Glycogen Synthase Kinase 3 Regulates NMDA Receptor Channel Trafficking and Function in Cortical Neurons

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Abbreviations: GSK, glycogen synthase kinase; AD, Alzheimer’s disease; NR2A, NMDA receptor subunit 2A; NR2B, NMDA receptor subunit 2B; KIF17, kinesin superfamily member 17; TDZD, 4-Benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione; PKB, protein kinase B.
ABSTRACT
Emerging evidence has suggested that glycogen synthase kinase 3 (GSK-3) is a key regulatory kinase involved in a plethora of processes in the nervous system including neuronal development, mood stabilization and neurodegeneration. However, the cellular mechanisms underlying the actions of GSK-3 remain to be identified. In this study, we examined the impact of GSK-3 on the NMDA receptor channel, a central player involved in cognitive and emotional processes. We found that application of various structurally different GSK-3 inhibitors caused a long-lasting reduction of NMDA receptor-mediated ionic and synaptic current in cortical pyramidal neurons. Cellular knockdown of GSK-3β in neuronal cultures with a small interfering RNA led to smaller NMDAR current and loss of its regulation by GSK-3 inhibitors. The NR2B subunit-containing NMDA receptor was the primary target of GSK-3, but the GSK-3 modulation of NMDAR current did not involve the motor protein KIF17-based transport of NR2B-containing vesicles along microtubules. Combined electrophysiological, immunocytochemical and biochemical evidence indicated that GSK-3 inhibitors induced the down-regulation of NMDAR current through increasing the Rab5-mediated and PSD-95-regulated NMDAR internalization in a clathrin/dynamin-dependent manner.
INTRODUCTION

Glycogen synthase kinase 3 (GSK-3) was initially identified as an enzyme that regulates glycogen synthesis in response to insulin (Welsh et al., 1996). Two distinct but closely related GSK-3 forms, GSK-3α and GSK-3β, have been discovered. GSK-3, which is usually active in resting cells, is a critical downstream element of the phosphoinositide 3-kinase/Akt pathway, and its activity can be inhibited by Akt-mediated phosphorylation at serine residues (Ser-21 for GSK-3α and Ser-9 for GSK-3β) on their N-terminal domain (Cross et al., 1995). Recently GSK-3 has emerged as a multifunctional serine/theronine kinase involved in many cellular processes (Frame and Cohen, 2001), and pharmacological inhibitors of GSK-3 offer great potential for the treatment of a variety of disorders (Meijer et al., 2004). Considerable studies have shown that GSK-3β is a crucial player in regulating axon growth and neuronal polarity during development (Zhou and Snider, 2005). GSK-3 has also been implicated in the pathogenesis of mood disorders (Coyle and Duman, 2003), since it is one of the main targets of lithium (Phiel and Klein, 2001), the most established treatment for manic-depression illness. Moreover, GSK-3 is proposed as an important mediator of dopamine actions in vivo (Beaulieu et al., 2004), and converging evidence suggests that impaired Akt/GSK-3β signaling contributes to schizophrenia (Emamian et al., 2004). In addition, GSK-3 is a potential target of Alzheimer’s disease (AD), because inhibition of GSK-3 reduces the production of Aβ peptides in amyloid plaques (Phiel et al., 2003) and the hyperphosphorylation of tau protein in neurofibrillary tangles (Hong et al., 1997), two pathological hallmarks of AD.

To understand the cellular mechanisms underlying the broad physiological effects of GSK-3, it is essential to identify the molecular targets of GSK-3. The NMDA glutamate receptor, a principal subtype of excitatory ligand-gated ion channel, has been implicated in multiple neuronal functions ranging from synapse formation, synaptic plasticity, learning and memory to mental disorders (Tsai and Coyle, 2002) and Alzheimer’s disease (Selkoe, 2002). It prompts us to hypothesize that one important target of GSK-3 could be the NMDA receptor. To test this, we examined the impact of GSK-3 on NMDA receptor function in cortical pyramidal neurons. We found that inhibiting GSK-3 activity suppresses NMDAR current through a
mechanism involving the NMDAR internalization.

MATERIALS AND METHODS

Whole-cell recordings. Acutely dissociated frontal cortical pyramidal neurons from young adult (3-5 weeks postnatal) Sprague-Dawley rats or cortical cultures from 18 d rat embryos were prepared using procedures described previously (Wang et al., 2003). Recordings of whole-cell ion channel current employed standard voltage clamp techniques (Yuen et al., 2005a). The internal solution consisted of (in mM): 180 N-methyl-d-glucamine (NMG), 40 HEPES, 4 MgCl₂, 0.1 BAPTA, 12 phosphocreatine, 3 Na₂ATP, 0.5 Na₂GTP, 0.1 leupeptin, pH = 7.2-7.3, 265-270 mosm/L. The external solution consisted of (in mM): 127 NaCl, 20 CsCl, 10 HEPES, 1 CaCl₂, 5 BaCl₂, 12 glucose, 0.001 TTX, 0.02 glycine, pH = 7.3-7.4, 300-305 mosm/L. Recordings were obtained with an Axon Instruments 200B patch clamp amplifier that was controlled and monitored with an IBM PC running pCLAMP (v. 8) with a DigiData 1320 series interface (Axon instruments). Electrode resistances were typically 2-4 MΩ in the bath. After seal rupture, series resistance (4-10 MΩ) was compensated (70-90%) and periodically monitored. The cell membrane potential was held at -60 mV. The application of NMDA (100 µM) evoked a partially desensitizing inward current that could be blocked by the NMDA receptor antagonist D-APV (50 µM). NMDA was applied for 2 seconds every 30 seconds to minimize desensitization-induced decrease of current amplitude. Drugs were applied with a gravity-fed 'sewer pipe' system. The array of application capillaries (ca. 150 µm i.d.) was positioned a few hundred microns from the cell under study. Solution changes were effected by the SF-77B fast-step solution stimulus delivery device (Warner Instrument).

GSK-3 inhibitors 4-Benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD, Calbiochem), SB216763 (Tocris), α-4-Dibromoacetophenone (Calbiochem), and phosphatase inhibitors okadaic acid, its inactive analog okadaic acid methyl ester, and microcystin (Calbiochem) were made up as concentrated stocks in DMSO or water and stored at -20°C. Stocks were thawed and diluted immediately prior to use. The amino acid sequence for the dynamin inhibitory peptide is: QVPSRPNRAP. The polyclonal anti-Rab5 antibody (Santa Cruz) was raised against the full-length human Rab5A.
Data analyses were performed with AxoGraph (Axon instruments), Kaleidagraph (Albeck Software), Origin 6 (OriginLab) and Statview (Abacus Concepts). For analysis of statistical significance, Mann-Whitney U tests were performed to compare the current amplitudes in the presence or absence of GSK-3 inhibitors. ANOVA tests were performed to compare the differential degrees of current modulation between groups subjected to different treatment.

