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# Ethanol Inhibition of Recombinant NMDA Receptors Is Not Altered by Co-Expression of CaMKII-α or CaMKII-β

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## Abstract

Previous studies have shown that the N-methyl-D-aspartate (NMDA) receptor is an important target for the actions of ethanol in the brain. NMDA receptors are glutamate-activated ion channels that are highly expressed in neurons. They are activated during periods of significant glutamatergic synaptic activity and are an important source of the signaling molecule calcium in the post-synaptic spine. Alterations in the function of NMDA receptors by drugs or disease are associated with deficits in motor, sensory and cognitive processes of the brain. Acutely, ethanol inhibits ion flow through NMDA receptors while sustained exposure to ethanol can induce compensatory changes in the density and localization of the receptor. Defining factors that govern the acute ethanol sensitivity of NMDA receptors is an important step in how an individual responds to ethanol. In the present study, we investigated the effect of calcium-calmodulin dependent protein kinase II (CaMKII) on the ethanol sensitivity of recombinant NMDA receptors. CaMKII is a major constituent of the post-synaptic density and is critically involved in various forms of learning and memory. NMDA receptor subunits were transiently expressed in human embryonic kidney 293 cells (HEK 293) along with CaMKII-α or CaMKII-β tagged with the green fluorescent protein (GFP). Whole cell currents were elicited by brief exposures to glutamate and were measured using patchclamp electrophysiology. Neither CaMKII-α or CaMKII-β had any significant effect on the ethanol inhibition of NR1/2A or NR1/2B receptors. Ethanol inhibition was also unaltered by deletion of CaMKII binding domains in NR1 or NR2 subunits or by phospho-site mutants that mimic or occlude CaMKII phosphorylation. Chronic treatment of cortical neurons with ethanol had no significant effect on the expression of CaMKII-α or CaMKII-β. The results of this study suggest that CaMKII is not involved in regulating the acute ethanol sensitivity of NMDA receptors.

### Keywords

electrophysiology; alcohol; ion channel; kinase; phosphorylation

## Introduction

Results from previous studies conducted in this laboratory and others over the last 15–20 years have demonstrated that ethanol alters the function of a wide variety of ion channels expressed in brain neurons (reviewed by Lovinger, 1997; Narahashi, 2000). Among these is the family

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of N-methyl-D-aspartate (NMDA) receptors that are activated in vivo by the neurotransmitter glutamate. NMDA receptors are calcium-permeable ion channels critically involved in fast glutamatergic synaptic transmission (Dingledine et al., 1999). During acute exposure, ethanol reduces current flow through NMDA receptors and this may contribute to some of neurobehavioral signs and symptoms that follow alcohol ingestion.

The mechanisms and sites of action of ethanol on native and recombinant NMDA receptors have been extensively studied. For example, it is known that ethanol does not act as a competitive antagonist at the site for glutamate or the co-agonist glycine (Gonzales and Woodward, 1990; Peoples and Weight, 1992). It also appears that ethanol's inhibition of NMDA current does not resemble that of open channel blockers such as ketamine or MK-801 (Mirshahi and Woodward, 1995; Weight et al., 1993). Ethanol's actions are more consistent with an effect on channel gating (Wright et al., 1996) and sites within transmembrane domains of both NR1 and NR2 NMDA subunits have been identified that regulate the overall sensitivity of the receptor to ethanol (Ren et al., 2003; Ronald et al., 2001). It is also known that the overall sensitivity of NMDA receptors to ethanol varies across brain regions and developmental stages (Lovinger, 1995; Simson et al., 1991; Swartzwelder et al., 1995; Yang et al., 1996). Some of this variation may arise from differences in subunit stochiometry as ethanol inhibition of recombinant NMDA receptors is subtly altered by changes in the expression of either NR1 or NR2 subunits (Jin and Woodward, 2006). However, it is also possible that factors other than subunit composition contribute to determining the magnitude of ethanol inhibition of NMDA receptors.

One important process that may be involved in regulating the ethanol sensitivity of NMDA receptors is kinase-dependent phosphorylation. NMDA receptors contain consensus phosphorylation sites for both serine/threonine and tyrosine kinases (reviewed by Dingledine et al., 1999). Phosphorylation of these sites can affect both channel function (Lan et al., 2001) and trafficking of receptor subunits from intracellular compartments to the plasma membrane (Scott et al., 2003; Tingley et al., 1993). Previous studies from this laboratory examined the effect of Src family kinases (Src/Fyn) and the serine/threonine kinase PKA on ethanol inhibition of recombinant and native NMDA receptors (Anders et al., 1999a; Anders et al., 1999b; Xu and Woodward, 2006). Fyn kinase was shown to reduce the ethanol inhibition of NR2A but not NR2B containing receptors while PKA had no significant effect on any NMDA receptor subtype studied.

