Identification and characterization of a novel phosphorylation site on the GluR1 subunit of AMPA receptors

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Phosphorylation of various AMPA receptor subunits can alter synaptic transmission and plasticity at excitatory glutamatergic synapses in the central nervous system. Here, we identified threonine-840 (T840) on the GluR1 subunit of AMPA receptors as a novel phosphorylation site. T840 is phosphorylated by protein kinase C (PKC) in vitro and is a highly turned-over phosphorylation site in the hippocampus. Interestingly, the high basal phosphorylation of T840 in the hippocampus is maintained by a persistent activity of a protein kinase, which is counter-balanced by a basal protein phosphatase activity. To study the function of T840, we generated a line of mutant mice lacking T840, in addition to two previously identified phosphorylation sites S831 and S845. Using this mouse, we demonstrate that T840 may regulate synaptic plasticity in an age-dependent manner.

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Introduction

Many of the brain functions critically depend on plasticity of the glutamatergic excitatory synaptic transmission, which can be accomplished postsynaptically by modulation of glutamate receptors. AMPA-type glutamate receptors mediate the majority of fast excitatory synaptic transmission, and functional changes to these receptors are implicated in synaptic plasticity (Song and Huganir, 2002; Collingridge et al., 2004). One way to alter the function of AMPA receptors is by changing phosphorylation of its subunits. All four subunits of AMPA receptors, GluR1–4, which have several identified phosphorylation sites on their intracellular carboxy-terminus (Song and Huganir, 2002).

Among the four subunits, GluR1 has three identified phosphorylation sites, serines 818 (S818) (Boehm et al., 2006), 831 (S831) and 845 (S845) (Roche et al., 1996). S831 is phosphorylated by calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC), S845 is a cAMP-dependent protein kinase (PKA) substrate (Roche et al., 1996; Barria et al., 1997a; Mammen et al., 1997), while S818 is phosphorylated by PKC (Boehm et al., 2006). Changes in phosphorylation of GluR1 at S845 and S831 affect AMPA receptor mediated currents (Derkach et al., 1999; Banke et al., 2000) and are involved in long-term potentiation (LTP) and long-term depression (LTD) in the hippocampus (Barria et al., 1997b; Kameyama et al., 1998; Lee et al., 1998, 2000, 2003). Recent data suggest that S845 and S818 affect the trafficking AMPA receptors to synapses and/or stabilize synaptic AMPA receptors (Esteban et al., 2003; Lee et al., 2003; Boehm et al., 2006).

Phosphorylation of GluR2 subunit at serine-880 has been implicated in LTD both in hippocampus and in cerebellum (Daw et al., 2000; Matsuda et al., 2000; Xia et al., 2000; Kim et al., 2001; Chung et al., 2003). Phosphorylation of this site can regulate synaptic localization of AMPA receptors by altering its interaction with intracellular binding partners: glutamate receptor interacting protein (GRIP)/AMPA receptor binding protein (ABP) and protein interacting with C-kinase-1 ( PICK-1) (Matsuda et al., 1999; Chung et al., 2000).

Among several identified GluR4 subunit phosphorylation sites (Carvalho et al., 1999), S842, which is a PKA phosphorylation site, was shown to be involved in activity-dependent trafficking of AMPA receptors to synapses (Esteban et al., 2003). Therefore, phosphorylation of different subunits of AMPA receptor seems to play an important role in regulating receptor function, which can alter synaptic transmission and/or synaptic plasticity.

We had recognized early on from generating phosphopeptide maps of the GluR1 subunit that there remain additional unidentified phosphorylation sites on GluR1 (Blackstone et al., 1994; Roche et al., 1996; Mammen et al., 1997). Here, we report that threonine-
840 (T840) is one of the major phosphorylation sites on GluR1. This site is phosphorylated by PKC in vitro and can be regulated by altering PKC activity in hippocampal slices. Interestingly, phosphorylation of T840 undergoes rapid turnover under basal conditions in the hippocampus, sustained by a balance between persistently active protein kinase(s) and protein phosphatase(s). To study the functional role of GluR1 T840, we generated a line of mice lacking T840 and show that it may be involved in regulating synaptic plasticity mechanisms in an age-dependent fashion.