**Electrophysiological Recordings in Slices.** To evaluate the regulation of NMDAR-mediated excitatory postsynaptic current in cortical slices, the whole-cell voltage-clamp recording technique was used (Wang et al., 2003; Yuen et al., 2005a). Electrodes (5-9 MΩ) were filled with the following internal solution (in mM): 130 Cs-methanesulfonate, 10 CsCl, 4 NaCl, 10 HEPES, 1 MgCl₂, 5 EGTA, 2.2 QX-314, 12 phosphocreatine, 5 MgATP, 0.2 Na₂GTP, 0.1 leupeptin, pH = 7.2-7.3, 265-270 mosm/L. The slice (300 µm) was placed in a perfusion chamber attached to the fixed-stage of an upright microscope (Olympus) and submerged in continuously flowing oxygenated artificial cerebrospinal fluid (ACSF) containing CNQX (20 µM) and bicuculline (10 µM) to block AMPA/kainate receptors and GABA_A receptors. Cells were visualized with a 40X water-immersion lens and illuminated with near infrared (IR) light and the image was detected with an IR-sensitive CCD camera. A Multiclamp 700A amplifier was used for these recordings. Tight seals (2-10 GΩ) from visualized pyramidal neurons were obtained by applying negative pressure. The membrane was disrupted with additional suction and the whole cell configuration was obtained. The access resistances ranged from 13-18 MΩ and were compensated 50-70%. Evoked currents were generated with a 50 µs pulse from a stimulation isolation unit controlled by a S48 pulse generator (Astro-Med). A bipolar stimulating electrode (FHC) was positioned ~100 µm from the neuron under recording. Before stimulation, cells (voltage-clamped at -70 mV) were depolarized to +60 mV for 3 seconds to fully relieve the voltage-dependent Mg²⁺ block of NMDAR channels. Clampfit Program (Axon Instrument) was used to analyze evoked synaptic activity.

**Biochemical Measurement of Surface-Expressed Receptors.** The surface NMDA receptors were detected as described previously (Wang et al., 2003). Briefly, after treatment, cortical slices were incubated with ACSF containing 1 mg/ml Sulfo-NHS-LC-Biotin (Pierce Chemical Co., Rockford, IL) for 20 min on ice. The slices
were then rinsed three times in TBS to quench the biotin reaction, followed by homogenization in 300 µl of modified RIPA buffer (1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, 50 mM NaPO₄, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, and 1 mg/ml leupeptin). The homogenates were centrifuged at 14,000 x g for 15 min at 4°C. 15 µg of protein were removed to measure total NR1. For surface protein, 150 µg of protein were incubated with 100 µl 50% Neutravidin Agarose (Pierce Chemical Co.) for 2 hr at 4°C, and bound proteins were resuspended in 25 µl of SDS sample buffer and boiled. Quantitative Western blots were performed on both total and biotinylated (surface) proteins using anti-NR1 (1:1000, Upstate Biotechnology, Lake Placid, NY).

**Immunocytochemistry.** Cultured cortical neurons (12-15 DIV) were used for immunocytochemical experiments. NR2B or NR2A tagged with GFP at the extracellular N-terminus (Luo et al., 2002) was used to transfect cortical cultures. For the detection of GFP-NR2B or GFP-NR2A on the cell surface, cultured neurons (48 hrs following transfection) were treated with different agents, then they were fixed in 4% paraformaldehyde, but not permeabilized. After background blocking in BSA, the cells were incubated with the anti-GFP antibody (Chemicon, 1:100) at RT for 1 hour. After washing off the primary antibodies, the cells were incubated with a rhodamine-conjugated secondary antibody (Sigma, 1:200) for 50 min at room temperature. After washing in PBS for three times, the coverslips were mounted on slides with VECTASHIELD mounting media (Vector Laboratories, Inc., Burlingame, CA).

Labeled cells were imaged using a 40x or 100x objective with a cooled CCD camera mounted on a Nikon microscope. All specimens were imaged under identical conditions and analyzed using identical parameters. Images were first thresholded to subtract the average background fluorescence, then the level of surface NMDAR immunoreactivity on the same length of dendrites in treated vs. untreated cells was compared. Three to five independent experiments were performed. On each coverslip, the immunofluorescence intensity of 5-8 neurons was quantified. For each neuron, the immunoreactivity of four neurites (100 µm each, with similar diameters and distances from the soma) was measured. Quantitative analyses were conducted blindly (without knowledge of experimental treatment).
Small Interfering RNA and Antisense. To suppress the expression of GSK-3 in cultured neurons, we used the small interfering RNA (siRNA), a potent agent for sequence-specific gene silencing. The GSK-3 siRNA oligonucleotide sequences (Phiel et al., 2003) selected from GSK-3α mRNA were: 5’-UUCUACUCCAGUGGAGAdTdT-3’ (sense) and 5’-UCUCACCACUGGAGUAGAAdT dT-3’ (antisense), and from GSK-3β mRNA were: 5’-AUCUUUGGAGCCACU-GAUUdTdT-3’ (sense) and 5’-AAUCAGUGGCUCCAAAGAUdTdT-3’ (antisense). siRNA was synthesized (Ambion), and co-transfected with EGFP into cultured cortical neurons (11 DIV) using the Lipofectamine 2000 method. Two to three days after transfection, electrophysiological recordings were performed. To test the effectiveness of GSK-3αβ siRNAs, immunocytochemical experiments were performed as previously described (Yuen et al., 2005a) with antibodies against GSK-3α or GSK-3β (1:100, Cell Signaling).

To knockdown the expression of KIF17 in cultured cortical neurons, we used the antisense oligonucleotide approach as previously described (Yuen et al., 2005a). The antisense oligonucleotide against KIF17 cDNA was 5’-CAGAGGCTCACCACCGAA-3’, and the corresponding sense oligonucleotide was 5’-TTCGGTGGTGAGCCTCTG-3’. After 8-11 days of culture, 1 µM of oligonucleotides were added directly to the culture medium. Two to three days after being exposed to these oligonucleotides, electrophysiological recordings were performed on the cultured neurons.