In the present study, we continued this line of study and investigated the effects of CaMKII on the ethanol inhibition of recombinant NMDA receptors. CaMKII is the major serine/threonine kinase found at glutamatergic synapses and is activated in a calmodulin-dependent manner following calcium entry through NMDA receptors. CaMKII is a critical regulator of glutamatergic synapses and is essential for many forms of synaptic plasticity including long-term potentiation; a cellular correlate of memory (Lisman et al., 2002; Malenka and Bear, 2004). Of special interest is the finding that NR2B subunits possess both binding and phosphorylation sites for CaMKII suggesting that this receptor's sensitivity to ethanol may be affected by an interaction with CaMKII. We explored this possibility by recording whole-cell currents from recombinant NMDA receptors co-expressed with CaMKII in human embryonic kidney (HEK) 293 cells and by examining changes in CaMKII expression following treatment of neurons with ethanol.

## Materials and Methods

### Molecular Biology, Cell Culture and Transfection

The NMDA receptor cDNAs used in these experiments were kindly provided by Drs. S. Nakanishi (Kyoto Univ, Kyoto, Japan) and P. Seeburg (Max-Planck Institute for Medical

Research, Heidelberg, Germany). Plasmids encoding CaMKII-α and GFP-tagged CaMKII-α and CaMKII-β were kindly provided by Dr. R. Colbran (Vanderbilt Univ. Nashville, TN) and Dr. T. Meyer (Stanford Univ., Palo Alto, CA), respectively. Site-directed mutagenesis was performed using the Quik-Change mutagenesis kit (Invitrogen, Carlsbad, CA) and mutants were confirmed by DNA sequencing. Human embryonic kidney (HEK) 293 cells were obtained from ATCC (Manassas, VA). Cells were maintained in feeder flasks containing serum-supplemented DMEM in a humidified incubator supplied with 5% $CO_2$ and were split weekly. For recordings, cells were plated onto poly-ornithine coated 35 mm dishes and transfected with plasmids encoding various NMDA receptor subunits using Lipofectamine 2000 (Invitrogen, Inc., Carlsbad, CA) according to the manufacturer's recommendation. In each set of transfections, at least one cDNA encoded the enhanced green fluorescent protein (eGFP) allowing for detection of transfected cells. Plasmids were used at a ratio of 1:1:1 unless otherwise indicated. Following transfection, the NMDA antagonist AP5 (200 μM) was added to the media to prevent glutamate-mediated excitotoxicity (Cik et al., 1994). AP5 was removed by extensive washing prior to recording. Primary cultures of rat cortical neurons were prepared from postnatal day 1 Sprague-Dawley rat pups and maintained as previously described (Chandler et al., 1997). All experimental protocols involving animals were approved by the MUSC IACUC committee and conformed to guidelines listed inn the NIH Guide for the Care and Use of Laboratory Animals. Briefly, cell suspensions were prepared after trypsin treatment and plated at a density of 3×106 cells in poly-lysine coated 35 mm culture dishes. Cultures were incubated in DMEM media supplemented with plasma-derived horse serum and treated after 3 days with cytosine arabinoside to reduce glial proliferation. After 2 days treatment, the media was replaced with serum containing DMEM and cultures were kept for 14–16 days.

## Electrophysiology

Dishes containing transfected cells were mounted on the stage of an Olympus IX50 inverted microscope and perfused with extracellular recording solution at 1–2 ml/min. The recording solution contained (in mM); NaCl (135), KCl (5.4), $CaCl_2$ (1.8), HEPES (5), glucose (10), (pH adjusted to 7.4 and osmolarity adjusted to 310–325 mOsm with sucrose. Patch pipettes (2–5 mOhms) were pulled from borosilicate glass (1.5 × 0.86 mm) and filled with internal solution containing (in mM); KCl 140, MgCl2 6, CaCl2 1, EGTA 5, HEPES 10, tetraethylammonium chloride 2, and NaATP 4, (pH adjusted to 7.2 with KOH). Transfected cells were identified by eGFP fluorescence and whole-cell voltage clamp recordings were carried out at room temperature using an Axon 200B microamplifier (Molecular Devices, Union City, CA). Cells were held at −60 mV to monitor seal breakthrough and maintained at this potential unless otherwise noted. Whole-cell capacitance and series resistance were compensated for and access resistance was monitored over the course of the experiment. Cells with unstable holding currents were not used for analysis. NMDA receptor currents were evoked using a Warner FastStep multi-barrel perfusion system to switch between normal extracellular solution to one containing agonist (glutamate plus glycine (both at 10 μM)) or agonist plus ethanol (25–100 mM). Each cell tested was exposed to duplicate solutions containing agonist (− and + ethanol) with or without added magnesium. The order of solutions was alternated between cells. Data were filtered at 1–2 kHz and acquired at 5 kHz using an Instrutech ITC-16 digital interface (Instrutech Corp., Port Washington, NY) controlled by IgorPro software (Wavemetrics, Lake Oswego, OR) running the Pulse control acquisition module. Data were analyzed offline using Axograph software (Axograph, Sydney, New South Wales, Australia). Agonist-evoked currents were baseline subtracted and amplitudes were measured during the last 0.5 seconds of agonist application when currents had reached steady-state levels. Ethanol inhibition was calculated using the formula $(1-(I_{Glutamate+Etoh}/I_{Control})) \times 100$, where $I_{Glutamate+Etoh}$ represents the response to co-application of agonist + ethanol, and $I_{Control}$ represents the mean of two responses to agonist, one before and one after the co-application of ethanol. Ethanol

