Interactions between the NR2B Receptor and CaMKII Modulate Synaptic Plasticity and Spatial Learning

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The NR2B subunit of the NMDA receptor interacts with several prominent proteins in the postsynaptic density, including calcium/calmodulin-dependent protein kinase II (CaMKII). To determine the function of these interactions, we derived transgenic mice expressing a ligand-activated carboxy-terminal NR2B fragment (cNR2B) by fusing this fragment to a tamoxifen (TAM)-dependent mutant of the estrogen receptor ligand-binding domain LBD G521R. Here, we show that induction by TAM allows the transgenic cNR2B fragment to bind to endogenous CaMKII in neurons. Activation of the LBD G521R-cNR2B transgenic protein in mice leads to the disruption of CaMKII/NR2B interactions at synapses. The disruption decreases Thr286 phosphorylation of αCaMKII, lowers phosphorylation of a key CaMKII substrate in the postsynaptic membrane (AMPA receptor subunit glutamate receptor 1), and produces deficits in hippocampal long-term potentiation and spatial learning. Together our results demonstrate the importance of interactions between CaMKII and NR2B for CaMKII activity, synaptic plasticity, and learning.

Key words: CaMKII; NMDA receptor; synapse; plasticity; learning; memory

Introduction

NMDA receptors (NMDARs) are heteromeric complexes containing both NR1 and NR2 subunits (Cull-Candy et al., 2001), and more rarely NR3 subunits (Prybylowski and Wenthold, 2004). The NR2 subunits (NR2A–2D) are encoded by four distinct genes. NR2A and NR2B subunits are the predominant NR2 subunits in adult hippocampus and neocortex (Monyer et al., 1994).

Mutations of NR2A and NR2B indicate that both are important for plasticity and memory performance in adult mice (Sakimura et al., 1995; Kiyama et al., 1998; Kohr et al., 2003). Consistently, previous pharmacological studies, including in vivo recordings, showed that NR2A and NR2B subunits are critical for the expression of long-term potentiation (LTP) in hippocampal and cortical regions (Berberich et al., 2005; Weitlauf et al., 2005; Fox et al., 2006). In addition, overexpression of the NR2B subunit in the hippocampus increases the amplitude of LTP in the CA1 region and enhances learning (Tang et al., 1999). Pharmacological blockade of the NR2B subunit has also been shown to impair fear learning in the anterior cingulate cortex and the amygdala (Rodrigues et al., 2001; Zhao et al., 2005). These latter findings suggest that the NR2B subunit is particularly important for plasticity and learning. The goal of the current study was to determine the signaling pathways through which the NR2B subunit affects these processes.

There are multiple downstream signaling pathways linked to the NMDARs that may play important roles in plasticity and memory (Thomas and Huganir, 2004; Barria and Malinow, 2005; Kennedy et al., 2005; Kim et al., 2005). Several important postsynaptic density (PSD) proteins, including calcium/calmodulin-dependent protein kinase II (CaMKII) and PSD-95 are known to interact with NR2B in vitro, but little is known about how these interactions influence their functions (Kornau et al., 1995; Leonard et al., 1999; Kennedy, 2000). Interactions with CaMKII are particularly interesting because of its well-established role in plasticity and learning (Silva et al., 1992; Mayford et al., 1996) and also because of its high-affinity binding to the C-terminal tail of the NR2B subunit (Strack and Colbran, 1998; Leonard et al., 1999; Strack et al., 2000). Previous studies suggested that NR2B binding targets active CaMKII to signaling networks in the postsynaptic density and supports persistent activity of this kinase in specific subcellular compartments (Bayer et al., 2001; Robison et al., 2005a,b). Given that introduction of persistently active CaMKII into neurons produces LTP at synapses (Pettit et al., 1994; Lledo et al., 1995), the association between NR2B and active CaMKII could be a key component mediating synaptic plasticity and certain types of learning (Lisman et al.,...
2002). Consistent with this idea, previous studies from organotypic hippocampal slices (7–8 in culture) demonstrated that the association between NR2B and active CaMKII is required for LTP (Barria and Malinow, 2005). However, the precise physiological role of the association between NR2B and active CaMKII, as well as the association between NR2B and other synaptic proteins in adult animals has not been elucidated. Nor has it been shown whether these associations with NR2B are important for learning and memory at the systems level.

The current study addresses this issue by demonstrating that inducible and reversible disruption of NR2B/CaMKII interactions in transgenic mice downregulates Thr286 phosphorylation of αCaMKII, disrupts LTP in the hippocampus, and impairs spatial learning.

Materials and Methods

Transgene construction and generation of cNR2B mice. The transgene used in these studies contains an αCaMKII promoter, a hybrid intron in the 5′ untranslated leader, a Kozak sequence, a hemiaglutamin virus (HA)-tag, an LBD G321R cDNA, and a polyadenylation signal (Kida et al. 2002). The coding region of the NR2B c terminus (839–1482 aa) was subcloned into the pMM-LBD G321R-CREBS A vector; we used PCR and restriction enzymes to replace the CREBS A gene by the NR2B c terminus gene, resulting in the pNN-LBD G321R-NR2B plasmid. The NR2B cDNA (1–1482 aa) clone was kindly provided by Dr. Gary Lynch (University of California, Irvine, Irvine, CA). The pNN-LBD G321R-NR2B plasmid was digested with SfiI and transgenic mice were generated by injecting the purified insert into pronuclei of C57BL/6NHsd zygotes. Studies were performed in F1 mice (aged 3–6 months) that were offspring of a cross between the founders and C57BL/6N (Tacf) mice (Taconic Farms, Germantown, NY). All experiments were performed blind with respect to genotype and treatment. All animal protocols were approved by the Chancellor’s Animal Research Committee at the University of California at Los Angeles, in accordance with National Institutes of Health guidelines.