Results
Mapping of a “basal” phosphorylation site on GluR1

To identify phosphorylation sites on GluR1 subunit of AMPA receptors, we generated phosphopeptide maps using trypsin digested GluR1 immunoprecipitated from metabolically labeled hippocampal slices. We were specifically interested in a large phosphopeptide spot (#1 in Fig. 1) that had not been previously characterized. This phosphopeptide was highly phosphorylated under basal conditions and was not noticeably upregulated by either forskolin (50 μM, 30 min) or phorbol ester (TPA: 1 μM, 30 min) treatment to the slices. Treatment of forskolin to slices upregulated phosphorylation on a phosphopeptide (#5 in Fig. 1) that contains S845, a previously identified PKA phosphorylation site (Roche et al., 1996), without affecting much the other phosphopeptides. On the other hand, phorbol ester treatment increased the signal of two phosphopeptides (#3 and #6 in Fig. 1), which contain S831, a known CaMKII and PKC phosphorylation site (Roche et al., 1996; Mammen et al., 1997).

To identify the phosphorylation site responsible for the phosphorylation of phosphopeptide #1, we transfected HEK293 cells with various GluR1 constructs. Transfection of a wildtype GluR1 generated a similar phosphopeptide map to the one obtained from GluR1 immunoprecipitated from hippocampal slices (Fig. 1B, left panel). We identified T840 as a responsible phosphorylation site since mutation of T840 to an alanine (GluR1-T840A) eliminated phosphopeptide #1 (Fig. 1B, middle panel). As shown in Fig. 1, phosphopeptide #1 migrates in close proximity to phosphopeptide #6, which includes S831. Therefore, we further confirmed that T840A indeed eliminates phosphopeptide #1 by generating a phosphopeptide map using GluR1 with both T840A and S831A mutations (Fig. 1B, right panel). In addition to phosphopeptide #1, phosphopeptides #3 and #6 corresponding to S831 were also absent. Our results indicate that T840 site is responsible for the phosphorylation of phosphopeptide #1.

Generation of T840 phosphospecific antibody

In order to characterize the role of GluR1-T840 phosphorylation site, we generated phosphorylation site-specific antibody (Ab)
against T840. The T840 phospho-Ab was specific to phosphorylated T840 since preincubation with the antigen phosphopeptide blocked its signal while the antibody was preincubated with the same sequence non-phosphorylated peptide or random sequenced peptide did not block the signal (Fig. 2A). To determine if the T840 phospho-Ab specifically recognizes phosphorylated GluR1, we performed immunoblots using hippocampal samples with or without lambda-phosphatase treatment. As shown in Fig. 2B, lambda-phosphatase treatment abolished the signal using GluR1-T840 phospho-Ab, but not with GluR1 C-terminal recognizing antibody.

The T840 phospho-Ab recognizes GluR1 in both hippocampal slices and in HEK293 cells transfected with GluR1 (Fig. 2C). However, the signal in HEK293 cells was much less compared to that obtained from hippocampal homogenates despite the fact that total GluR1 expression was much greater. This indicates that the basal phosphorylation level of T840 is higher in the hippocampus compared to that in transfected HEK293 cells. This is in line with our observation from the phosphopeptide maps where we saw a larger phosphorylation signal on phosphopeptide #1 in GluR1 isolated from hippocampal slices compared to that from transfected HEK293 cells (Fig. 1).