Cloning, Expression and Purification of Proteins. Wild-type Rab5 cDNA was cloned by RT-PCR using mouse brain total RNA. After sequence verification, the cDNA was subcloned into the bacterial expression vector pQE-80 (Qiagen), which added a His6 tag at the N-terminus of the protein. Rab5 mutants (S34N and Q79L) were generated by site-directed mutagenesis using the QuikChange Kit from Stratagene. Rab5 expression in the M15 strain of E. Coli (Qiagen) was induced by adding IPTG to 1 mM final concentration for 4–5 hr at 25°C (to minimize the formation of inclusion bodies). Rab5 in cleared E. Coli lysate was purified by affinity chromatography using the His Gravitrap column (GE Bioscience) according to the manufacturer’s protocol. His6-tagged Rab5 proteins were eluted from the column in a buffer containing 50 mM Tris, 500 mM NaCl, and 300 mM imidazole (pH 7.4). Fractions of eluate were analyzed by SDS-PAGE.
and Coomassie Blue staining to identify the peak fractions containing Rab5 proteins. Western blotting with the polyclonal anti-Rab5 antibody (Santa Cruz) was also performed to verify the expression of purified Rab5 protein. The most pure one or two fractions (shown as a single band by Coomassie blue staining) were dialyzed against PBS before being used in electrophysiological experiments.

**Western Blot and Co-Immunoprecipitation.** Western blotting procedures were similar to what was described previously (Gu et al., 2005). The phospho-GSK-3α/β (Ser21/9) antibody (1:1000, Cell Signaling) was used to detect inactivated GSK-3. For co-immunoprecipitation experiments, after treatment with indicated agents, each slice was collected and homogenized in 1 ml lysis buffer (50 mM Tris, 1% deoxycholic acid, 10 mM EDTA, 10 mM EGTA, 1 mM PMSF, and 1 mg/ml leupeptin). Lysates were ultracentrifuged (200,000 × g) at 4°C for 60 min. Supernatant fractions were incubated with an anti-NR1 antibody (2 µg, Upstate Biotechnology, Lake Placid, NY) for 1 hr at 4°C, followed by incubation with 50 µl of protein A/G plus agarose (Santa Cruz Biotechnology) for 1 hr at 4°C. Immunoprecipitates were washed for three times with lysis buffer containing 0.2 M NaCl, then boiled in 2x SDS loading buffer for 5 min, and separated on 7.5% SDS-polyacrylamide gels. Western blotting experiments were performed with an anti-PSD-95 antibody (Affinity BioReagents, 1:1000).

**RESULTS**

**GSK-3 inhibitors reduce NMDAR-mediated current in cortical pyramidal neurons.**

Since GSK-3 has a high basal activity and signaling pathways often proceed by inhibiting GSK-3 activity (Doble and Woodgett, 2003), we first examined the effect of GSK-3 inhibitors on NMDA receptor-mediated current in dissociated cortical pyramidal neurons to determine the potential influence of GSK-3 on NMDA receptors. Application of TDZD (10 µM), a highly selective non-ATP competitive inhibitor of GSK-3, caused a potent reduction in the amplitude of NMDA (100 µM)-evoked current, while the NMDAR current amplitude was stable throughout the recording period with application of the control vehicle DMSO. The time courses and current traces from representative cells are shown in **Figure 1A**. Dose response
experiments (Figure 1B) indicated that different concentrations of TDZD inhibited NMDR current to different extents with the EC50 ~1.7 µM.

To verify the effect of GSK-3 on NMDA receptor current, we further examined other structurally different GSK-3 inhibitors. As shown in Figure 1C, SB216763 (10 µM), another potent and selective GSK-3 inhibitor, produced a strong reduction of NMDAR current in the dissociated cortical neuron. Application of LiCl (5 mM), a known inhibitor of GSK-3 in vitro and in neurons (Phiel and Klein, 2001), also potently suppressed NMDAR current (Figure 1D). The effect of different GSK-3 inhibitors on NMDAR current is summarized in Figure 1E. TDZD had a significant inhibitory effect on the peak NMDAR current amplitude in freshly isolated cortical pyramidal neurons (22.0 ± 1.1%, n = 31, p < 0.001, Mann-Whitney). TDZD also inhibited the steady-state NMDAR current amplitude (19.3 ± 1.0%, n = 10, p < 0.001, Mann-Whitney).

Fitting the NMDAR current with a single exponential equation indicated that TDZD did not significantly alter the desensitization profile of NMDAR current (τ in control: 0.60 ± 0.05 sec; τ in TDZD: 0.59 ± 0.03 sec, n = 5). Similar to the effect of TDZD, other GSK-3 inhibitors, including SB216763, Dibromoacetophenone (Dibro, 15 µM), LiCl and Li2CO3 (1 mM), also significantly suppressed peak NMDAR current amplitude (SB216763: 21.0 ± 1.0%, n = 10; Dibro: 18.7 ± 3.0%, n = 9; LiCl: 19.7 ± 2.0%, n = 13; Li2CO3: 19.5 ± 6.0%, n = 8). Similar effects were found in cultured cortical pyramidal neurons with TDZD or LiCl (TDZD: 17.3 ± 2.1%, n = 20; LiCl: 19.4 ± 3.0%, n = 6, p < 0.001, Mann-Whitney).

The reduction of NMDAR current induced by all these GSK-3 inhibitors was robust and irreversible following 10-15 min of washing. Partial (40-50%) or near complete (70-80%) recovery could happen with shorter application of GSK-3 inhibitors and more prolonged washing (n = 6, data not shown). It suggests that persistent inhibition of GSK-3 could produce a long-lasting effect on NMDA receptors.

GSK-3α and GSK-3β are inactivated through phosphorylation of serine residues (Ser-21 for GSK-3α and Ser-9 for GSK-3β) on their N-terminal domain (Cross et al., 1995; Frame and Cohen, 2001), so we used an antibody selective for Ser-21/9 phosphorylated GSK-3 to determine whether the GSK-3 inhibitors can cause the inhibition of GSK-3 activity. As shown in Figure 1F, treatment of cortical slices with LiCl (5 mM)
or TDZD (2-10 µM) markedly increased the level of phosphorylated GSK-3α/β without changing the level of total GSK-3α/β, indicating that the GSK-3 inhibitors indeed resulted in GSK-3 inactivation.

Next we tested whether the pharmacological agents we used converge on GSK-3 to regulate NMDA receptor current. To do so, we co-applied different GSK-3 inhibitors. As shown in Figure 2A, following TDZD suppression of NMDAR current, subsequent addition of LiCl did not cause further reduction of NMDAR current. Similarly, combined application of TDZD with LiCl after sole application of LiCl did not have further effect on NMDAR current (Figure 2B). As summarized in Figure 2C, the percent reduction of NMDAR current by co-application of TDZD and LiCl was similar to that by TDZD alone or LiCl alone, suggesting that one GSK-3 inhibitor occludes the effect of another GSK-3 inhibitor.