was purchased from Aaper Alcohol and Chemical Company (Shelbyville, KY) while all other chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

### Immunohistochemistry and Western Blot Analysis

HEK 293 cells transfected with empty vector or cDNAs encoding CaMKII were fixed in 4% paraformaldehyde and exposed to anti-CaMKII-α or –βantibodies (Invitrogen, Carlsbad, CA). After incubation, cells were washed and then incubated with Alexa594 anti-mouse secondary antibody (Molecular Probes, OR). Dishes were then rinsed, dried and mounted with 1,4-diazabicyclo(2,2,2)octane containing mounting media. Cells were imaged using an inverted Olympus IX-50 microscope equipped with fluorescent optics, CCD camera and IPLab image acquisition software (BD Biosciences, Rockville, MD). For western blot analysis of CaMKII, primary cultures of cortical neurons were treated with ethanol (100 mM) for 4 days beginning at day 10 in culture. Cells were scraped into 100 μL of ice-cold homogenization buffer containing (in mM unless noted otherwise): Tris-HCl 50, NaCl 50, EGTA 10, EDTA 5, sodium pyrophosphate 2, sodium orthovanadate 1, AEBSF 0.1, aprotinin 1 μg/ml, benzamide 1, leupeptin 10 μg/ml, pepstatin 1 μg/ml, pH 7.5. Cells from 3 dishes were combined, probe sonicated for 5 s and centrifuged at 15,000 × g for 30 min at 4oC. Protein concentration was determined using the bicinchoninic acid assay (BCA; Pierce Biotechnology Inc., Rockford, IL). An aliquot of each sample was diluted with an equal volume of 2x sample buffer yielding final concentrations of 50 mM Tris-HCl, 4% glycerol (w/v), 4% SDS, 1% 2-mercaptoethanol and Bromophenol Blue, pH 6.7. Samples were boiled for 5 min, and 20 μg of sample were separated on a 10% SDS-polyacrylamide gel using the buffer system of Laemmli and transferred to a Millipore Immobilon-P PVDF membrane (Bedford, MA). After transfer, blots were washed with PBS containing 0.05% Tween-20 and then blocked with PBST containing 5% nonfat dry milk for 1 h at room temperature. Membranes were incubated overnight with anti-CaMKII-α or –β (Invitrogen, Carlsbad, CA) diluted 1:2500 in PBST. The membranes were washed in PBST and then incubated with horseradish peroxidase-conjugated goat anti-mouse secondary diluted 1:1000 in PBST. Antigen-antibody complex was determined using enhanced chemiluminescence (Amersham Biosciences, Arlington Heights, IL). Film autoradiograms were quantified by computer-assisted densitometry. For detection of auto-phosphorylated CaMKII, HEK 293 cells were transfected with wild-type or mutant isoforms of CaMKII and collected 48 hrs later as described above for neurons. Blots were incubated with an antibody that recognizes phosphorylated threonine 286 in the auto-inhibitory domain of CaMKII (1:1000 dilution; PhosphoSolutions, Aurora, CO) followed by a horseradish peroxidase-conjugated goat anti-rabbit secondary diluted 1:1000 in PBST. Antigen-antibody complex was determined as described above.

### Data Analysis

Data are expressed as mean ± SEM. and were analyzed by analysis of variance or t-test (as indicated) using Prism 4.0 software (Graphpad Software, San Diego, CA).

## Results

### *Effects of* CaMKII-α and CaMKII-β *on NR1/NR2 Receptors*

HEK 293 cells transfected with NMDA receptor subunits showed robust inward currents when briefly exposed to glutamate and glycine (Figure 1B). These currents were reliably inhibited by ethanol and showed full recovery upon washout. For example, 100 mM ethanol inhibited currents from cells expressing NR1/2A or NR1/2B receptors by approximately 25–30% (Figure 1B). These values are similar to those published in previous reports from this laboratory (Blevins et al., 1997; Jin and Woodward, 2006; Xu and Woodward, 2006).