Fig. 2. Generation of T840 phosphorylation site-specific antibody. (A) T840 phospho-antibody signal is blocked specifically when the antibody was preincubated with the antigen phosphopeptide (lane 2), while the signal is intact when preincubated with the same sequence non-phosphorylated peptide (lane 3) or random sequenced peptide (lane 4). (B) Signal generated by T840 phospho-antibody disappears when hippocampal samples were preincubated with lambda phosphatase (top panel). Note that GluR1 C-terminus recognizing antibody still recognized GluR1 in lambda phosphatase treated samples (bottom panel). (C) Comparison of GluR1-T840 phosphorylation level in membrane (P2) fractions prepared from HEK293 cells expressing GluR1 and hippocampal slices. Note that T840 phosphorylation level in HEK293 cells is much less compared to that in hippocampus (left panel) despite the higher expression of GluR1 in HEK293 cells (shown by probing with GluR1 C-Ab; right panel). (D) T840 phospho-antibody specifically recognizes T840 phosphorylation site. HEK293 cells were transfected with cDNAs of wildtype GluR1, GluR1 with S831A mutation, and GluR1 with T840A mutation. T840 phospho-antibody signal disappeared in cells transfected with GluR1-T840A construct (top blot, lane 4), while S831 phospho-antibody signal is absent in cells transfected with GluR1-S831A construct (middle blot, lane 3). Expression levels of each GluR1 constructs were equivalent as shown using GluR1 C-terminal antibody (bottom blot).
To further examine the specificity of the T840 phospho-Ab, we tested it against various mutations of GluR1 expressed in HEK293 cells. Mutation at T840A eliminated the signal using T840 phospho-Ab, while mutation at S831A abolishes signal using S831 phospho-Ab but not with T840 phospho-Ab (Fig. 2D).

T840 is phosphorylated by PKC in vitro and is regulated by PKC and protein phosphatases in hippocampal slices.

Next we wanted to determine the protein kinase responsible for the phosphorylation of GluR1-T840 site. To do this we first performed an in vitro phosphorylation assay using GluR1 C-terminal GST fusion proteins and purified protein kinases. Phosphorylation of wildtype GluR1 fusion proteins with PKC increased T840 phosphorylation detected using T840 phospho-antibody, while CaMKII and PKA did not (Fig. 3A). Mutating T840 to an alanine in the GluR1 fusion protein (R1-T840A) abolished the phosphorylation by PKC, further confirming that PKC was affecting T840 phosphorylation (Fig. 3A). CaMKII and PKA were active in our assay since they were able to phosphorylate their respective substrates, S831 and S845 (Fig. 3A).

To examine whether PKC activation is responsible for T840 phosphorylation in vivo, we treated hippocampal slices with a PKC activator phorbol ester (TPA, 1 μM). As shown in Fig. 2B, TPA treatment increased T840 phosphorylation in parallel to S831, a previously identified PKC site. Since T840 phosphorylation is basally high in hippocampal slices, we next examined if an ongoing PKC activity is required to maintain this. We tested this by treating hippocampal slices with a PKC inhibitor, Go6976 (1 μM). We found that T840 phosphorylation was decreased by Go6976.
treatment, which indicates that a persistent PKC activity is needed to keep the basal phosphorylation at T840. This suggests that the basal level of T840 phosphorylation should be susceptible to alterations in basal protein phosphatase activity. To test this we incubated slices with a broad spectrum protein phosphatase inhibitor okadaic acid (1 μM). As shown, okadaic acid treatment enhanced phosphorylation of T840 from early time points (Fig. 3D). This is in contrast with S831 and S845, where phosphorylation detectably increases only after incubation in okadaic acid for more than 2 h (Kameyama et al., 1998) (data not shown). Collectively, our results suggest that the high basal phosphorylation of GluR1-T840 in hippocampal slices is maintained by a persistent activity of PKC, which is counter-balanced by a basal protein phosphatase activity.