Given the effect of GSK-3 inhibitors on NMDAR current, we would like to know what the normal stimulus is that could activate this pathway. Insulin has been found to induce GSK-3 inhibition via protein kinase B (PKB, also called AKT) signaling (Cohen and Frame, 2001). Thus, we examined the effect of insulin on NMDAR current. As shown in Figure 2D, application of insulin (2 µM) caused a decrease of NMDAR current (17.5 ± 1.2%, n = 22, Figure 2F), and occluded the effect of subsequently applied GSK-3 inhibitor SB216763 (Figure 2E, 17.8 ± 2.2%, n = 9, Figure 2F). The effect of TDZD on NMDAR current was also significantly attenuated in the presence of insulin (n = 4, data not shown). These results suggest that activation of insulin signaling could down-regulate whole-cell NMDAR current via inhibiting GSK-3.

Because the NMDA-evoked current in isolated neurons is mediated by both synaptic and extrasynaptic NMDA receptors, we further examined the effect of GSK-3 on NMDAR-EPSC evoked by stimulation of synaptic NMDA receptors in cortical slices. As shown in Figure 3A and 3B, application of TDZD (20 µM) induced a significant reduction in the amplitude of NMDAR-EPSC. In parallel control measurements where no TDZD was administrated, NMDAR-EPSC remained stable throughout the length of the recording. In a sample of cortical pyramidal neurons we examined, TDZD decreased the mean amplitude of NMDAR-EPSC by 30.2 ± 2.1% (n = 10, p < 0.001, Mann-Whitney).
Cellular knockdown of GSK-3 leads to smaller NMDAR current and loss of its regulation by GSK-3 inhibitors in cultured cortical pyramidal neurons.

To further test the role of GSK-3 in the regulation of NMDA receptors, we suppressed GSK-3 protein expression in cultured cortical neurons (11 DIV) by transfecting a small interfering RNA (siRNA) directed against GSK-3α or GSK-3β (Phiel et al., 2003). GFP was co-transfected with GSK-3 siRNA. In all the GFP-positive neurons we observed, the GSK-3α/β siRNA caused efficient and specific down-regulation of GSK-3α/β expression (Figure 4A and 4B, GSK-3α: n = 12; GSK-3β: n = 15), which is likely to cause functional inactivation of GSK-3. So we examined NMDAR current in GSK-3 siRNA-transfected neurons. Control neurons were transfected with GFP alone or with a scrambled siRNA. As shown in Figure 4C, in GSK-3β siRNA-transfected neurons, the distribution of NMDAR current density (pA/pF) was shifted to the left with smaller values, compared to non-transfected neurons or neurons transfected with GFP alone or GSK-3α siRNA. Transfecting both GSK-3α and GSK-3β siRNAs also caused a leftward shift of the NMDAR current density. The average NMDAR current density (pA/pF) in neurons transfected with different agents is summarized in Figure 4D. Significantly (p < 0.001, ANOVA) smaller values were found in neurons transfected with GSK-3β siRNA (16.3 ± 0.8, n = 18) or both GSK-3α and GSK-3β siRNAs (16.7 ± 1.1, n = 19), compared to non-transfected neurons (22.5 ± 1.0, n = 12), GFP-transfected neurons (23.1 ± 0.8, n = 18), scrambled siRNA-transfected neurons (20.8 ± 1.5, n = 8) or GSK-3α siRNA-transfected neurons (20.5 ± 0.9, n = 15). These results suggest that cellular knockdown of GSK-3β results in depressed basal NMDAR current.

We next examined the effect of GSK-3 inhibitors on NMDAR current in neurons transfected with GSK-3 siRNA. As shown in Figure 4E, TDZD had little effect on NMDAR current in the GFP-positive neuron transfected with both GSK-3α and GSK-3β siRNAs, while it produced a potent reduction of NMDAR current in the control neuron transfected with GFP alone. As summarized in Figure 4F, in cultured neurons transfected with GSK-3α and GSK-3β siRNAs, TDZD reduced NMDAR currents by 6.0 ± 1.7% (n = 11, p > 0.05, Mann-Whitney), which was significantly (p < 0.001, ANOVA) smaller than the effect of
TDZD in control neurons transfected with GFP alone (21.0 ± 1.1%, n = 6, p < 0.001, Mann-Whitney). These results indicate that suppression of GSK-3 expression prevents exogenously applied GSK-3 inhibitors from regulating NMDAR current.

The GSK-3 regulation of NMDAR current is not dependent on the kinesin motor protein or cytoskeleton stability.

In the next series of experiments, we sought to determine the potential mechanism underlying the reduction of NMDAR current by GSK-3 inhibitors. In mature cortical synapses, the primary NMDA receptors are composed of NR1/NR2A or NR1/NR2B, which differ in subcellular localization. To determine which subpopulation(s) of NMDARs is modulated by GSK-3, we applied the selective inhibitor of NR2B subunit, ifenprodil. As shown in Figure 5A, in the presence of ifenprodil, TDZD had almost no effect on the remaining NMDAR current (2.3 ± 1.0%, n = 19), suggesting that GSK-3 primarily target NR2B subunit-containing NMDA receptors.

After NMDA receptors leave ER, they are transported along microtubules in dendrites via the kinesin motor protein KIF17, which is linked to NR2B-containing vesicles (Setou et al., 2000). It has been found that GSK-3 phosphorylates kinesin light chains and causes the detachment of kinesin from transported cargoes, leading to a reduction in kinesin-based motility (Morfini et al., 2002). To test whether the KIF17-mediated transport of NMDA receptors is involved in the GSK-3 regulation of NMDAR current, we performed cellular knockdown of KIF17 by treatment of cortical cultures with antisense oligonucleotides, and examined the effect of TDZD on NMDAR current in these cultures. Our previous studies have shown that KIF17 antisense oligonucleotides, but not sense oligonucleotides, totally inhibited KIF17-mediated functions (Yuen et al., 2005a, b). However, in KIF17 antisense (1 µM)-treated neurons, TDZD had an intact inhibitory effect on NMDAR current, similar to what was found in neurons exposed to KIF17 sense oligonucleotides (1 µM, Figure 5B and 5C). These results suggest that the GSK-3 modulation of NMDAR current does not involve the motor protein KIF17-based transport of NR2B-containing NMDA receptors.

