflects the increased activity of CREB Ser133 kinase/kinases (3, 9, 56). GSK3β inhibition with LiCl or SB216763 did not affect the early increase of pSer133 levels (Fig. 7D). These suggest that GSK3β does not regulate the phosphorylation of CREB Ser133. To determine GSK effects on CREB pSer133 dephosphorylation, neurons were treated for 60 min with 100 μM NMDA in the presence or absence of LiCl or SB216763. For instance, NMDA reduced pSer133 levels to 0.3-fold of controls (Fig. 7E). In NMDA-stimulated neurons with GSK3β inhibited by LiCl, pSer133 decline was reduced to 1.4-fold of controls (Fig. 7E). Similar results were obtained with a GSK3β drug inhibitor, SB216763 (Fig. 7F). These data suggest that GSK3β enhances PP1-mediated inhibition of CREB.

To test if NR2B-activated GSK3β inhibits non-NR2B-mediated stimulation of CRE transcription, we determined the effects of GSK3β overexpression on the activity of a CRE-driven luciferase reporter gene. Hippocampal neurons were transfected with this plasmid and 72 h later were stimulated with 20 μM NMDA. After 20 h, luciferase activity was determined. We have used NMDA at 20 μM because this concentration did not significantly reduce neuronal survival (data not shown). Therefore, changes in luciferase activity in neurons stimulated with 20 μM NMDA are because of transcriptional regulation but not cell death. NMDA increased CRE-driven transcription to 350% of control levels (Fig. 8A). The increase was abolished by the non-selective NMDAR antagonists, MK801 or APV, but not by the NR2B-specific blocker, ifenprodil, supporting the involvement of non-NR2B NMDARs in the activation of CRE transcription (Fig. 8A).

**DISCUSSION**

Our data demonstrate that: (i) stimulation of the NR2B NMDAR causes a PP1-mediated disinhibition of GSK3β in cultured primary neurons and in the adult mouse brain, (ii) GSK3β provided a stimulatory feedback to PP1 further enhancing the activation of this phosphatase by NMDA, and (iii) GSK3β increased PP1-mediated inhibition of CREB in NR2B NMDAR-stimulated neurons. Collectively, these data identify a novel role for GSK3β in the regulation of phosphorylation-dependent signaling activated by NMDARs. GSK3β appears to serve as an amplifier of NR2B NMDAR-mediated activation of PP1. As a consequence, GSK3β antagonizes the NMDA-triggered signaling events that involve increased phosphorylation of PP1 targets including non-NR2B-dependent CRE-driven transcription.

We found that stimulation of the NR2B subtype of glutamate NMDA receptors activates GSK3β by PP1-mediated decrease in pSer9 levels. In addition to glutamatergic neurotransmission, GSK3β is also modulated by dopamine and serotonin. For instance, drugs enhancing serotonergic neurotransmission increase pSer9 levels and inhibit GSK3β activity in mouse brain (57). This effect was reported to involve 5HT1A receptors. On the other hand, activation of 5HT2 receptors activated GSK3β by decreasing the phosphorylation at Ser9. Also, stimulation of dopamin-
Protein phosphorylation is normally regulated by the opposing activities of phosphorylating kinases and dephosphorylating phosphatases (11). However, the reported cases of decreased pSer9 in neurons were usually associated with reduced activity of Akt rather than increased activity of pSer9 phosphatases (30, 58, 59). Others have reported a possible role of pSer9 in regulation of Ser9 levels in unstimulated cells. The graphs represent the mean of four independent experiments ± S.E.; statistical significance is indicated (***, p < 0.001; **, p < 0.01; *, p < 0.05; ns, not significant, ANOVA). In D, the numbers under the blot indicate relative pSer9 levels. E, cortical neurons were treated with 100 μM NMDA in the presence or absence of 500 nM tautomycin for 60 min. The NMDA-induced decline of pSer9 was abolished by tautomycin suggesting PP1 involvement. The increase in basal phosphorylation of Ser9 in neurons treated with OA at 100 nM increases basal pERK1/2 levels suggesting PP2A inhibition. C and D, cortical or hippocampal neurons were treated with 100 μM NMDA for 60 or 20 min, respectively, in the presence or absence of OA. Note that the NMDA-mediated dephosphorylation of GSK3β pSer9 was inhibited by 1000 nM but not by the lower OA concentrations suggesting PP1 involvement. The increase in basal phosphorylation of Ser9 in neurons treated with OA at 100 nM increases basal pERK1/2 levels indicating activation of pSer9 kinase pathways, Akt, and/or ERK1/2. Therefore, our results imply that a selective increase in activity of a Ser9 phosphatase, PP1 may serve as an important mechanism to control GSK3β activity in the nervous system. This is supported by the recent report that GSK3β activation by PP1-mediated pSer9 dephosphorylation is involved in the inhibitory action of a cyclin-dependent kinase 5 blocker, olomucin on axonal transport in cortical neurons (61). Also, PP1 was shown to dephosphorylate pSer9 in several cell lines, including mouse neuroblastoma N2A (26).