To examine whether CaMKII affects ethanol's inhibition of NMDA receptor current, HEK 293 cells were transfected with cDNAs encoding various NMDA subunits and either GFP-tagged CaMKII-α or CaMKII-β. Calmodulin was not included in the transfection mixture as HEK 293 cells express appreciable amounts of this protein (Black et al., 2004). Under control conditions, non-transfected HEK 293 cells or those expressing just NMDA subunits showed no evidence of endogenous CaMKII expression as determined by immunohistochemistry. In contrast, cells transfected with CaMKII-α showed high levels of expression after labeling with a monoclonal CaMKII antibody (Figure 1A, left panel). Similar results were observed with CaMKII-β (data not shown). To test whether expressed CaMKII is active, cells were transfected with NR1/2A subunits and either CaMKII-α or CaMKII-β or a mutant form of CaMKII in which threonine 286 was replaced with alanine (T286A). Cells were maintained for 48 hrs, collected and analyzed by western blotting using an antibody that recognizes phosphorylated threonine 286 within the auto-inhibitory domain of CaMKII. As shown in Figure 1A, untransfected cells or those transfected with the mutant CaMKII(T286A) showed no signal for activated CaMKII while those transfected with either CaMKII-α or CaMKII-β showed robust staining for auto-phosphorylated CaMKII. Interestingly, treatment of cells with glutamate and glycine (10 μM each) for five minutes prior to sample collection did not appear to significantly increase the intensity of the phospho-CaMKII signal. Cells transfected with NMDA subunits and GFP-tagged CaMKII cDNAs generated inward currents when exposed to glutamate and glycine (Figure 1B). In contrast to a previously published report (Sessoms-Sikes et al., 2005), there appeared to be no significant effect of CaMKII co-expression on the amplitude or macroscopic kinetics of the glutamate response in cells transfected with NR1 and any of the NR2 subunits tested (data not shown). This is likely due to the use of different concentrations of agonists used in the two studies and the fact that adherent cells were recorded from in the present study. Importantly, the ability of 100 mM ethanol to inhibit NR1/2A or NR1/2B receptors was not altered by coexpression of CaMKII-α or CaMKII-β (Figure 1B). A similar lack of effect of CaMKII was observed at lower concentrations of ethanol (Figure 1C).

The C-terminus of the NR1 subunit contains three intracellular domains termed C0, C1, and C2 (Figure 2A). The C0 domain is expressed by all NR1 splice variants while the C1 and C2 regions are subject to alternative splicing events (reviewed by Dingledine et al., 1999). CaMKII binds to both the C0 and C1 domains of the NR1 subunit (Leonard et al., 1999). To investigate whether these domains may mask an effect of CaMKII on ethanol sensitivity, a series of experiments were conducted using naturally occurring or engineered C-terminal deletion variants of the NR1 subunit. Neither CaMKII-α nor CaMKII-β had any significant effect on the ethanol inhibition of NMDA receptors lacking the C0 (NR1-C0) or C1 (NR1-2a) domain (Figure 2B–C).

NR2B subunits also show high affinity for CaMKII binding and residues required for this binding have been identified. These include amino acids that flank Serine 1303, the major CaMKII phosphorylation site on the NR2B subunit (Figure 3A). For example, binding of CaMKII to NR2B can be almost completely eliminated by mutation of leucine 1298 to alanine (Strack et al., 2000). In addition, phosphorylation of S1303 reduces CaMKII binding while binding is enhanced when this site is occupied by an alanine residue. To test whether these sites have any impact on the ethanol sensitivity of NMDA-mediated currents, a series of mutants in the NR2B C-terminus were generated. Cells expressing the NR1-1a subunit and NR2B(L1298A) generated currents in response to glutamate and glycine that were similar to those observed for wild-type receptors (data not shown). Ethanol (100 mM) inhibited currents in NR1-1a/NR2B(L1298A) receptors by approximately 37%, a value that was not significantly different from that of the matched wild-type control. Co-expression of either CaMKII-α or CaMKII-β had no significant effect on ethanol's inhibition of these receptors (Figure 3B). Cells expressing NR1-1a and a CaMKII phosphorylation-deficient NR2B mutant (S1303A) were inhibited by 100 mM ethanol to the same extent as wild-type controls. Co-expression of either

CaMKII-α or CaMKII-β with NR2B(S1303A) had no significant effect on the magnitude of this effect. Similar findings were observed when CaMKII phosphorylation of NR2B was mimicked by replacing S1303 with a negatively charged aspartate residue (S1303D).

Although co-expression of CaMKII had no discernable effects on the acute ethanol inhibition of recombinant NMDA receptors, it is possible that chronic exposure of brain neurons to ethanol may alter the expression of CaMKII isoforms. To examine this possibility, primary cultures of cortical neurons were treated with 100 mM ethanol for 4 days beginning at day 10 in vitro and then examined for levels of CaMKII-α and CaMKII-β by western blotting. As shown in Figure 4, ethanol treatment had no significant effect on the amounts of either isoform of CaMKII expressed or on the ratio of CaMKII-α to CaMKII-β.