GSK-3, by targeting different microtubule binding proteins, has been found to regulate several
aspects of microtubule assembly, including microtubule dynamics, microtubule stability and microtubule polymerization (Zhou and Snider, 2005). Our recent studies have shown that microtubule stability plays a role in controlling the trafficking and function of NMDA receptor channels in cortical pyramidal neurons (Yuen et al., 2005a, b). To test whether GSK-3 regulates NMDAR current by affecting microtubule assembly, we examined the effect of TDZD in the presence of agents that depolymerize or stabilize microtubules. As shown in Figure 5D, application of the microtubule-depolymerizing agent nocodazole (30 µM) suppressed NMDAR currents, but subsequent application of TDZD in the presence of nocodazole produced a further reducing effect on NMDAR current. On the other hand, dialysis with taxol (40 µM), a microtubule-stabilizing agent, failed to block the effect of TDZD on NMDAR current (Figure 5E). As summarized in Figure 5F, the modulation of NMDAR current by TDZD was not affected by microtubule depolymerizer nocodazole or colchicine (30 µM), nor by the microtubule stabilizer taxol, suggesting the lack of involvement of microtubules in the GSK-3 regulation of NMDAR current.

A previous study has found that an inactive phosphorylated pool of GSK-3 colocalizes with F-actin in neurons and plays a key role in regulating axonal growth (Eickholt et al., 2002). To determine whether the GSK-3 modulation of NMDAR current is affected by the integrity of F-actin, we used the potent actin depolymerizing agent latrunculin B (Gu et al., 2005). As shown in Figure 5G, application of latrunculin (5 µM) resulted in a gradual decrease of NMDAR current. Subsequent application of TDZD in the presence of latrunculin still induced a marked reduction of NMDAR current, indicating that latrunculin did not occlude the effect of TDZD. To further test the involvement of actin cytoskeleton, we dialyzed neurons with phalloidin (5 µM), an actin-stabilizing compound. As shown in Figure 5H, the TDZD-induced reduction of NMDAR current was not prevented when actin filaments were stabilized by phalloidin (Gu et al., 2005). The unaltered effects of TDZD on NMDAR current in the presence of actin manipulating agents (Figure 5I) suggest that the GSK-3 regulation of NMDAR current is not dependent on actin filaments.

The GSK-3 regulation of NMDAR current involves the clathrin/dynamin-dependent internalization of NMDA receptors that is mediated by Rab5.
To test whether clathrin-dependent endocytosis of NMDA receptors (Roche et al., 2001) is involved in the GSK-3 modulation of NMDAR current, we dialyzed neurons with a dynamin inhibitory peptide, QVPSRPNRAP, which interferes with the binding of amphiphysin with dynamin and therefore prevents endocytosis through clathrin-coated pits (Gout et al., 1993). We found that the effect of TDZD was largely abolished in neurons loaded with the dynamin inhibitory peptide (50 μM, Figure 6A and 6B, 4.0 ± 1.2%, n = 15), compared to neurons loaded with a scrambled control peptide (22.4 ± 1.0%, n = 10). It suggests that the mechanism underlying the GSK-3 inhibitor-induced down-regulation of NMDAR current is a decrease of functional surface NMDA receptors mediated by clathrin/dynamin-dependent endocytosis.

Given the potential involvement of GSK-3 in NMDAR internalization, we further examined the role of Rab5, a key mediator of protein transport from plasma membrane to early endosomes during clathrin-dependent endocytosis (Bucci et al., 1992), in the GSK-3 regulation of NMDAR current. To do so, we injected neurons with the purified dominant-negative (DN) mutant form of Rab5 protein (Rab5S34N) or the purified constitutively-active (CA) variant of Rab5 protein (Rab5Q79L), which inhibits or accelerates clathrin-mediated endocytosis, respectively (Stenmark et al., 1994). While most endogenous Rab5 is associated to membrane compartments, it has been shown that recombinant WT-Rab5 overexpressed in hippocampal slice cultures is also membrane bound, but DN-Rab5 is predominantly cytosolic (Brown et al., 2005). We found that the effect of TDZD on NMDAR current was diminished in cells loaded with DN-Rab5 or CA-Rab5 (4 μg/ml, Figure 6C). Similarly, another GSK-3 inhibitor SB216763 also failed to regulate NMDAR current with the dialysis of DN-Rab5 (Figure 6D). In a sample of neurons tested (Figure 6E), the basal NMDAR current was significantly reduced with CA-Rab5 injection (580 ± 124 pA, n = 5), compared to control cells (1100 ± 90 pA, n = 7) or cells injected with DN-Rab5 (1005.5 ± 61.5 pA, n = 9). The effect of GSK-3 inhibitors on NMDAR current was markedly blocked by DN-Rab5 (Figure 6F, TDZD: 7.2 ± 1.1%, n = 6; SB216763: 3.0 ± 0.8%, n = 10), and largely occluded by CA-Rab5 (TDZD: 8.0 ± 1.6%, n = 8). Moreover, loading cells with the Rab5 antibody (2 μg/ml), which binds to and inhibits Rab5, significantly blocked the effect of SB216763 on NMDAR current (Figure 6F, 5.0 ± 1.1%, n = 15), while the heat-
inactivated Rab5 antibody was ineffective (21.0 ± 2.1%, n = 5). These results suggest that Rab5 activity is required for the clathrin-dependent NMDAR internalization, which is regulated by GSK-3.

To provide more direct evidence showing that the GSK-3 regulation of NMDAR current can be accounted for by the altered number of NMDA receptors on the cell membrane, we first performed surface biotinylation experiments to measure levels of surface NR1 in cortical slices. Surface proteins were labeled with sulfo-NHS-LC-biotin, and then biotinylated surface proteins were separated from non-labeled intracellular proteins by reaction with Neutravidin beads. Surface and total proteins were subjected to electrophoresis and probed with an antibody against the NR1 subunit. As shown in Figure 7A, treatment of cortical slices with TDZD (10 µM, 10 min) reduced the level of surface NR1, with no change in the total NR1 protein. Furthermore, the TDZD effect on the surface expression of NR1 was blocked by pretreatment with the myristoylated (cell permeable) dynamin inhibitory peptide (50 µM, 30 min). Quantitative analysis (Figure 7B) in a sample of experiments indicated that TDZD decreased the level of surface NR1 to 58 ± 6.8% of control (n = 8; p < 0.01, ANOVA), but failed to do so in the presence of the dynamin inhibitory peptide (95 ± 4.5% of control, n = 3; p > 0.05, ANOVA). Another GSK-3 inhibitor, SB216763 (10 µM, 15 min), also significantly decreased the level of surface NR1 (54 ± 3.9% of control, n = 4; p < 0.01, ANOVA).