Generation of a GluR1 “penta” phosphorylation site mutant that lacks GluR1-T840

In order to test the in vivo function of GluR1-T840 phosphorylation site, we wanted to generate mice lacking T840 on GluR1 using a gene knock-in technique. We have previously characterized a line of mice that lack previously identified phosphorylation sites S831 and S845 (Lee et al., 2003). LTP was reduced while LTD was abolished in this “double” phosphomutants. Our initial question was whether the residual LTP could be due to T840 phosphorylation. To test this hypothesis, we generated mice lacking S831, S845 and T840 phosphorylation sites. This was accomplished by combining the following mutations on the GluR1 target construct: S831A, T838A, S839A, T840A and S845A (Fig. 4A). This “penta” mutation was necessary since mutating T840A alone might allow the responsible protein kinase to phosphorylate upstream T838 or S839, which might substitute the function of T840.

We confirmed the mutations in the knock-in mice using both PCR and immunoblot analysis (Figs. 4A and B). The mutants did not show any gross abnormalities in anatomy and behavior. Hippocampal cytoarchitecture was normal as shown using Nissl stain, and GluR1 distribution was normal as shown using immunohistochemistry (Fig. 4C).

Deficits in LTP and LTD in adult “penta” phosphomutant mice

Next we tested synaptic plasticity in the CA1 region of hippocampal slices made from adult (2-3 months old) wildtype and homozygote littermates. LTP induced by a theta burst stimulation (TBS) was reduced in the homozygotes (128±11% of baseline measured at 2 h post-TBS, n=11, 7 mice) compared to the wildtypes (172 ±9% of baseline, n=13, 7 mice; Student’s t-test: P<0.01; Fig. 5A). This result is similar to the previously described “double” phosphomutant, which only lacks S831 and S845, suggesting that T840 may not contribute additionally to LTP. Similar to the “double” phosphomutants, LTD induced with paired-pulse 1 Hz protocol (PP-1 Hz, paired pulses at interstimulus interval of 50 ms repeated at 1 Hz for 15 min) was abolished in the “penta” homozygotes (94±3% of baseline at 1 h post-onset of PP-1 Hz, n=12, 6 mice) compared to wildtypes (82±4% of baseline, n=11, 5 mice; Student’s t-test: P<0.02; Fig. 5B).

Normal LTP and LTD in young “penta” phosphomutants

There is now accumulating evidence that molecular mechanisms of LTP and LTD may differ in young versus adult hippocampus.
(Zamanillo et al., 1999; Jensen et al., 2003; Lee et al., 2003; Wikstrom et al., 2003; Yasuda et al., 2003; Palmer et al., 2004; Nosyreva and Huber, 2005). In particular, the involvement of GluR1 in LTP has been reported to occur only in adults (Zamanillo et al., 1999; Jensen et al., 2003). Similarly, we have reported using the “double” phosphomutants that LTP is normal in young mutants despite a deficit seen in adults (Lee et al., 2003). Therefore, we next looked at LTP and LTD in young “penta” phosphomutants. We found that LTP is normal in the young “penta” mutants (homozygotes: 138±9% of baseline, n=16, 6 mice; wildtype: 149±12% of baseline, n=15, 7 mice; Fig. 5C). In addition, LTD induced with 1 Hz stimulation was normal in the young “penta” mutants (homozygotes: 77±5% of baseline, n=10, 6 mice; wildtype: 85±3% of baseline, n=12, 6 mice; Fig. 5C). This result is different from our previous observation from “double” mutants, where LTD was absent in the young homozygotes (Lee et al., 2003).

Normal basal synaptic transmission in adult “penta” phosphomutants

To rule out the possibility that the observed deficits in LTP and LTD in the adult “penta” mice were due to abnormalities in basal synaptic function, we compared basal synaptic transmission between homozygotes and wildtype littermates. Basal AMPA receptor-mediated synaptic responses and pharmacologically isolated NMDA receptor-mediated synaptic responses were both normal in the “penta” mutants (Figs. 6A and B). In addition, presynaptic function assessed by measuring the paired-pulse facilitation (PPF) ratio at various interstimulus intervals (ISIs) did not differ between the “penta” homozygotes and wildtypes (Fig. 6C). Therefore, lacking T840, in addition to S831 and S845, does not seem to alter basal synaptic transmission measured using our assays.