## Discussion

The results of this study strongly suggest that the ethanol sensitivity of recombinant NMDA receptors is not regulated by CaMKII and that sustained exposure of neurons to ethanol does not affect the expression of CaMKII. Co-expression of either α- or β-CaMKII had no significant effect on the ethanol inhibition of NR2A or NR2B containing NMDA receptors. The lack of a significant effect was not due to problems with expression as GFP-tagged constructs ensured that cells expressing functional NMDA receptors also expressed the kinase. Expressed CaMKII was capable of activation as shown by the strong signal obtained with a phospho-specific antibody that recognizes phosphorylated threonine 286 of CaMKII. This residue lies within the auto-inhibitory domain that normally occludes the catalytic site of CaMKII. Calcium/calmodulin dependent auto-phosphorylation at this site relieves this inhibition and induces a persistent calcium-independent form of kinase activity (Lisman et al., 2002). To further investigate the role of CaMKII in regulating ethanol sensitivity of NMDA receptors, site-directed mutagenesis was used to mimic the state of CaMKII phosphorylation of the NR2B subunit (S1303A, S1303D). These receptors also showed normal ethanol inhibition. The results of these manipulations are similar to those previously reported by this laboratory for PKA, another serine/threonine kinase (Xu and Woodward, 2006). In that study, a variety of experiments were performed in order to enhance or inhibit PKA-mediated phosphorylation of recombinant NMDA receptors. No significant differences in ethanol inhibition were noted in that study suggesting that PKA, like CaMKII does not directly influence the ethanol sensitivity of NMDA receptors.

Despite these negative findings, results from previous studies have suggested that phosphorylation does appear to change the ethanol sensitivity of certain ion channels. For example, CaMKII-mediated phosphorylation of calcium-activated potassium channels (BK) shifts the effects of ethanol on channel function from activation to inhibition (Liu et al., 2006). Findings from similar studies have implicated other kinases as important determinants of the ethanol sensitivity of channel function. In particular, tyrosine phosphorylation has been suggested to reduce the ethanol sensitivity of both recombinant (Anders et al., 1999b) and native (Alvestad et al., 2003; Miyakawa et al., 1997) NMDA receptors. However, subsequent studies suggested that in some cases, these kinase-mediated alterations in ethanol inhibition may be indirectly mediated. For example, the effect of Fyn kinase on recombinant NMDA receptors is likely due to relief of tonic zinc inhibition rather than a reduction in ethanol inhibition. This is supported by the observation that Fyn kinase only affected zinc sensitive NR2A containing receptors whereas those composed of zinc insensitive NR2B subunits were unaffected (Anders et al., 1999b). In addition, when the effects of zinc were removed either by the use of chelators or zinc-insensitive NR2A mutants, Fyn kinase no longer reduced ethanol inhibition (Woodward, 2004; Woodward and Smothers, 2003). In neurons, tyrosine phosphorylation mediated changes in ethanol inhibition may reflect ethanol-induced increases in the surface expression of functional NMDA receptors (Yaka et al., 2003). This change, which

occurs over minutes, is manifested by a rebound increase in the magnitude of current flow following washout of the ethanol containing solution (Li et al., 2005; Yaka et al., 2003). More recently, PKCε has been shown to reduce the potentiation of both native and recombinant γ2 containing $GABA_A$ receptor subtypes to ethanol and the benzodiazepine flunitrazepam (Qi et al., 2007). Overall, these results illustrate the usefulness of recombinant expression and recording techniques and suggest that phosphorylation may modulate the ethanol sensitivity of ion channels in both kinase and receptor subtype dependent ways.

Although the results from the present study suggest that phosphorylation of NMDA subunits by CaMKII does not actively regulate the acute ethanol sensitivity these receptors, it is possible that by regulating the expression of CaMKII itself, ethanol could indirectly affect NMDA receptor function or localization. However, using standard western blotting techniques, there were no obvious differences in CaMKII expression in cultured cortical neurons following a 4-day exposure to a relatively high concentration of ethanol (100 mM). These findings suggest that total levels of CaMKII may be relatively stable in the presence of ethanol although they do not rule out the possibility that ethanol may induce changes in the sub-cellular localization of CaMKII that could affect NMDA receptor activity. Similar findings have been made with respect to NMDA subunits (Carpenter-Hyland et al., 2004) and studies to examine whether CaMKII may be similarly affected are currently underway in this laboratory.

In summary, the results of the present study suggest that while CaMKII is an important regulator of glutamatergic synaptic transmission, it appears unlikely to play a major role in determining the acute ethanol sensitivity of brain NMDA receptors.