To further evaluate the changes of surface NMDAR subunits induced by GSK-3 inhibitors, we transfected neurons with GFP-tagged NR2B or NR2A (the GFP tag is placed at the extracellular N terminus of NR2), which has been shown to exhibit similar properties and localization as endogenous NR2B or NR2A subunit (Luo et al., 2002). The surface distribution of recombinant NR2B or NR2A was assessed by immunostaining with anti-GFP primary antibody followed by rhodamine-conjugated secondary antibody in nonpermeabilized conditions. As shown in Figure 7C, punctate red fluorescence was clearly visible on dendritic branches of GFP-NR2B transfected cells under control conditions, while in neurons treated with SB216763 (10 µM, 10 min) or TDZD (10 µM, 10 min), the fluorescent GFP-NR2B surface clusters on dendrites were significantly reduced (control: 65.6 ± 11.3 clusters/50µm dendrites; SB216763: 44.1 ± 8.4 clusters/50µm dendrites; TDZD: 40.3 ± 8.9 clusters/50µm dendrites; p < 0.01, ANOVA, compared to
control). In contrast, GFP-NR2A surface clusters on dendrites were not significantly affected by GSK-3 inhibitors (Figure 7D, control: 58.8 ± 10.7 clusters/50µm dendrites; SB216763: 55.6 ± 9.1 clusters/50µm dendrites; TDZD: 53.3 ± 9.5 clusters/50µm dendrites; p > 0.05, ANOVA, compared to control). The total amount of NR2B or NR2A (GFP channel) was not altered by GSK-3 inhibitor treatment (data not shown). Taken together, these results have demonstrated the involvement of GSK-3 in regulating NMDAR internalization.

GSK-3 regulates the clathrin-dependent NMDAR internalization that is affected by the scaffolding protein PSD-95.

Since the clathrin-dependent internalization of NMDARs is prevented by the postsynaptic scaffolding protein PSD-95 (Roche et al., 2001), we further examined the involvement of PSD-95 in GSK-3 regulation of NMDARs. As shown in Figure 8A and 8B, SB216763 or TDZD treatment caused a significant reduction of NR1 that bound to PSD-95 (SB216763: 44.7 ± 8.3% of control, n = 6; TDZD: 40.8 ± 7.5% of control, n = 6; p < 0.01, ANOVA). The decreased association between NR1 and PSD-95 is consistent with the increased NMDAR internalization in response to GSK-3 inhibitors.

Next, we dialyzed neurons with a peptide derived from the NR2B C-terminal nine residues, NR2B9c, which contains the binding region for PSD-95 and has the capability to disrupt preformed NR2B-PSD-95 complexes (Aarts et al., 2002). As shown in Figure 8C and 8D, injection with the NR2B9c peptide (5 µM) caused a gradual decline of NMDAR current (23 ± 3.3%, n = 10) and occluded the effect of subsequently applied GSK-3 inhibitor SB216763 (NR2B9c peptide + SB216763: 25 ± 1.5%, n = 8). In contrast, a scrambled control peptide (5 µM) had little effect on basal NMDAR current and failed to alter the effect of SB216763 (25 ± 1.3%, n = 6). Dialysis with low concentrations of NR2B9c peptide (1 µM or 100 nM) did not affect the basal NMDAR current or GSK-3 regulation (data not shown), suggesting that high concentrations of NR2B9c peptide (5 µM or 10 µM) are needed to perturb the association between NMDA receptors and PSD-95. Taken together, these results indicate that inhibiting GSK-3 increases the clathrin-
dependent NMDAR internalization, which is affected by the level of PSD-95-bound NMDA receptors.

DISCUSSION

Discovered originally as a protein involved in regulating the glucose level in skeletal muscle cells, GSK-3 is also expressed at a very high level in the CNS. On one hand, this distribution pattern might be related to the brain’s high demand of glucose as its primary energy source. The high level of GSK-3 could prevent glycogen synthesis and therefore maintain glucose molecules in the accessible form. On the other hand, the high level of GSK-3 in CNS also suggests that it may be critically involved in regulating neural physiological processes. Currently, GSK-3 is known to play important roles in the cell fate and differentiation, cellular architecture and motility, cell survival and apoptotic signaling, and is related to the pathogenesis of a diverse array of diseases (Welsh et al., 1996; Jope and Johnson 2004). Particularly GSK-3 has been implicated in AD, because it is associated with the production of Aβ (Phiel et al. 2003) and hyperphosphorylated Tau protein (Hong et al., 1997), both of which are prominent features of AD. In this study, we found that the whole-cell NMDAR current was reduced in the presence of various GSK-3 inhibitors. Additional evidence is obtained when the cellular GSK-3β protein is specifically knocked down by a siRNA, in which the basal NMDAR current density is smaller and the modulatory effect of GSK-3 inhibitors is abolished. Since GSK-3β knockdown alone is sufficient to down-regulate NMDAR current, it suggests that constitutively active endogenous GSK-3β is playing an important role in maintaining the expression of functional NMDARs on the surface of cortical neurons under basal conditions. Given the key role of NMDARs in synaptic plasticity, our data indicate that inactivating GSK-3 will have direct impact on synaptic functions, which provides a new mechanism underlying the role of GSK-3 in AD, a disease linked to synaptic failure (Selkoe, 2002).

Emerging evidence suggests that NMDAR trafficking involves multiple steps that are tightly regulated, including exiting from ER, transporting along microtubules on dendrites by the kinesin motor protein KIF17, delivery to actin-enriched postsynaptic density, internalization from the cell surface and lateral diffusion at synaptic and extrasynaptic sites in the plasma membrane (Wenthold et al., 2003). GSK-
3’s abilities to regulate microtubule dynamics (Zhou and Snider, 2005) and unload vesicles from kinesin protein family (Morfini et al., 2002) make it a good candidate potentially involved in the microtubule/KIF17-based transport of NMDARs (Yuen et al., 2005b). However, our data with microtubule manipulating agents and KIF17 antisense oligonucleotides suggest that GSK-3 is not regulating NMDAR trafficking through interference with NMDAR transport along dendritic microtubules. Moreover, actin manipulating agents also fail to affect GSK-3 regulation of NMDAR channels.

Inactivating GSK-3 has been shown to promote the recycling of internalized integrins through an unclear mechanism (Roberts et al. 2004). To test the possible involvement of GSK-3 in regulating NMDAR internalization, we examined the effect of GSK-3 inhibitors on the clathrin-mediated endocytosis of NMDARs, which is by far the most prevalent form of regulated endocytosis. In the presence of a dynamin inhibitory peptide to prevent endocytosis through clathrin-coated pits (Gout et al., 1993), GSK-3 inhibitors fail to regulate NMDAR current, which supports the role of GSK-3 in NMDAR internalization. The involvement of GSK-3 in the clathrin/dynamin-dependent NMDAR internalization is further demonstrated in the series of experiments with Rab5. Rab5, a member of the Rab family of small GTPases that function as specific regulators of vesicle transport between organelles (Zerial and McBride, 2001), is a key coordinator of early endocytic trafficking events including early endosome fusion, internalization and clathrin-coated vesicle formation. Dominant negative Rab5 blocks the effect of GSK-3 inhibitors on NMDAR current, while constitutively active Rab5 decreases basal NMDAR current and occludes the effect of GSK-3 inhibitors, suggesting that GSK-3 regulates NMDAR current via affecting Rab5/clathrin-mediated NMDAR internalization. More direct evidence comes from biochemical and immunocytochemical studies. In response to GSK-3 inhibitor treatment, cortical neurons show markedly reduced level of surface NR1 and NR2B, as well as significantly increased level of internalized NR1 and NR2B. In contrast, GSK-3 inhibitors do not have a significant effect on surface NR2A clusters, consistent with the electrophysiological finding that NR2B-containing NMDARs are the major target of GSK-3.