Discussion

Here we identified and described T840 as a novel phosphorylation site on GluR1 subunit of AMPA receptors. GluR1-T840 is phosphorylated by PKC in vitro and is a highly turned-over phosphorylation site under basal conditions in the hippocampus. The basal phosphorylation of GluR1-T840 in vivo is likely due to a persistent activity of a protein kinase, which is counter-balanced by a basal protein phosphatase activity. In order to test the in vivo role of GluR1-T840, we generated a GluR1 “penta” phosphomutant, which lacks not only T840 but also S831 and S845, two previously characterized phosphorylation sites (Roche et al., 1996; Mammen et al., 1997). We found that adult “penta” mice show similar phenotypes as the previously described “double” phosphomutants lacking only S831 and S845 phosphorylation sites, in that they show deficits in both LTP and LTD (Lee et al., 2003). However, in the young “penta” mice, LTP and LTD were both intact. This is different from the “double” mutants, which show normal LTP but have deficits in LTD (Lee et al., 2003).

Our results show that GluR1-T840 is highly phosphorylated under basal conditions in the hippocampus compared to GluR1 transfected HEK293 cells. This could be interpreted that the activity of protein kinase(s) responsible for T840 phosphorylation is higher in the hippocampus. Alternatively, the protein kinase responsible for the phosphorylation of T840 may not be present in the HEK293 cells. Although our in vitro phosphorylation data suggest that PKC can phosphorylate T840, whether PKC is indeed the protein kinase responsible for in vivo phosphorylation is unclear. Our data indicate that the protein kinase responsible for phosphorylating T840 in vivo should be affected by PKC activator phorbol ester and inhibitor Go6976 (Fig. 3), either because the drugs directly affect its activity or indirectly through alterations in PKC activity. In any case, our results indicate that the activity of this protein kinase is likely to be high in the hippocampus under basal conditions.

An interesting finding is that the basal phosphorylation of T840 seems to undergo a rapid turnover, which is due to persistent activity of a protein kinase and a protein phosphatase. This is quite different from previously characterized S831 and S845 on GluR1. Blockade of basal protein phosphatase activity by okadaic acid

![Fig. 6. Normal basal synaptic transmission in "penta" phosphomutants.](image)
quickly upregulated T840 (Fig. 3D), while S831 and S845 only increased with prolonged (>3 h) application (Kameyama et al., 1998). This suggests that S831 and S845 may not be readily accessible to protein phosphatases. In support of this the same PKC inhibitor (Go6976) that reduced T840 phosphorylation (Fig. 3C) did not affect S831 (data not shown). The mechanism in which protein phosphatases preferentially affect T840 is unclear at this point, but could be due to conformation of the GluR1 C-tail or by interacting proteins that mask the other sites.