RT-PCR. Hippocampus and cortex were acutely dissociated from mouse brains. Total RNA (1 μg) was reverse-transcribed with an oligo dT primer. The PCR s were performed for 35 cycles (1 s a 1 min cycle at each of the following temperatures: 94, 56, and 72°C. PCR products were amplified using two primers for LBD G321R-NR2B mRNA (LBD G321R, cNR2B, S′-ATT GCC TGG CTC GCA GGC CCC, and LBD G321R, cNR2B, 5′-TCA TGG AGG GTC AAA TCC AC). The transgene for LBD G321R-cNR2B contains an intron (235 bp) in the region between sequences corresponding to 5′ and 3′ ends, such that the PCR product from LBD G321R-cNR2B mRNA is distinct from transgene. PCR products were analyzed by agarose gel electrophoresis and then stained by ethidium bromide.

Tamoxifen solution and pellet. Because of the extended nature of behavior studies (see below), tamoxifen citrate (TAM; Sigma, St. Louis, MO) was administered orally as pellets, whereas in other less-extensive studies, TAM [in dimethyl sulfoxide (DMSO)] was injected. Pellets for oral administration were prepared by mixing TAM powder with melted peanut butter chips (H. B. Reese Candy, Hershey, PA). The mixture was molded into 200 mg pellets containing 0.6 mg TAM each. The TAM pellets were fed to mice once daily for 5 d before training and continuously during training and testing. All behavioral studies were performed using adult male mice (aged 4–6 months). In other studies (i.e., biochemical studies), TAM solution (20 mg/ml) was injected intraperitoneally daily (20 mg/kg body weight) for 5 d and mice were killed on the fifth day, 6 h after the final injection.

Protein isolation. PSD-enriched fractions were prepared from adult mice (3 to 4 months old) with a protocol modified from Dunkley et al. (1988). In brief, the forebrain was rapidly dissected and homogenized in ice-cold homogenization buffer containing 4 mM HEPES-NaOH, pH 7.4, 0.32 M sucrose, fresh protease inhibitor mixture, and optional phosphatase inhibitor mixtures; Roche, Welwyn Garden City, UK). Supernatant (S1) was collected after centrifugation of homogenates (1000 × g for 10 min at 4°C). S1 was diluted with the same volume of 10% Percoll (GE Healthcare Bio-Sciences, Piscataway, NJ) and then laid on top of a 10 and 20% discontinuous Percoll gradient. The interface between 10 and 20% Percoll was collected after centrifugation (33,000 × g for 5 min at 4°C) and then diluted with PSD buffer (PSDB; containing 40 mM HEPES-NaOH, pH 8.1, fresh protease inhibitor mixture, and optional phosphatase inhibitor mixtures). After centrifugation of mouse 20,000 × g for 20 min at 4°C, the pellet (synaptosome (SS)) was resuspended thoroughly in PSDB. Then, 0.5% Triton X-100 was added to SS lysates to enhance solubilization. After stirring for 15 min (4°C) and centrifugation for 40 min (40,000 × g at 4°C), the final pellet (PSD) was resuspended in 100 μl of PSDB and stored at −80°C for use. A small amount of SS and S1 were also saved for additional analysis during preparation of the PSD fraction. Protein concentrations were determined by the method used by Bradford (1976) using a Bio-Rad (Hercules, CA) Protein Assay Kit.

Coimmunoprecipitation. Freshly purified synaptosomal lysates (400 μg each for immunoprecipitation) were solubilized in 500 μl of 1% deoxycholate (DOC) buffer (pH 8.5, containing 1% deoxycholate, 50 mM TrisCl, 10 mM EGTA, 10 mM EDTA, 137 mM NaCl, protease inhibitor cocktails) for 30 min at 37°C, and then for 30 min on ice with occasional mixing. After centrifugation at 15,000 × g for 10 min (4°C), the supernatants were saved for immunoprecipitation and subsequent immunoblotting. All steps were performed at 4°C.

For each immunoprecipitation (IP), 500 μl of PBSA (0.1% BSA in PBS, pH 7.4) and 5 μg of specific antibody were added first to prewashed Dynabeads (protein G beads; 30 μl; anti-mouse IgG beads, 60 μl; Invitrogen, Eugene, OR). Five microliters of mouse or rabbit IgG were used instead of the specific experimental antibody in parallel as a negative control for the IP. After incubation for 1 h at 4°C, beads were washed to remove unbound antibody. Five hundred microliter (400 μg) supernatants of synaptosomal lysates were then added to the beads and incubated for 3 h at 4°C with stirring. Beads were washed three times and collected on Magnet (Bio-Rad), then eluted in 1× SDS sample buffer and analyzed by Western blot. Thirty micrograms of SS lysate were eluted simultaneously as a positive control (input) for IP.

Western blotting and protein expression analysis. Protein samples were separated by electrophoresis on a 7% SDS-PAGE gel (Bio-Rad). Gels were blotted to nitrocellulose membranes and then blocked for 1 h at room temperature with Tris-buffered saline containing 0.1% (v/v) Tween 20 and 5% (w/v) nonfat dry milk (TBST). After washing in TBST, membranes were hybridized at room temperature with antibodies of interest diluted in the blocking solution. The membranes were then incubated with IRIye-conjugated anti-mouse or anti-goat IgG (1:100,000). Protein signals were visualized and quantified with the Odyssey Imaging System (LI-COR Biosciences, Bad Homburg, Germany), using Odyssey fluorescent scanning channels fluorescing with Cy3 or Cy5. Protein bands were boxed, and the integrated intensity of all the pixels within that band was calculated above average background levels of a box of the same size. Membranes were stripped in stripping buffer and re-probed with other antibodies, and finally reprobed with β-actin as a control for protein loading. The amount of phosphorylated protein was expressed as a ratio of (phosphorylated protein)/actin or (phosphorylated protein)/(total protein). The ratio of the wild-type treated with vehicle (wt/veh) group was used as a standard and other groups were normalized to it before statistical analysis. To make weak bands easier to see in some of our figures, we adjusted the contrast and brightness so that some other bands may appear saturated. However, this manipulation did not affect the quantification results because quantification was performed under linear conditions on the initial 16-bit images without background modification.