The regulatory pathway underlying the endocytosis of different NMDAR subunits is quite complex. Previous studies have found that extrasynaptic NMDARs (NR2B-predominated) have a higher rate of use-
dependent turnover from the surface than synaptic NMDARs (NR2A-predominated), presumably resulting from an altered balance in their interactions with tyrosine kinases/phosphatases, and/or lack of interaction with PDZ proteins (Li et al., 2002). Consistently, it has been shown that NR2A and NR2B have distinct endocytic motifs and endocytic sorting, with NR2B undergoing more robust endocytosis than NR2A in mature cultures (Lavezzari et al., 2004). Thus, the lack of GSK-3 effect on NR2A may be due to the lack of very active endocytosis of NR2A in native cortical neurons.

The anchoring protein PSD-95 has been found to affect NMDAR internalization (Roche et al., 2001). An AP-2 binding motif (YEKL) located on the distal C-terminus of NR2B is normally covered up by PSD-95 to prevent internalization (Roche et al. 2001). PDZ protein-mediated stabilization and AP-2-mediated internalization actually controls the synaptic localization of NR2B-containing NMDA receptors (Prybylowski et al., 2005). Since GSK-3 regulates NMDAR internalization, we further examined the involvement of PSD-95. Biochemical evidence indicates that the association of NMDARs with PSD-95 is reduced in response to GSK-3 inhibitors. Moreover, electrophysiological data demonstrate that perturbing NR2-PSD-95 complexes causes a gradual decline of NMDAR current and occludes the effect of subsequently applied GSK-3 inhibitors. Hence, constitutively active GSK-3 is important for stabilizing and/or promoting the expression of surface NMDARs that is affected by the binding between PSD-95 and NMDARs.

The mechanism underlying how GSK-3 inhibitors regulate NMDAR internalization depends on what protein(s) is actually targeted by GSK-3. One possibility is that the dynamin-like protein, a putative GSK-3 substrate (Chen et al., 2000), is the target of GSK-3 inhibitors in the regulation of NMDA receptors. This possibility is supported by the finding that GSK-3 represents a potent and unique clathrin-coated vesicle-associated protein kinase (Yu and Yang, 1993). Another possibility is that the putative GSK-3 substrate protein phosphatase 1 (Jope and Johnson, 2004), which participates in the regulation of the association and dissociation cycle of the clathrin-based endocytic machinery (Slepnev et al., 1998), is a potential target of GSK-3 involved in its regulation of NR2B/AP-2 interactions and the ensuing NMDAR internalization.

A recent study shows that stimulation of NR2B-containing NMDA receptor disinhibits GSK-3 by
protein phosphatase 1-mediated dephosphorylation of GSK-3 (Szatmari et al., 2005). Our present study indicates that GSK-3 inhibition leads to the suppression of NR2B function. Therefore it provides a potential mechanism for the protective role of GSK-3 inhibitors against the excessive activation of NR2B-GSK-3 loop and NMDAR-mediated excitotoxicity (Facci et al., 2003). Since pharmacological inhibitors of GSK-3 could potentially be used to treat several diseases, including AD and bipolar affective disorder (Meijer et al., 2004), our results provide an important framework to understand their actions on key targets involved in these diseases, such as NMDAR channels.
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FOOTNOTES

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FIGURE LEGENDS

**Figure 1.** GSK-3 inhibitors reduce NMDA receptor-mediated ionic current in dissociated cortical pyramidal neurons. **A, C, D.** Plot of peak NMDAR current showing that the selective GSK-3 inhibitors TDZD (10 µM, A), SB216763 (10 µM, C), LiCl (5 mM, D), but not the control vehicle DMSO (A), decreased NMDA (100 µM)-evoked current in acutely isolated neurons. **Inset:** Representative current traces (at time points denoted by #). Scale bars: 250 pA, 1 sec. **B.** Dose response data showing the percentage reduction of NMDAR current by different concentrations of TDZD. **E.** Cumulative data (mean ± SEM) showing the percentage reduction of NMDAR current by various GSK-3 inhibitors in freshly dissociated neurons. The number of cells tested in each condition is shown in each bar. **F.** Representative Western blotting and quantitative analysis from 4-6 experiments showing the effect of LiCl (5 mM) or TDZD (2, 5, 10 µM) on Ser-21/9 phosphorylated GSK-3α/β and total GSK-3α/β in cortical slices. *: p < 0.001, ANOVA.

**Figure 2.** The effects of different GSK-3 inhibitors on NMDAR current occlude each other, and are mimicked and occluded by insulin application. **A, B.** Plot of peak NMDAR current showing the effect of TDZD (10 µM) alone followed by co-application of TDZD and LiCl (5 mM, A) or the effect of LiCl alone followed by co-application of LiCl and TDZD (B) in representative neurons. **C.** Cumulative data (mean ± SEM) showing the percentage reduction of NMDAR current by individual or combined GSK-3 inhibitors. **D, E.** Plot of peak NMDAR current showing the effect of insulin (2 µM, D) or insulin followed by co-application of insulin and SB216763 (10 µM, E). **Inset (A, B, D):** Current traces (at time points denoted by #). Scale bars: 250 pA, 1 sec. **F.** Cumulative data (mean ± SEM) showing the percentage reduction of NMDAR current by insulin or combined application of insulin and SB216763.

**Figure 3.** GSK-3 inhibitors reduce NMDA receptor-mediated synaptic current in cortical slices. **A.** Plot of normalized peak evoked NMDAR-EPSC as a function of time and TDZD (20 µM) application. Each point represents the average peak (mean ± SEM) of 3 consecutive NMDAR-EPSCs. **B.** Representative current traces (average of 10 trials) taken from the records used to construct A (at time points denoted by #). Scale
bars: 100 pA, 200 ms.