In addition to PP1, pSer9 may be dephosphorylated by PP2A (24, 25). However, in the present study, the low OA concentrations that selectively inhibit PP2A failed to affect NMDA-stimulated dephosphorylation of pSer9. Conversely, the NMDA effect on pSer9 was blocked by 1 μM OA, a concentration that inhibits both PP1 and PP2A (49). Furthermore, 1 μM OA, but not the lower concentrations, reduced NMDA-induced dephosphorylation of CREB, indicating activation of PP1 following NMDAR stimulation. Similarly, a PP1-specific inhibitor, tautomycin blocked both NMDA-induced pSer9 and pSer133 dephosphorylations without affecting pERK1/2-targeted PP2A activity. Noteworthy, the low OA concentrations increased basal pSer9 levels in cultured neurons, suggesting that PP2A may

ergic system in mouse brain was shown to activate the striatal GSK3β (58). The mechanism of activation involved D2 receptor-mediated decrease in activity of the Ser9 kinase, Akt. Thus, GSK3β may be regulated in the brain by several neurotransmitters including glutamate, serotonin, and dopamine.

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participate in pSer\(^9\) dephosphorylation under basal, non-NMDA-stimulated conditions.

We demonstrate that in primary neurons, I2 undergoes phosphorylation that becomes evident if PP1 activity is blocked with okadaic acid. The phosphorylation produced a mobility shift that was removed if GSK3\(\beta\) was inhibited. Because GSK3\(\beta\) is known to directly phosphorylate I2 at Thr\(^72\), GSK3\(\beta\) is likely to be an I2 kinase in neurons. However, we cannot exclude an indirect regulation of I2 phosphorylation by GSK3\(\beta\).

For instance, GSK3\(\beta\) could stimulate alternative Thr\(^72\) kinases including ERK1/2 or CDK5 (15, 16) or it could enhance casein kinase 2 (CK2)-mediated phosphorylation of I2 at serines 86, 120, and/or 121 (62).

We did not detect pI2 in either NMDA-stimulated or unstimulated neurons if OA was not included in the medium (Fig. 6A and data not shown). This indicates that in neurons, I2 phosphorylation is transient because of rapid dephosphorylation and reactivation by PP1. As PP1 contributes to NMDAR-mediated activation of GSK3\(\beta\), it is expected that OA will perturb GSK3\(\beta\) activation by NMDA. Indeed, previous studies showed that OA inhibits GSK3\(\beta\) activity in brain slices (63). Consequently, it is difficult to directly test that NMDA-activated GSK3\(\beta\) phosphorylates I2. Nevertheless, the presence of I2 in neurons as well as its ability to undergo transient phosphorylation that is regulated by GSK3\(\beta\) indicate that I2 is a reasonable candidate mediator of the feedback between GSK3\(\beta\) and PP1 in NMDA-stimulated neurons.

We show that PP1-mediated activation of GSK3\(\beta\) further amplifies PP1 activity. This may be at least in part via phosphorylation and inhibition of I2. A similar positive feedback loop between PP1 and GSK3\(\beta\) was recently demonstrated in several cell lines (26). A transient character of phosphorylation-mediated inhibition of I2 (14, 64) suggests that I2 may affect the activity of pre-existing I2/PP1 complexes at specific subcellular sites. In neurons, I2 was found in dendritic spines as well as in the cell bodies (65). As accumulation of I2 in the nucleus occurs only during the S and M phases of the cell cycle (66), postmitotic cells including neurons may have low levels of nuclear I2. Therefore, GSK3\(\beta\)/I2/PP1 signaling may modify neuronal PP1 activity locally, in the perikarial cytosol and/or in the dendritic spines.

Our data suggest that in NMDA-stimulated neurons, inhibition of GSK3\(\beta\) reduces PP1-mediated dephosphorylation of a nuclear protein CREB at the Ser\(^133\) residue. Therefore, it appears that in addition to increasing PP1 activity in the cytosol and/or dendritic spines, GSK3\(\beta\) may also stimulate nuclear PP1. It is unclear what may be the mechanisms underlying GSK3\(\beta\) regulation of PP1 in the nucleus. The possibilities include (i) existence of a nuclear GSK3\(\beta\)/I2/PP1 complex that targets CREB Ser\(^133\) and/or (ii) a potential stimulatory effect of GSK3\(\beta\)-mediated phosphorylation of CREB Ser\(^129\) (67) on CREB Ser\(^133\) dephosphorylation.