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## References

Alvestad RM, Grosshans DR, Coultrap SJ, Nakazawa T, Yamamoto T, Browning MD. Tyrosine dephosphorylation and ethanol inhibition of N-Methyl-D-aspartate receptor function. J. Biol. Chem 2003;278:11020–11025. [PubMed: 12536146]

Anders DL, Blevins T, Sutton G, Chandler LJ, Woodward JJ. Effects of c-Src tyrosine kinase on ethanol sensitivity of recombinant NMDA receptors expressed in HEK 293 cells. Alcohol. Clin. Exp. Res 1999a;23:357–362. [PubMed: 10069568]

Anders DL, Blevins T, Sutton G, Swope S, Chandler LJ, Woodward JJ. Fyn tyrosine kinase reduces the ethanol inhibition of recombinant NR1/NR2A but not NR1/NR2B NMDA receptors expressed in HEK 293 cells. J. Neurochem 1999b;72:1389–1393. [PubMed: 10098840]

Black DJ, Tran QK, Persechini A. Monitoring the total available calmodulin concentration in intact cells over the physiological range in free Ca2+ Cell. Calcium 2004;35:415–425. [PubMed: 15003851]

Blevins T, Mirshahi T, Chandler LJ, Woodward JJ. Effects of acute and chronic ethanol exposure on heteromeric N-methyl-D-aspartate receptors expressed in HEK 293 cells. J. Neurochem 1997;69:2345–2354. [PubMed: 9375665]

Carpenter-Hyland EP, Woodward JJ, Chandler LJ. Chronic ethanol induces synaptic but not extrasynaptic targeting of NMDA receptors. J. Neurosci 2004;24:7859–7868. [PubMed: 15356198]

Chandler LJ, Sutton G, Norwood D, Sumners C, Crews FT. Chronic ethanol increases N-methyl-D-aspartate-stimulated nitric oxide formation but not receptor density in cultured cortical neurons. Mol. Pharmacol 1997;51:733–740. [PubMed: 9145911]

Cik M, Chazot PL, Stephenson FA. Expression of NMDAR1-1a (N598Q)/NMDAR2A receptors results in decreased cell mortality. Eur. J. Pharmacol 1994;266:R1–R3. [PubMed: 7909752]

Dingledine R, Borges K, Bowie D, Traynelis SF. The glutamate receptor ion channels. Pharmacol. Rev 1999;51:7–61. [PubMed: 10049997]

Gonzales RA, Woodward JJ. Ethanol inhibits N-methyl-D-aspartate-stimulated [3H]norepinephrine release from rat cortical slices. J. Pharmacol. Exp. Ther 1990;252:1138–1144. [PubMed: 2162947]

Jin C, Woodward JJ. Effects of 8 different NR1 splice variants on the ethanol inhibition of recombinant NMDA receptors. Alcohol. Clin. Exp. Res 2006;30:673–679. [PubMed: 16573586]

Lan J-Y, Skeberdis VA, Jover T, Grooms SY, Lin Y, Araneda RC, Zheng X, Bennett MVL, Zukin RS. Protein kinase C modulates NMDA receptor trafficking and gating. Nat. Neurosci 2001;4:382–390. [PubMed: 11276228]

Leonard AS, Lim IA, Hemsworth DE, Horne MC, Hell JW. Calcium/calmodulin-dependent protein kinase II is associated with the N-methyl-Daspartate receptor. Proc. Natl. Acad. Sci. USA 1999;96:3239–3244. [PubMed: 10077668]

Li HF, Mochly-Rosen D, Kendig JJ. Protein kinase Cgamma mediates ethanol withdrawal hyper-responsiveness of NMDA receptor currents in spinal cord motor neurons. Br. J. Pharmacol 2005;144:301–307. [PubMed: 15655532]

Lisman J, Schulman H, Cline H. The molecular basis of CaMKII function in synaptic and behavioural memory. Nat. Rev. Neurosci 2002;3:175–190. [PubMed: 11994750]

Liu J, Asuncion-Chin M, Liu P, Dopico AM. CaM kinase II phosphorylation of slo Thr107 regulates activity and ethanol responses of BK channels. Nat. Neurosci 2006;9:41–49. [PubMed: 16341213]

Lovinger DM. Developmental decrease in ethanol inhibition of N-methyl-D-aspartate receptors in rat neocortical neurons: Relation to the actions of ifenprodil. J. Pharmacol. Exp. Ther 1995;274:164–172. [PubMed: 7616394]

Lovinger DM. Alchohols and neurotransmitter gated ion channels: past, present and future. Naunyn-Schmiedeberg's Arch. Pharmacol 1997;356:267–282. [PubMed: 9303562]

Malenka RC, Bear MF. LTP and LTD: an embarrassment of riches. Neuron 2004;44:5–21. [PubMed: 15450156]