**Figure 4.** Suppressing GSK-3 expression leads to smaller NMDAR current and loss of its regulation by GSK-3 inhibitors. **A, B.** Representative immunocytochemical images stained with anti-GSK-3α (A) or anti-GSK-3β (B) in cultured cortical pyramidal neurons co-transfected with GFP and GSK-3α siRNA (A), GFP and GSK-3β siRNA (B), or transfected with GFP alone (control). Note that GSK-3α/β siRNA suppressed the expression of GSK-3α/β in GFP-positive neurons. **C, D.** Distribution (C) and cumulative average (D) of NMDAR current density (pA/pF) in cultured neurons transfected with GFP, scrambled siRNA, GSK-3α siRNA, GSK-3β siRNA or both GSK-3α and GSK-3β siRNAs. *: p < 0.001, ANOVA. **E.** Plot of peak NMDAR current showing the effect of TDZD (20 µM) in a GFP-positive neuron transfected with GSK-3α&β siRNA and a GFP-positive neuron without GSK-3 siRNA transfection. Inset: Representative current traces (at time points denoted by #). Scale bars: 250 pA, 1 sec. **F.** Cumulative data (mean ± SEM) showing the percentage reduction of NMDAR current by TDZD in a sample of GFP-positive neurons transfected with or without GSK-3α&β siRNA. *: p < 0.001, ANOVA.

**Figure 5.** GSK-3 regulation of NMDAR current is independent of KIF17-based transport of NR2B-containing vesicles, microtubule stability or F-actin integrity. **A.** Plot of peak NMDAR current showing that the effect of TDZD (10 µM) was abolished in the presence of ifenprodil (3 µM), the selective inhibitor of NR2B subunit. **B.** Plot of peak NMDAR current showing the effect of TDZD in neurons treated with KIF17 antisense or sense oligonucleotides. **C.** Cumulative data (mean ± SEM) showing the percentage reduction of NMDAR current by TDZD in a sample of cultured neurons treated with KIF17 antisense or sense oligonucleotides. **D, G.** Plot of peak NMDAR currents showing that the effect of TDZD (10 µM) was not occluded by the microtubule-depolymerizing agent nocodazole (30 µM, D) or the actin-depolymerizing agent latrunculin B (5 µM, G). **E, H.** Plot of peak NMDAR currents showing that dialysis with the microtubule-stabilizing agent taxol (40 µM, E) or the actin-stabilizing agent phallolidin (5 µM, H) did not
block the effect of TDZD. Inset (A, B, D, E, G, H): Representative current traces (at time points denoted by #). Scale bars: 250 pA, 1 sec. F, I. Cumulative data (mean ± SEM) showing the percentage reduction of NMDAR current by TDZD in the absence or presence of various agents that interfere with microtubule (F) or actin (I) network.

**Figure 6.** GSK-3 regulation of NMDAR current involves the clathrin/dynamin-dependent endocytosis of NMDA receptors that is mediated by Rab5. A. Plot of normalized peak NMDAR current as a function of time and TDZD (10 μM) application in neurons dialyzed with the dynamin inhibitory peptide (50 μM) or a scrambled control peptide (50 μM). B. Cumulative data (mean ± SEM) showing the percentage reduction of NMDAR current by TDZD in neurons injected with different peptides. *: p < 0.001, ANOVA. C, D. Plot of normalized peak NMDAR current as a function of time and TDZD (10 μM, C) or SB216763 (10 μM, D) application in neurons dialyzed with the purified dominant-negative Rab5 (DN-Rab5, 4 μg/ml) or constitutively-active Rab5 (CA-Rab5, 4 μg/ml) protein. Inset (A, C): Representative current traces (at time points denoted by #). Scale bars: 250 pA, 1 sec. E. Cumulative data (mean ± SEM) showing the amplitude of peak NMDAR current in neurons loaded with or without DN-Rab5 or CA-Rab5. *: p < 0.005, ANOVA. F. Cumulative data (mean ± SEM) showing the percentage reduction of NMDAR current by TDZD or SB216763 in neurons injected with different agents. *: p < 0.001, ANOVA.

**Figure 7.** GSK-3 inhibitors reduce surface NMDA receptors in a clathrin/dynamin-dependent mechanism. A, Immunoblots showing the surface NR1 subunit and total NR1 subunit in cortical slices treated with TDZD (TD, 10 μM, 10 min) in the absence or presence of the membrane-permeable dynamin inhibitory peptide (50 μM, 30 min pretreatment), or cortical slices treated with SB216763 (SB, 10 μM, 15 min). B, Quantitation of the surface NR1 subunit expression with various treatments. *: p < 0.01, ANOVA. C, D. Left panel: Immunocytochemical images of surface GFP-NR2B (C) or GFP-NR2A (D) in transfected cortical cultures either untreated (control) or treated with SB216763 (10 μM, 10 min) or TDZD (10 μM, 10 min). Enlarged
versions of the boxed regions of dendrites are shown beneath each of the images. Right panel: Quantitation
of the density of surface GFP-NR2B (C) or GFP-NR2A (D) clusters on neuronal dendrites with various
treatments. *: p < 0.01, ANOVA.

**Figure 8.** The association between NMDA receptors and PSD-95 is involved in GSK-3 regulation of
NMDAR current. **A.** Effect of GSK-3 inhibitors on the interaction of NMDA receptors with PSD-95. Cell
lysates from cortical slices were incubated with TDZD (10 µM, 10 min) or SB216763 (10 µM, 15 min),
followed by immunoprecipitation with anti-NR1 antibody and Western blot analysis for PSD-95. Note that a
control IgG resulted in no signal in the co-immunoprecipitation experiments. **B.** Bar graphs showing the level
of NR1 bound to PSD-95 in the absence or presence of GSK-3 inhibitors. *, p < 0.01, ANOVA. **C.** Plot of
normalized peak NMDAR current as a function of time and SB216763 (10 µM) application in neurons
dialyzed with the NR2B9c peptide (5 µM) or a scrambled control peptide (5 µM). **D.** Cumulative data (mean
± SEM) showing the percentage reduction of NMDAR current by different peptides without or with
subsequently added SB216763. *: p < 0.001, ANOVA.
Figure 6

A

Normalized NMDA Current

- ctl
- TDZD
- wash

- △ Dynamin Inh. pep.
- ○ scrambled pep.

Time (min)

B

Modulation of NMDA Current by TDZD (%)

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C

Normalized NMDA Current

- ctl
- TDZD
- wash

- ○ control
- △ DN-Rab5
- □ CA-Rab5

Time (min)

D

Normalized NMDA Current

- ctl
- SB 216763
- wash

- ○ control
- △ DN-Rab5

Time (min)

E

Baseline NMDA Current (pA)

- control
- DN-Rab5
- CA-Rab5

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F

Modulation of NMDA Current (%)

- TDZD
- SB 216763

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