Western blots were probed with antibodies against human estrogen receptor (ER; HC-20, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA), NR2BC (c terminus, 1:10,000, Xandria; prepared in the Kennedy laboratory), NR2B N terminus (NR2BN, 1:2000; prepared in the Kennedy laboratory), NR2A (1:5000; Evj; prepared in the Kennedy laboratory), NR1 (1:5000; ABR), SynGAP (1:2000; Affinity Bioreagents, Golden, CO), PSD-95 (7E3, 1:5000; Millipore, Temecula, CA), Densin-180 (CT245, c terminus prepared in the Kennedy laboratory, 1:5,000), synaptophysin (1:2000, Millipore), Thr286-P-CaMKII (22B1, 1:1000; Affin-
ity Bioreagents, Golden, CO), CaMKII (6G9, 1:5000; Millipore), β-actin (AC-15, 1:5000; Sigma), Serr381-P-GluR1 (1:50; from the Helli laboratory), and glutamate receptor 1 (Glur1; 1:1000, from the Helli laboratory), respectively.

Chemical LTP and Western blot analysis on hippocampal CA1. Sagittal hippocampal slices (400 μm thick) were prepared from adult mice (3 to 4 months old). Slices recovered in artificial CSF (ACSF; containing 1 μM 4-hydroxytanszeaxifen or vehicle) at room temperature for at least 1 h before the start of recordings. Stable baseline field EPSPs (fEPSPs) were recorded for at least 10 min. Chemical LTP (cLTP) was subsequently induced by chemical treatment consisting of a 5 min application of a high K+/Ca2+/ACSF (30 mM KCl, 10 mM CaCl2, and 0 mM MgSO4) preceded by 50 μM forskolin (FSK) in the absence of electrical presynaptic fiber stimulation (Makhinson et al., 1999). Test stimulation and normal ACSF were used for the experiments at times 100 μM 4-hydroxy-2,5-anisoxifen (from reference 1) in vehicle (at least 1 h before the start of recordings. Stable baseline field EPSPs (fEPSPs) were recorded for at least 10 min. Chemical LTP (cLTP) was subsequently induced by chemical treatment consisting of a 5 min application of a high K+/Ca2+/ACSF (30 mM KCl, 10 mM CaCl2, and 0 mM MgSO4) preceded by 50 μM forskolin (FSK) in the absence of electrical presynaptic fiber stimulation (Makhinson et al., 1999). Test stimulation and normal ACSF were applied after a 5 min perfusion with high K+/Ca2+/ACSF. Field fEPSPs recorded at least 50 min after chemical treatments were compared with those measured before treatment to ensure that LTP was induced.

For Western analysis, five to six slices were chemically treated whereas an additional two untreated slices from the same animal served as controls. The tissue from four mice with the same genotype and treatment (4-hydroxytanszeaxifen or vehicle) were pooled together for each Western blot. Slices were quickly frozen before 10 min, and then 40 min after perfusion with high K+/Ca2+/ACSF, and CA1 were isolated. These frozen CA1 slices were then transferred to microcentrifuge tubes in a dry ice/ethanol bath where they remained frozen. The tissue was thawed and homogenized with 100 μl of ice-cold 0.3 M sucrose buffer (containing 300 mM sucrose, 10 mM Tris-base, pH 7.4, 10 mM MgCl2, pH 7.4, and fresh protease and phosphatase inhibitor mixtures). After centrifugation (5000 rpm, 2 min) at 4°C, supernatant was centrifuged again at 45,000 rpm for 30 min at 4°C. The resulting pellet was solubilized in Trion X-100 buffer. Ten micrograms of protein each were electrophoresed on a 10% SDS-PAGE gels. Protein quantification analysis was done as described above. Percentage changes attributable to chemical treatment were calculated relative to the optical density volume of the corresponding untreated (base) protein bands within a single experiment, and statistical significance was based on six independent Western analyses.

Electrophysiology. Sagittal hippocampal slices (400 μm thick) were prepared from adult mice (3 to 4 months old) as described previously (Giese et al., 1998). Field fEPSPs were evoked alternatively in separate statistical significance was based on six independent Western analyses.

Results

Generation of cNR2B transgenic mouse

To engineer a transgenic protein that could be used to repress endogenous CaMKII/NR2B interactions in an inducible and reversible manner, we fused the NR2B carboxy terminus (839–1482 aa, cNR2B) to the ligand-binding domain of a human ER with a G521R mutation (LBDG21R). This mutant LBD does not bind estrogen and is instead regulated by the synthetic ligand tamoxifen (Kida et al., 2002). We found that administration of TAM allows the LBDG21R-cNR2B fusion protein to interact with its binding partner CaMKII. The LBDG21R-cNR2B fusion gene was expressed under the control of the αCaMKII promoter (Fig. 1A), which is known to be active only in postnatal neurons of forebrain areas, including the hippocampus, amygdala, cortex, and striatum (Mayford et al., 1996). The resulting transgenic mice bred normally and showed no overt behavioral abnormalities.

Reverse transcriptase (RT)-PCR and Western blot analyses confirmed the presence of LBDG21R-cNR2B messenger RNA (Fig. 1B) and protein (Fig. 1C,D) in the brain of the transgenic mice, especially in the hippocampus and cortex. Both an anti-ER (HC-20) antibody and an anti-NR2B C-terminal antibody (NR2BC) detected the LBDG21R-cNR2B protein (110 kDa) in the transgenic mice (Fig. 1C,D). To induce the function of the LBDG21R-cNR2B protein, transgenic mice and their wild-type littermates were injected intraperitoneally with TAM (or an equivalent volume of DMSO; vehicle) daily for 5 d, and killed on the fifth day, 6 h after the final injection. To avoid the toxic effects of DMSO, individual animals received no >50 μl of the TAM (or vehicle) solution each time they were injected. Although TAM (20 mg/kg/d for 5 consecutive days) did not affect the gross expression levels of the fusion protein in forebrain, it did affect its subcellular distribution; TAM treatment resulted in an increase in the levels of LBDG21R-cNR2B in the SS and PSD fractions, perhaps because the active LBDG21R-cNR2B protein, but not the inactive form, interacts with targets (i.e., CaMKII) in these fractions (Fig. 1E).