Mirshahi T, Woodward JJ. Ethanol sensitivity of heteromeric NMDA receptors: Effects of subunit assembly, glycine and NMDAR1 Mg2+-insensitive mutants. Neuropharmacology 1995;34:347–355. [PubMed: 7630488]

Miyakawa T, Yagi T, Kitazawa H, Yasuda M, Kawai N, Tsuboi K, Niki H. Fyn-kinase as a determinant of ethanol sensitivity: Relation to NMDA-receptor function. Science 1997;278:698–701. [PubMed: 9381182]

Narahashi T. Neuroreceptors and ion channels as the basis for drug action: past, present, and future. J. Pharmacol. Exp. Ther 2000;294:1–26. [PubMed: 10871290]

Peoples RW, Weight FF. Ethanol inhibition of N-methyl-D-aspartate-activated ion current in rat hippocampal neurons is not competitive with glycine. Brain Res 1992;571:342–344. [PubMed: 1377089]

Qi ZH, Song M, Wallace MJ, Wang D, Newton PM, McMahon T, Chou WH, Zhang C, Shokat KM, Messing RO. Protein Kinase C{epsilon} Regulates {gamma}-Aminobutyrate Type A Receptor Sensitivity to Ethanol and Benzodiazepines through Phosphorylation of {gamma}2 Subunits. J. Biol. Chem 2007;282:33052–33063. [PubMed: 17875639]

Ren H, Honse Y, Peoples RW. A site of alcohol action in the fourth membrane-associated domain of the N-methyl-D-aspartate receptor. J. Biol. Chem 2003;278:48815–48820. [PubMed: 14506267]

Ronald KM, Mirshahi T, Woodward JJ. Ethanol inhibition of N-methyl-D-aspartate receptors is reduced by site-directed mutagenesis of a transmembrane domain phenylalanine residue. J. Biol. Chem 2001;276:44729–44735. [PubMed: 11572853]

Scott DB, Blanpied TA, Ehlers MD. Coordinated PKA and PKC phosphorylation suppresses RXR-mediated ER retention and regulates the surface delivery of NMDA receptors. Neuropharmacology 2003;45:755–767. [PubMed: 14529714]

Sessoms-Sikes S, Honse Y, Lovinger DM, Colbran RJ. CaMKIIalpha enhances the desensitization of NR2B-containing NMDA receptors by an autophosphorylation-dependent mechanism. Mol. Cell. Neurosci 2005;29:139–147. [PubMed: 15866054]

Simson PE, Criswell HE, Johnson KB, Hicks RE, Breese GR. Ethanol inhibits NMDA-Evoked electrophysiological activity in vivo. J. Pharmacol. Exp. Ther 1991;257(No 1):225–231. [PubMed: 1826926]

Strack S, McNeill RB, Colbran RJ. Mechanism and regulation of calcium/calmodulin-dependent protein kinase II targeting to the NR2B subunit of the N-methyl-D-aspartate receptor. J. Biol. Chem 2000;275:23798–23806. [PubMed: 10764765]

Swartzwelder HS, Wilson WA, Tayyeb MI. Differential sensitivity of NMDA receptor-mediated synaptic potentials to ethanol in immature versus mature hippocampus. Alcoholism Clin. Exp. Res 1995;19:320–323.

Tingley WG, Roche KW, Thompson AK, Huganir RL. Regulation of NMDA receptor phosphorylation by alternative splicing of the C-terminal domain. Nature 1993;364:70–73. [PubMed: 8316301]

Weight FF, Peoples RW, Wright JM, Lovinger DM, White G. Ethanol action on excitatory amino acid activated ion channels. Alcohol Alcohol 1993:353–358. [PubMed: 8394713]

Woodward JJ. Fyn kinase does not reduce ethanol inhibition of zinc-insensitive NR2A-containing N-methyl-D-aspartate receptors. Alcohol 2004;34:101–105. [PubMed: 15902902]

Woodward JJ, Smothers C. Ethanol inhibition of recombinant NR1/2A receptors: effects of heavy metal chelators and a zinc-insensitive NR2A mutant. Alcohol 2003;31:71–76. [PubMed: 14615013]

Wright JM, Peoples RW, Weight FF. Single-channel and whole-cell analysis of ethanol inhibition of NMDA-activated currents in cultured mouse cortical and hippocampal neurons. Brain Res 1996;738:249–256. [PubMed: 8955520]

Xu M, Woodward JJ. Ethanol inhibition of NMDA receptors under conditions of altered protein kinase A activity. J. Neurochem 2006;96:1760–1767. [PubMed: 16539691]

Yaka R, Phamluong K, Ron D. Scaffolding of Fyn kinase to the NMDA receptor determines brain region sensitivity to ethanol. J. Neurosci 2003;23:3623–3632. [PubMed: 12736333]