To further elucidate the role of T840 phosphorylation site, we generated a line of “penta” phosphomutants that have alanine substitutions at five residues: S831A, T838A, S839A, T840A and S845A. We had reported previously that adult “double” phosphomutant mice (S831A and S845A) display residual LTP (Lee et al., 2003). Therefore, we wanted to test whether the remaining T840 could have contributed to the phenotype. Hence our strategy was to mutate T840 in addition to the two previously characterized phosphorylation sites. Our assumption was that several phosphorylation sites may interact with each other to regulate GluR1 function, which is in line with a recent study (Boehm et al., 2006). Despite the fact that the basal phosphorylation of GluR1-T840 is high in the hippocampus, we did not observe a difference in basal AMPA receptor-mediated synaptic transmission in our “penta” mutants. Therefore, the function of the GluR1-T840 is not likely to maintain basal synaptic transmission. In addition, it is also unlikely that GluR1-T840 participates in LTP or LTD in the adult hippocampal CA1 region since the phenotype we saw is similar to our “double” phosphomutants lacking only S831 and S845 (Lee et al., 2003). An interesting observation that may lead to the function of GluR1-T840 comes from our data using young “penta” mice. We found that both LTP and LTD are intact in the young “penta” mice. This is qualitatively different from the “double” mutants, where LTP was intact but LTD was defective (Lee et al., 2003). This suggests that the lack of T840 can somehow “rescue” LTD in young mice when both S831 and S845 phosphorylation sites are missing. Whether this “rescue” is due specifically to lacking T840 in addition to S831 and S845 or due to the additional mutations at T838 and S839 cannot be determined at this point. However, we surmise that the lack of T840 allows LTD to be expressed via another pathway not dependent on S831 and/or S845 phosphorylation site(s). One possibility is a recruitment of GluR2-dependent mechanisms for LTD (Daw et al., 2000; Kim et al., 2001; Seidenman et al., 2003). However, the link between GluR1-T840 and GluR2-dependent LTD mechanisms is presently not clear and awaits further exploration. The “rescue” of LTD by T840 mutation only occurred in young animals indicating that this putative alternative pathway for LTD is no longer accessible in the adults.

**Experimental methods**

**Hippocampal slice preparation and metabolic labeling**

Hippocampal slices from adult Sprague–Dawley rats (150–250 g, males) were prepared as previously described (Lee et al., 2000). In brief, 400 μm thick hippocampal slices were dissected using ice-cold dissection buffer (212.7 mM sucrose, 2.6 mM KCl, 1.23 mM NaH2PO4, 26 mM NaHCO3, 10 mM dextrose, 3 mM MgCl2 and 1 mM CaCl2) and recovered at room temperature in ACSF (124 mM NaCl, 5 mM KCl, 1.25 mM NaH2PO4, 26 mM NaHCO3, 10 mM dextrose, 1.5 mM MgCl2 and 2.5 mM CaCl2) inside a Plexiglas chamber supplied with 95% O2/5% CO2. For metabolic labeling, hippocampal slices were incubated with 1 μCi/ml [32P]orthophosphate in ACSF for 2 h. Slices were then collected and quickly homogenized in 1 ml volume of ice-cold 1% Triton X-100 in IP buffer (10 mM NaPO4, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 10 mM sodium pyrophosphate, 50 mM NaF, 1 mM NaVO3, 1 μM okadaic acid and 10 U/ml aprotinin, pH 7.4). Insoluble fraction was removed by centrifugation at 14,000× g for 10 min at 4 °C. The GluR1 subunit was immunoprecipitated using anti-GluR1 C-terminal antibody as described previously (Roche et al., 1996; Mammen et al., 1997). Samples were analyzed on a 7.5% SDS–PAGE gel and visualized by autoradiography.

Phosphorylation of 1 μg of GST-fusion proteins of GluR1 C-terminal 80 amino acids was performed by incubation with 300 ng of purified kinases at 30 °C for 30 min in a 100 μl total volume. Phosphorylation reaction buffer for PKC and CaMKII reactions contained 100 mM HEPES, pH 7.5, 20 mM MgCl2, 200 μM CaCl2 and 250 μM ATP. For PKC reactions, 50 μg/ml phosphatidylserine and 5 μg/ml diacylglycerol were added. Phosphorylation of fusion proteins by PKA was performed in 20 mM
HEPES, pH 7.0, 10 mM MgCl₂ and 250 μM ATP. Reactions were stopped with SDS–PAGE sample buffer, and the phosphorylated fusion proteins were resolved by SDS–PAGE and prepared for immunoblot analysis.