An alternative explanation for increased pSer\(^133\) levels in GSK3\(\beta\)-inhibited neurons is that GSK3\(\beta\) inhibits CREB phosphorylation by the CREB kinases rather than stimulates PP1-mediated CREB dephosphorylation. The increased CREB kinase activity is clearly noticeable at early time points after NMDAR stimulation when pSer\(^133\) levels are elevated (3, 9, 56). As the early increase of pSer\(^133\) levels is not enhanced if GSK3\(\beta\) is inhibited, CREB phosphorylation at Ser\(^133\) does not seem to be regulated by GSK3\(\beta\). In addition, in NMDA-stimulated neurons, GSK inhibition did not affect activities of several kinases implicated in Ser\(^133\) phosphorylation including PKA, Akt, or MSK1 (68, 69). Therefore, it seems more likely that GSK3\(\beta\) regulates pSer\(^133\) levels by modulating its dephosphorylation rather than phosphorylation.

\(^4\) E. Szatmari and M. Hetman, unpublished observation.
In concert with the observations, that GSK3β/H9252 reduces pSer133 levels, we found that overexpression of wild-type GSK3β/H9252 abolished the stimulation of CRE transcription by non-NR2B NMDARs. The non-NR2B-mediated increase of CRE transcription depends on Ser133 phosphorylation, while its inhibition by NR2B is via dephosphorylation of pSer133 (3). Therefore, GSK3β may be a part of the circuitry that sets the balance between phosphorylation- and dephosphorylation-dependent events in NMDAR signaling.

As Ser133 phosphorylation may be only one of several mechanisms controlling CRE-mediated transcription, one cannot exclude that GSK3β reduces CRE-driven transcription by affecting alternative determinants of CREB activity including such as (i) CREB phosphorylation/dephosphorylation at other sites than Ser133 and/or (ii) the activity of CREB-binding protein (CBP) (8, 67).

We demonstrate that inhibition of GSK3β with 20 mM LiCl reduced NMDA-induced decline of pSer133. LiCl produced rapid inhibition of Tau phosphorylation and also blocked accumulation of phospho-I2 in PP1-inhibited neurons. These data suggest that in cultured neurons, lithium effectively inhibited GSK3β in NMDAR-stimulated cerebellar granule neurons, PP1 was shown to participate in CREB dephosphorylation, and prolonged but not acute lithium treatment affected this process (10). The concentration of LiCl used in that study was significantly lower than in our experiments (3 mM versus 20 mM). Therefore, it is possible that longer treatment was needed to reach intracellular concentrations of Li+ that will be sufficient to inhibit PP1-GSK-PP1 signaling.

Inhibition of CRE transcription induces neuronal death and impairs learning and memory (5, 70, 71). On the other hand, stimulation of CRE by neurotrophins protects against trophic deprivation induced apoptosis (72, 73). Interestingly, excessive activation of GSK3β contributes to neuronal apoptosis following trophic deprivation (32, 74–77). In addition, GSK inhibition attenuates cell death in a cellular model of HD (21). These data indicate that GSK may affect neuronal survival by inhibition of CRE transcription. However, in our hands, overexpression of GSK3β did not affect the basal rate of CRE transcription. Moreover, it did not affect stimulation of CRE by a neurotrophin, BDNF or by KCl suggesting a specific effect on non-NR2B-mediated increase of CRE. These results argue against but do not exclude the possibility that GSK3β induces neuronal death by affecting CRE transcription.

In summary, we have identified a NR2B NMDAR-stimulated positive feedback loop that amplifies PP1 activation in neurons. In this circuitry, the PP1-mediated increase of GSK3β activity leads to further PP1 acti-
were stimulated with 20 μM NMDA for 20 h in the presence or absence of NMDAR antagonists. We have used NMDA at 20 μM because this concentration did not significantly reduce neuronal survival (data not shown). Therefore, changes in luciferase activity in neurons stimulated with 20 μM NMDA are caused by transcriptional regulation but not cell death. Note that the NMDA-mediated activation of the CRE transcription was abolished in the presence of the nonselective NMDAR antagonists (100 μM APV or 10 μM MK-801, MK, p < 0.01), but not in the presence of the NR2B-specific inhibitor, ifenprodil (ifenprodil, 10 μM). Data represent the mean of quadruplicate determinations from three independent experiments ± S.E.; **, p < 0.01; ns, not significant, ANOVA. GSK3β-overexpressing neurons stimulated with 20 μM NMDA for 20 h. Note the inhibition of NMDA-mediated activation of CRE-driven transcription in GSK3β-overexpressing neurons. Data represent the mean of quadruplicate determinations from four independent experiments ± S.E.; **, p < 0.001; ns, not significant, ANOVA.

Acknowledgments—We thank Drs. Scott R. Whittemore, Jacek Jaworski, and Richard Benton for critical reading of the manuscript and Drs. Antione Dunah and David Brautigan for providing reagents used in this study.

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