To test whether LBDG21R-cNR2B activation affects the levels of other PSD-enriched proteins, including NR1, NR2A, NR2B, and PSD-95, etc., we measured the levels of several important
PSD proteins by Western blot analysis using forebrain PSD-enriched fractions. No significant changes were observed in the level of these proteins among the four different treatment groups (Fig. 2).

TAM-activated LBD<sup>G521R</sup>-cNR2B protein binds to CaMKII in vivo
First, we immunoprecipitated Triton X-100-solubilized CaMKII complexes from forebrain homogenates with an antibody against CaMKII (6G9). Subsequent immunoblotting of the complexes with an antibody against the ER revealed the presence of the LBD<sup>G521R</sup>-cNR2B protein in complexes pulled down from transgenic mice treated with TAM injection (tg/tam), but not in the other three control groups [wt/veh, wild-type mice treated with TAM (wt/tam), and transgenic mice treated with vehicle (tg/veh)]. Conversely, immunoprecipitation of ER complexes with the same antibody against the ER resulted in the coprecipitation of CaMKII only in the TAM-treated transgenic group (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). These findings mean that TAM-activated LBD<sup>G521R</sup>-cNR2B binds to CaMKII in vivo, but its inactive form does not.

Second, to test whether TAM-activated LBD<sup>G521R</sup>-cNR2B binds to CaMKII in a fraction enriched in synaptic proteins, we used 1% Doc buffer (Robison et al., 2005a,b) to solubilize synaptosome lysates prepared from forebrain. Doc-solubilized synaptosomes were subjected to immunoprecipitation with an antibody against CaMKII only in the TAM-treated transgenic group, but not in the other three control groups (wt/veh, wt/tam, and tg/veh) (Fig. 3A). This result means that TAM-activated LBD<sup>G521R</sup>-cNR2B binds to synapse-associated CaMKII, but its inactive form does not.

Previous studies showed that endogenous NR2B binds to PSD-95 (Kornau et al., 1995). We therefore tested whether TAM-activated LBD<sup>G521R</sup>-cNR2B binds to PSD-95 in addition to CaMKII. Immunoblotting of the same ER complexes with antibodies against CaMKII revealed coprecipitation of CaMKII only in the TAM-treated transgenic group, but not in the other three control groups (wt/veh, wt/tam, and tg/veh) (Fig. 3A). However, no coprecipitation was observed in the other three control groups (wt/veh, wt/tam, and tg/veh) (Fig. 3A). This result means that TAM-activated LBD<sup>G521R</sup>-cNR2B binds to synapse-associated CaMKII, but its inactive form does not.

LBD<sup>G521R</sup>-cNR2B decreases the association between CaMKII and endogenous NR2B receptor complexes
Next, we tested whether the TAM-activated cNR2B fusion protein disrupts the association between CaMKII and endogenous NR2B receptor complexes. Doc-solubilized synaptosomes containing NR2B were immunoprecipitated with an antibody against NR2BN. Because this antibody was raised against the N terminus of NR2B, it can recognize the endogenous NR2B subunit (180 kDa), but not LBD<sup>G521R</sup>-cNR2B (110 kDa). Immunoblots of the immunoprecipitated NR2B complexes (Fig. 3B) demonstrated that the amount of αCaMKII coprecipitated was significantly reduced in the tg/tam group, compared with the other three control groups (tg/tam, 77.6 ± 7.8% of wt/veh group, p < 0.01, one-sample t test, n = 6; wt/tam, 99.3 ± 8.4%; tg/veh,
were not caused by differences in the immunoprecipitation of NR2B (tg/tam, 87.0 ± 4.8%; wt/tam, 89.2 ± 7.9%; and tg/veh, 96.7 ± 5.8% of wt/veh control group). As expected, both endogenous NR2B and LBDG521R-cNR2B were immunoprecipitated with the NR2BC antibody from tg/tam samples. Together these results show that TAM-activated LBDG521R-cNR2B binds to CaMKII and interferes with its interaction with endogenous NR2B.

**LBDG521R-cNR2B does not alter the association between CaMKII and densin**

Next, we tested whether the activated LBDG521R-cNR2B also interferes with the association between CaMKII and other potential binding partners such as densin (Walikonis et al., 2001). Immunoprecipitation with the αCaMKII antibody and Western blot analysis with the densin-180 antibody (CT245) (Apperson et al., 1996) showed equivalent levels of densin in all four groups (tg/tam, 105.8 ± 7.8%; wt/tam, 111.3 ± 7.4%; and tg/veh, 114.9 ± 16.6% of the wt/veh control group) (Fig. 3D). Altogether, these results demonstrate that activation of LBDG521R-cNR2B transgenic protein reduces the binding between CaMKII and endogenous NR2B in synapses, but does not alter binding between CaMKII and densin.

**Activation of LBDG521R-cNR2B affects Thr286 phosphorylation of αCaMKII associated with PSD**

We asked whether disruption of the interaction between CaMKII and endogenous NR2B altered activity of αCaMKII. Because the phosphorylation of αCaMKII at Thr286 endows CaMKII with autonous activity and plays a major role in synaptic plasticity and learning, we examined the level and Thr286 phosphorylation state of αCaMKII by immunoblot analysis of fractions enriched in forebrain PSDs and forebrain homogenates (Fig. 4). In the PSD-enriched fraction, αCaMKII autophosphorylation at Thr286 (detected with the anti-Thr286-P-CaMKII antibody) was significantly reduced specifically in the TAM-treated transgenic group (tg/tam, 57.0 ± 6.8%, p < 0.01; n = 6) (Fig. 4A) (in contrast with wt/tam, 97.4 ± 10.8% and tg/veh, 102.3 ± 12.2% of wt/veh group). This difference was not observed in the forebrain homogenate S1 fraction (wt/tam, 101.6 ± 14.5%; tg/veh, 102.1 ± 15.1%; and tg/tam, 94.4 ± 13.2% of wt/veh group; one-sample t test, p > 0.05; n = 6 per group) (Fig. 4B). Nor were significant changes observed in the total expression level of either αCaMKII or β-actin (data not shown). These results indicate that induction of the LBDG521R-cNR2B protein decreases the steady state level of activation of αCaMKII.