Generation of GluR1 “penta” phosphorylation site mutant mice

Gene targeting was carried out as reported previously (Lee et al., 2003). Serines 831, 839 and 845 and threonines 838 and 840 were mutated to alanine by site directed PCR mutagenesis (Stratagene). Correct homologous recombiant ES clones were injected into C57BL/6 blastocysts, and chimeras were crossed to C57BL/6 mice. These mice were crossed to CMV-Cre (129 and C57BL/6 hybrid genetic background) to remove the neomycin resistant gene (Neo') cassette from the germ line through Cre/loxP mediated excision (Nagy et al., 1998). After confirmation of Neo' cassette excision by PCR and Southern blot analysis, heterozygous mice were bred to produce homozygotes, and mutants in which the Cre gene was absent were used for the following breeding. The intercross of heterozygotes resulted in production of wildtype, heterozygous and homozygous offspring at the expected 1:2:1 Mendelian ratio. For all analyses, mice with 129 and C57BL/6 hybrid genetic background were used. All procedure relating to animal care and treatment conformed to the institutional and NIH guidelines.

Hippocampal slice preparation and electrophysiology

Hippocampal slices from mice were prepared as previously described (Lee et al., 2003). In brief, 400 μm thick hippocampal slices were dissected using ice-cold dissection buffer and recovered at room temperature in ACSF bubbled with 95% O₂:5% CO₂. All recordings were done in a submersion recording chamber perfused with ACSF (29–30 °C, 2 ml/min) bubbled with 95% O₂:5% CO₂. Synaptic responses were digitized and stored online using Igor™ software (Wave Metrics). LTP was induced using a theta burst stimulation (TBS: four trains, each consisting of ten 100 Hz bursts (four pulses) given at 5 Hz, repeated at 10 s intervals) (Larson et al., 1986). LTD was elicited using a paired-pulse 1 Hz protocol (PP-P: paired-pulses of 50 ms interstimulus interval (ISI) repeated at 1 Hz for 15 min) (Lee et al., 2003) or a standard 1 Hz (15 min) protocol (Dudek and Bear, 1992). For measurement of paired-pulse facilitation (PPF), we used ISIs of 25, 50, 100, 200, 400, 800 and 1600 ms. Pharmacologically isolated NMDA receptor-mediated synaptic responses were measured using 0 mM MgCl₂ ACSF with 10 μM NBQX. At the end of each experiment, 100 μM t-APV was added to confirm the NMDA receptor-mediated response.

Immunoblot analysis

Proteins on SDS–PAGE gel were transferred to PVDF membranes (Immobilion™, Millipore), and the blots were blocked in blocking buffer (1% bovine serum albumin and 0.1% Tween 20 in PBS, pH 7.4) for ~1 h. The blots were then incubated for ~1 h in primary antibodies diluted in blocking buffer and washed 5 × 5 min with blocking buffer. Secondary antibody (for ECL: linked to horseradish peroxidase, for ECF: linked to alkaline phosphatase) diluted in blocking buffer was applied for ~1 h followed by 5 × 5 min washes with blocking buffer. The blots were developed using either enhanced chemiluminescence (ECL) or enhanced chemifluorescence (ECF, Amersham). ECL blots were exposed to BioMax film (Kodak) and digitized using a densitometer (Molecular Dynamics). ECF blots were scanned using a Storm 860 scanner (Molecular Dynamics), and the fluorescence intensity of each band was quantified using ImageQuant™ software (Molecular Dynamics).

Immunohistochemistry

Immunohistochemistry was performed as described (Lee et al., 2003). In brief, brains were fixed in 4% paraformaldehyde by transcardiac perfusion and transferred to a 30% sucrose solution to sink. Coronal sections were cut at 40 μm thickness on a freezing microtome (Leica) and collected into ice-cold 0.1 M PBS (pH 7.4). Sections were stained with cresyl violet and observed under the light microscope or processed for immunohistochemistry using anti-GluR1 C-terminal Ab or anti-GluR2 C-terminal Ab.

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