To confirm whether αCaMKII activity is decreased in parallel with the decreased autophosphorylation, we measured the phosphorylation of one of its key substrates, the GluR1 subunit of the GluR1. Immunoblots with the NR2BC antibody and Western blot analysis with the densin-180 antibody (CT245) (Apperson et al., 1996) showed equivalent levels of densin in all four groups (tg/tam, 105.8 ± 7.8%; wt/tam, 111.3 ± 7.4%; and tg/veh, 114.9 ± 16.6% of the wt/veh control group) (Fig. 3D). Altogether, these results demonstrate that activation of LBDG521R-cNR2B transgenic protein reduces the binding between CaMKII and endogenous NR2B in synapses, but does not alter binding between CaMKII and densin.

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To confirm whether αCaMKII activity is decreased in parallel with the decreased autophosphorylation, we measured the phosphorylation of one of its key substrates, the GluR1 subunit of the
AMPAR-type glutamate receptors. Previous studies have shown that serine 831 (Ser831) of GluR1 is specifically phosphorylated by CaMKII and protein kinase C (PKC) (Roche et al., 1996; Barria et al., 1997). We found that phosphorylation of Ser831 was reduced in the PSD fraction of tg/tam mice (69.7 ± 9.3% of wt/veh group; p < 0.05, one-sample t test; n = 8) (Fig. 4C), but not in forebrain homogenates (Fig. 4D). The presence or activation of LBDG521R-cNR2B did not alter the level of GluR1. These results indicate that the kinase activity of CaMKII in the PSD is reduced after induction of LBDG521R-cNR2B.

Together, these data demonstrate that activation of the LBDG521R-cNR2B transgenic protein inhibits interaction between CaMKII and NR2B, leading to a decrease in the autophosphorylation and kinase activity of aCaMKII in the PSD in vivo, and to a subsequent decrease in GluR1 phosphorylation at serine 831, a site known to be critical for the function of this receptor (Lee et al., 2003).

Activation of LBDG521R-cNR2B impairs phosphorylation of aCaMKII at Thr286 after chemical induction of LTP

The results presented above indicate that the continued activation of the LBDG521R-cNR2B protein can interfere with the interaction between CaMKII and NR2B, leading to a decrease in the basel levels of active aCaMKII. To determine whether the fusion protein can also interfere with the increase in autophosphorylation of aCaMKII after LTP, we measured autophosphorylation of Thr286 after LTP induction in transgenics and controls in the presence or absence of TAM induction. Chemical LTP was used to ensure that a very large proportion of the synapses and spines examined were potentiated, allowing for a more robust biochemical analysis. Previous studies have shown that, like tetanus-induced LTP, cLTP triggers a persistent increase in autophosphorylation of aCaMKII (Makhinson et al., 1999; Otmakhov et al., 2004).

Chemical LTP was induced in CA1 of acute hippocampal slices by a brief application of high K+/Ca2+-ACSF after FSK treatment. Immediately after FSK-treated slices were exposed to high K+/Ca2+-free ACSF for 5 min, synaptic transmission was nearly abolished; however, at 10 min after application of high K+/Ca2+-ACSF, fEPSPs were dramatically potentiated (>2 fold of baseline). The cLTP was stable 30 min after induction and lasted for at least 50 min in all tested groups. High K+/Ca2+-free ACSF was applied for 5 min in the absence of FSK. There was no difference between groups. One way ANOVA (Fig. 5B, mixed con). Induction of cLTP did not change the total levels of aCaMKII. The presence or activation of the LBDG521R-cNR2B protein blocks the long-lasting increase in phosphorylation of aCaMKII after cLTP induction, a result that attests to the functional importance of the interaction between the tail of NR2B and CaMKII.

We found that exposure of tg/veh slices to FSK followed by high K+/Ca2+ produced a significant increase in autophosphorylation of aCaMKII at Thr286 at both 10 min (181.7 ± 19.2% of base level; one-sample t test, p < 0.01; n = 6 per group) and 40 min (141.0 ± 13.6% of base level, one-sample t test, p < 0.05; n = 6 per group) compared with that in control slices (base level, 100%) exposed only to normal ACSF (Fig. 5). However, TAM treatment blocked the increase in phosphorylation of Thr286 observed 40 min after induction of cLTP in tg/tam group (95.4 ± 14.8% of base level, one-sample t test, p > 0.05; n = 6) (Fig. 5). There is no difference among the wt/tam, wt/veh and wt/veh groups (p > 0.05; thus, we pooled the data together for analysis (Fig. 5B, mixed con). Induction of cLTP did not change the total levels of aCaMKII in any group. The control exposed to high K+/Ca2+ alone did not show increases in phosphorylation of Thr286. These results demonstrate that activation of the LBDG521R-cNR2B protein blocks the long-lasting increase in phosphorylation of aCaMKII after cLTP induction, a result that attests to the functional importance of the interaction between the tail of NR2B and CaMKII.
phorylation of αCaMKII, affects synaptic transmission, LTD or LTP in hippocampal area CA1 of adult mice. First, we looked at basal synaptic transmission by measuring EPSPs after stimulation with a range of stimulus intensities. To control for the different recruitment of presynaptic axons in different slices, we plotted synaptic responses against presynaptic fiber volley amplitudes. Analysis of the input-output curves from the four groups (wt/veh, wt/tam, tg/veh and tg/tam) showed that they were indistinguishable, indicating that there are no obvious changes in basal synaptic strength. Additionally, PPF across different interstimulus ISIs revealed no differences among genotype/treatment groups (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). Altogether these data suggest that, as we had expected, activation of the LBD<sup>G521R</sup>-cNR2B does not disrupt presynaptic function or basal synaptic transmission.

In contrast, activation of LBD<sup>G521R</sup>-cNR2B impaired LTP in area CA1 induced by two different patterned tetani. LTP triggered by a 100 Hz tetanus for 1 s was significantly reduced, albeit not completely absent, in hippocampal slices prepared from transgenic mice treated with TAM (tg/tam, 129.1 ± 5.3% measured 50–60 min post tetanus, n = 16 slices, 8 mice; tg/veh, 152.9 ± 6.8% at 50–60 min post, n = 16 slices, 8 mice; wt/tam, 148.2 ± 6.8% at 50–60 min post, n = 16 slices, 8 mice; wt/veh, 151.6 ± 6.2% at 50–60 min post, n = 14 slices, 7 mice; one-way ANOVA, F<sub>3,56</sub> = 3.58, Fisher’s PLSD, p < 0.05) (Fig. 6A). Similarly, a 10 Hz tetanus for 10 s also revealed significant deficit in LTP specifically in tg/tam mice (114.5 ± 6.0% at 50–60 min post tetanus, one-way ANOVA, F<sub>3,44</sub> = 4.02, Fisher’s PLSD, p < 0.05; n = 12 slices, 6 mice per group), but not in other mice (tg/veh, 137.5 ± 6.0%; wt/tam, 139.6 ± 7.3%; wt/veh 139.2% ± 5.2%) (Fig. 6B). These results demonstrate that the activation of LBD<sup>G521R</sup>-cNR2B disrupts LTP, suggesting that the suppression of autophosphorylation of αCaMKII produced by disruption of the interaction between CaMKII and the tail of NR2B is sufficient to interfere with mechanisms of LTP. In contrast with LTP, the activation of the LBD<sup>G521R</sup>-cNR2B did not affect LTD induced with a traditional 900 pulse/1 Hz stimulus in juvenile mice (Fig. 6C).

Collectively, these results indicate that activation of the LBD<sup>G521R</sup>-cNR2B causes deficits in LTP without disrupting synaptic transmission or LTD. Because autophosphorylation of αCaMKII at Thr286 has been shown to be critical for induction of LTP under the conditions we have used here, the simplest explanation for our findings is that a decrease in autophosphorylation of Thr286 in the PSD produced by disruption of the interaction between the tail of NR2B and CaMKII by LBD<sup>G521R</sup>-cNR2B prevents induction of LTP.

Loss of normal CaMKII/NR2B interaction impairs spatial learning in the Morris water maze

Mutations that disrupt CaMKII function lead to LTP impairments and spatial learning deficits in the Morris water maze task (Silva et al., 1992; Mayford et al., 1996). Training in this hippocampal-dependent task also results in the activation of CaMKII (Tan and Liang, 1996). To determine whether the activation of the LBD<sup>G521R</sup>-cNR2B affects spatial learning, we tested the mice in the hidden-platform version of the Morris water maze, a task in which animals are trained to search for a submerged platform in a pool of water. After 3, 5, and 7 d of training (four trials per day), spatial learning was assessed with probe trials in which the platform was removed from the pool, and the mice were allowed to search for 60 s. In the probe trial given at day 5, we found that tg/veh, wt/veh, and wt/tam mice spent significantly more time searching in the quadrant where the platform was during training (target quadrant) than in the other three quadrants. The tg/tam mice, in contrast, did not search selectively in the target quadrant (Fig. 7B). There were significant differences between tg/tam mice and the other three groups in three different measures of spatial learning: the time spent in the target quadrant (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). Altogether these data suggest that, as we had expected, activation of the LBD<sup>G521R</sup>-cNR2B does not disrupt presynaptic function or basal synaptic transmission.
that the activation of the LBDG521R-cNR2B impairs spatial learning without affecting motor performance in the maze.

To ensure that the spatial learning deficit observed in LBDG521R-cNR2B transgenic mice was not caused by changes in motivation, coordination or sensory processing, we tested the animals in the visible-platform task of the water maze. This is a nonspatial learning task known to be independent of hippocampal function, but with otherwise similar motivational, sensory and motor requirements. In this task, the platform is marked by a visible cue. A new group of cNR2B transgenic and controls were trained for 4 d (eight trials per day) with the visible-platform on a fixed location, but with a pseudo-random start position. Both cNR2B transgenic and WT mice performed similarly on this task and improved with training, compared with proximity to the opposite position in the pool. The data are analyzed from eight to 14 animals per group. *p < 0.05 (one-way ANOVA followed by Fisher’s PLSD) reveals learning deficits in the tg/tam mice, but not in any of the other groups.

Loss of normal CaMKII/NR2B interaction impairs performance in the delayed spatial win-shift eight-arm radial-maze task

The results presented above indicate that the activation of LBDG521R-cNR2B causes deficits in spatial learning. To confirm that the activation of LBDG521R-cNR2B disrupted hippocampal-dependent learning, and to further explore the nature of these deficits, we tested mice in the delayed eight-arm radial-maze task, which is a spatial working memory test that exploits rodents’ innate foraging behavior. Previous studies have shown that lesions or inactivations of the hippocampal formation (Olton et al., 1978; Floresco et al., 1997) cause severe impairments on this spatial task.

All four groups of mice (wt/veh, wt/tam, tg/veh, and tg/tam, eight to 10 mice per group) received 9 consecutive days of training. We averaged data collected from three consecutive days from each mouse as one block, and used these blocks for the final comparison among groups. There was no significant difference between the four groups in the percentage of total errors made on phase A across training days (repeated ANOVA, $F_{(0.96)} = 0.44; p > 0.05$) (data not shown). Together, these results indicate that impairments in spatial learning account for the Morris water maze deficits of the LBDG521R-cNR2B transgenic mice.

Figure 6. Reduced LTP and normal LTD in the cNR2B transgenic mice. A, LTP induced with 100 Hz (1 s) stimulation is reduced in the tg mice with tamoxifen induction compared with the other three control groups. The LTP of the other three groups is indistinguishable. B, LTP induced with 100 Hz (10 s) stimulation is also specifically reduced in the tg mice with tamoxifen induction. C, LTD (1 Hz, 15 min) in young tg mice (P14–P17) after tamoxifen induction is normal. Insets, Sample traces (average of five) taken at baseline and 50–60 min after tetanic stimulation.

Figure 7. Tamoxifen induction reveals spatial learning deficits in the cNR2B mice. A, Time to find the hidden platform plotted versus training day. B, Percentage time spent in each quadrant during a probe trial on day 5. Grouped bars from left to right indicate four different groups. Time spent in target quadrant (TQ), adjacent left quadrant (AL), opposite quadrant (OP), and adjacent right quadrant (AR) are labeled as indicated. C, Platform quadrant crossing times during the day 5 probe test. D, Average proximity to the exact position where the platform is during training, compared with proximity to the opposite position in the pool. The data are analyzed from eight to 14 animals per group. *p < 0.05 (one-way ANOVA followed by Fisher’s PLSD) reveals learning deficits in the tg/tam mice, but not in any of the other groups.

Figure 8. Learning deficits in the delayed spatial win-shift eight-arm radial-maze task. A, tg/tam mice are normal in phase A, indicating that they are not anxious and that they do not have visual or motor deficits. B, tg/tam mice learn the task at significantly slower rate as indicated by a poor percentage of correct choice in block 2 of phase B. C, tg/tam mice make significant more across errors in block 2, compared with wt/tam controls, revealing spatial memory defects. D, tg/tam mice made significant more within errors in block 2, compared with wt/tam controls, indicating that they may have spatial working memory deficits. Every experimental group includes 8–10 animals. *p < 0.05 (one-way ANOVA followed by Fisher’s PLSD) reveals learning deficits in the tg/tam mice.
we examined the types of errors made during the second testing block, we found that the tg/tam mice made more across-phase errors (across error; \( F_{(3,31)} = 3.4; p < 0.05 \) (Fig. 8C) and more within-phase errors (within error; \( F_{(3,31)} = 6.1; p < 0.05 \) (Fig. 8D)) than the other three groups of control mice. However, just as in the water maze, extended training remediated these deficits. In the third block of testing (days 7–9), the performance of tg/tam mice, measured as percentage of correct choices, across and within errors, was indistinguishable from the others (\( F_{(3,31)} < 1.0; \ p > 0.05 \)) (Fig. 8B–D). There were no significant differences among the groups in the latencies to reach the first food cup or in the average time per subsequent choice of arms on either the training or the test phases of the daily trials (\( F_{(3,31)} < 1.0; \ p > 0.05 \)).

After all four groups reached criterion performance (all four pellets were retrieved in five or fewer choices during phase B for 2 consecutive days), we increased the interphase interval to 20, 60, or 150 min randomly. Statistical analysis revealed an overall significant effect of delay (repeated ANOVA, \( F_{(3,42)} > 3.0; p < 0.05 \). However, there was no significant effect of genotype/treatment or an interaction between genotype/treatment and delay (repeated ANOVA, \( F_{(3,42)} < 1.0; \ p > 0.05 \)). The performance of all four groups decreased in the same manner and fell to chance level at the 150 min delay. This suggests that transgenic mice do not forget which arms were visited during phase A more quickly than WT animals.

Next, we examined whether the deficits described above were caused by impairments in spatial learning or to difficulties in acquiring the win-shift rule. To do this we tested the same groups of mice in a different environment (with distinct room cues but the same win-shift rules). Although both groups of mice took a shorter time (6 d) to achieve the previous criterion performance, the overall performance of the mice was similar in both mazes. Once again the tg/tam mice were slower to acquire the task and showed a reduction in the percentage of correct choices (\( F_{(1,20)} = 8.2; \text{PLSD,} \ p < 0.01 \). They also made more across errors (\( F_{(1,20)} = 9.4; \text{PLSD,} \ p < 0.01 \)) than control groups in the second block of testing (data not shown). This result suggests that deficits described for the cNR2B transgenic mice are unlikely to be caused by impairments in either working memory or rule learning; instead they are likely attributable to problems with developing and using a spatial map of the environment. Once a map is formed, their performance in the maze is normal. These results are consistent with the water maze findings and they indicate that the induction of LBD\(^{G521R}\)-cNR2B impairs the initial acquisition of spatial information.

### Discussion

Here we have shown that in the adult hippocampus, interactions with the c-terminal tail of NR2B help to modulate key post-translational synaptic events in the induction of LTP, including the autophosphorylation of αCaMKII and phosphorylation of GluR1. Our study also provides the first evidence that the association between CaMKII and NR2B affects the rate of acquisition of hippocampal-dependent spatial learning.

A key tool used in the present study is an inducible system in a transgenic mouse that allowed us to interfere with NR2B interactions in a temporal and neuron-specific manner. In our mice, the transgenic fusion protein LBD\(^{G521R}\)-cNR2B is highly expressed in forebrain regions, especially in the hippocampus, a structure important for spatial learning. Because it lacks a transmembrane domain, LBD\(^{G521R}\)-cNR2B diffuses freely in neuronal cytosol, as demonstrated by immunoblot analysis. We have shown that injection of TAM, the activator of the transgenic protein, specifically activates the binding function of LBD\(^{G521R}\)-cNR2B without other systemic effects on the mouse brain. First, CaMKII binds to LBD\(^{G521R}\)-cNR2B and reduces the association of CaMKII with endogenous full-length NR2B only after application of TAM. Second, after TAM administration LBD\(^{G521R}\)-cNR2B concentrates in the PSD fraction where it competes with endogenous NR2B for binding with CaMKII. Third, TAM administration does not affect the levels of other PSD-enriched proteins, including NR1, NR2A, NR2B and PSD-95. Fourth, induction by TAM also interferes with the autophosphorylation of PSD-associated αCaMKII and subsequent phosphorylation of GluR1 in the PSD fraction. Finally, the inducible disruption of the biochemical events listed above leads to impaired hippocampal synaptic plasticity and consequently to deficits in hippocampal-dependent spatial learning. We note that activation of LBD\(^{G521R}\)-cNR2B blocks only ~25% of the NR2B/CaMKII interaction (Fig. 3B). This incomplete block could be a reason why LTP and learning were not completely blocked. The fact that a 25% decrease in the NR2B/CaMKII interaction has such a pronounced impact on LTP and learning attests to the importance of the NR2B/CaMKII interaction and motivated our study of its effects on learning. The autophosphorylation of αCaMKII at Thr286 is postulated to play a major role in synaptic plasticity and learning, including spatial learning (Mayford et al., 1996; Giese et al., 1998; Irvine et al., 2005, 2006). This autophosphorylation is also critical for contextual fear conditioning: training in this task increases autophosphorylation of αCaMKII at Thr286 in spines in the lateral amygdala (LA), whereas blockade of activation of αCaMKII impairs both synaptic plasticity and fear conditioning in the LA (Rodrigues et al., 2004). Tetanic (Ouyang et al., 1997, 1999; Lengyel et al., 2004) or chemically (Makhinson et al., 1999; Lengyel et al., 2004) induced LTP in area CA1 results in long-lasting increases in autophosphorylation of αCaMKII (Lengyel et al., 2004). These findings suggest that a long-lasting increase in Thr286 autophosphorylations required for the full induction of LTP and learning.

In our present study, we examined both basal expression and dynamic changes in phosphorylation of Thr286 after induction of LTP in cNR2B transgenic mice and in wild-type littermates. We found that activation of the cNR2B transgenic protein results in the downregulation of the basal autophosphorylation of αCaMKII. Interestingly, the decrease in αCaMKII autophosphorylation after activation of the transgenic protein was only observed in the synaptosome fraction, but not in forebrain homogenates, suggesting that the impaired autophosphorylation of αCaMKII observed in PSD is not simply attributable to sterical hindrance after active cNR2B binding to CaMKII, but rather to disruption of NR2B interactions in the PSD. Although PSD-95 is known to bind to endogenous NR2B receptors (Kornau et al., 1995), PSD-95 is unlikely to be the target of the transgenic cNR2B fragment in the PSD because our studies did not show significant binding between the activated fusion protein and PSD-95. However, considering that under our IP conditions, the association between endogenous NR2B with PSD-95 does not seem to be as strong as that with CaMKII (Fig. 3C), the present results may not exclude the possibility that LBD\(^{G521R}\)-cNR2B binds to PSD-95 in vivo. Nevertheless, they do suggest that under certain conditions, TAM-activated LBD\(^{G521R}\)-cNR2B has a more stable association with CaMKII. The molecular mechanism mediating LBD\(^{G521R}\)-cNR2B translocation to the PSD fraction, where it competes with endogenous NR2B for binding with CaMKII, still needs to be identified.
Consistent with previous studies (Makhinson et al., 1999; Lengyl et al., 2004; Otmakhov et al., 2004), we also found that in wt mice, cLTP leads to a persistent increase (>40 min after treatment) in the Thr286 autophosphorylation of membrane-associated aCaMKII in CA1; however, activation of the cNR2B transgenic protein, and subsequent disruption of CaMkII/NR2B interactions impair the stability of Thr286 autophosphorylation triggered by cLTP induction. Therefore, our results indicate that binding to NR2B is critical to stabilize aCaMKII Thr286 phosphorylation in the PSD. Accordingly, we found that activation of the cNR2B transgenic protein dampened the amplitude of CA1 LTP and impaired hippocampal-dependent spatial learning.

There is considerable evidence that synaptic activity causes CaMkII to translocate from the dendritic cytoplasm to the synapse both in vitro and in vivo (Strack et al., 1997; Shen and Meyer, 1999; Gleason et al., 2003). Targeting of CaMkII to the PSD is required for effective phosphorylation of its membrane substrates, including the GluR1 subunit of the AMPA channel (Tsui et al., 2005), whose modulation is critical for synaptic plasticity. Notably, previous studies further demonstrated that CaMkII translocation to synapses is more persistent after LTP induction (Ouyang et al., 1997, 1999; Miller et al., 2002; Otmakhov et al., 2004). The available evidence suggests two mechanisms that may determine whether translocation is persistent or transient: T286 autophosphorylation (Lengyl et al., 2004) and binding to PSD proteins, such as NR2B (Bayer et al., 2001, 2006).

Compared with recent studies from organotypic hippocampal slices demonstrating that the association between NR2B and active CaMkII is required for LTP (Barria and Malinow, 2005), our studies provide in vivo evidence that interaction with NR2B may facilitate T286 autophosphorylation (Bayer et al., 2001, 2006). Because NR2A is the principal NR2 subunit in the adult hippocampus (Sheng et al., 1994), sufficient Ca2+ entry to produce LTP may require activation of the dominant NR2 species (NR2A), but modulation of downstream events may require NR2B. Synaptic inclusion of the NR2B subunit, as either NR2B/NR1 or NR2A/NR2B/NR1 heteromeric channels (Sheng et al., 1994), may be critical for the stabilization of long-lasting increases in Thr286 phosphorylation of aCaMkII in the PSD, and consequently for LTP induction and learning.

NR2B has also been previously proposed to be an important PSD docking site for CaMkII (Leonard et al., 1999; Bayer et al., 2001). Hence, it is surprising that the activation of the transgenic protein, which we showed affects the interaction between CaMkII and NR2B, did not alter the levels of total CaMkII in the PSD. Nevertheless, CaMkII interactions with other PSD proteins (including Densin-180) appear unaltered in the cNR2B mice, and because CaMkII is expressed at high basal levels in the PSD, small decreases in these levels, caused by interfering with CaMkII/NR2B interactions, may not be easily detectable by immunoblotting. An alternative and more likely interpretation is that in spines of cNR2B mice, CaMkII holoenzymes still remain associated with the PSD without association with NR2B and are, thus, copurified with the PSD during our biochemical fractionation. This scenario implies that the CaMkII holoenzymes that copurify with the PSD fraction in the TAM-treated cNR2B mice are positioned differently than in controls because of the deficit in association with the endogenous NR2B receptor. This difference could explain the reduced CaMkII autophosphorylation and reduced AMPA receptor phosphorylation.

Together, the data presented here indicate that the interaction between CaMkII and NR2B-containing NMDA receptors plays a crucial role in synaptic plasticity and spatial learning.
transiently increased following the induction of long-term potentiation in the rat hippocampus. Eur J Neurosci 20:3063–3072.