Yang XH, Criswell HE, Simson P, Moy S, Breese GR. Evidence for a selective effect of ethanol on N-methyl-D-aspartate responses: Ethanol affects a subtype of the ifenprodil-sensitive N-methyl-D-aspartate receptors. J. Pharmacol. Exp. Ther 1996;278:114–124. [PubMed: 8764342]

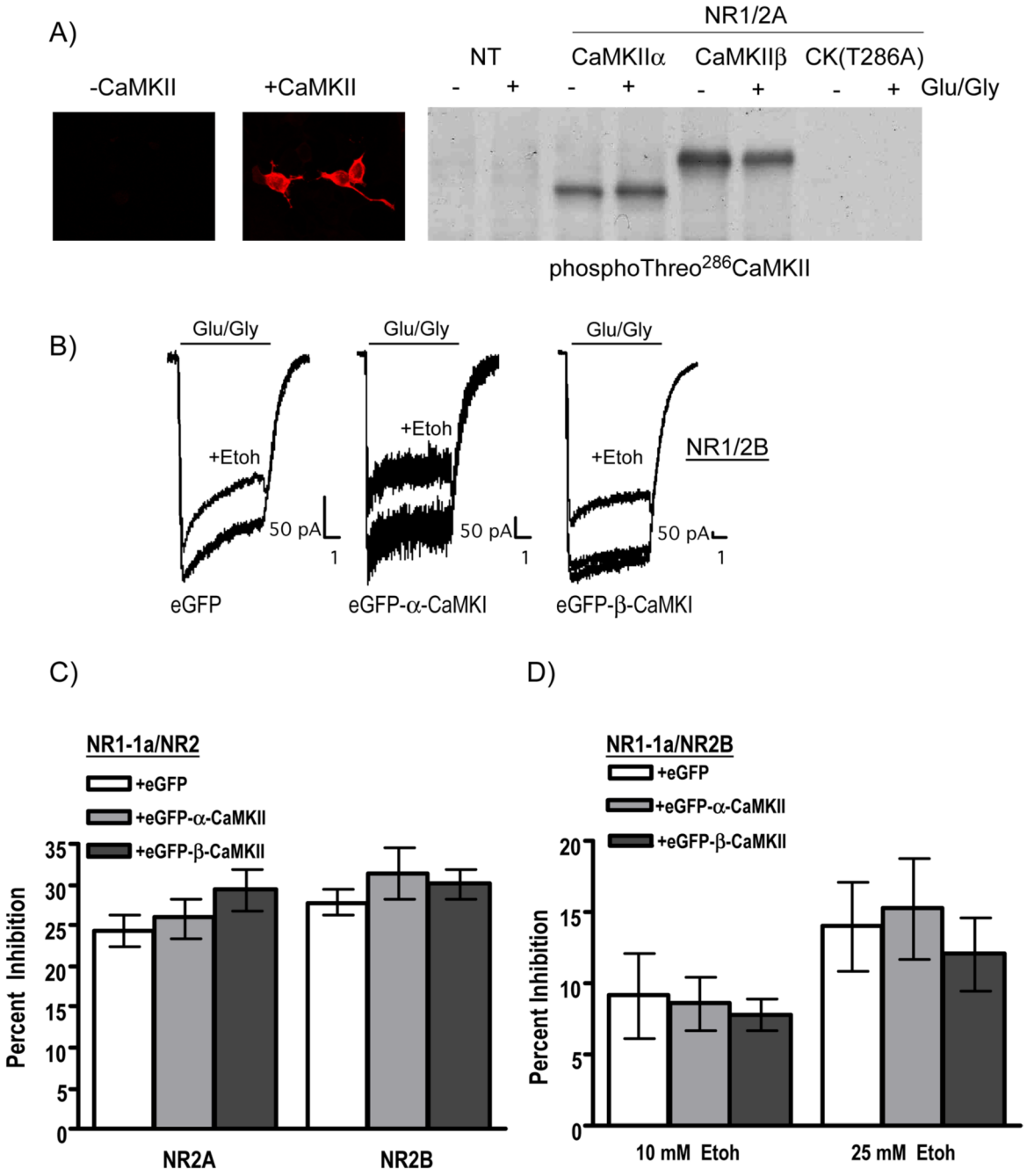


**Figure 1.**

CaMKII does not alter the acute ethanol inhibition of recombinant NMDA receptors. HEK 293 cells were transfected with NMDA subunits and either CaMKII-α or CaMKII-β and tested for sensitivity to ethanol using patch-clamp electrophysiology. Panels: A) Inset shows lack of CaMKII-α immunoreactivity in un-transfected cells (left panel) and robust staining in CaMKII-α transfected cells (right panel). Western blot shows expression of CaMKII-α or CaMKII-β before and after treatment of HEK 293 cells with agonist (10 μM glutamate and glycine, 5 min) using antibody directed against phosphorylated threonine residue 286. Note lack of signal for non-transfected (NT) cells or those expressing a mutant CaMKII-α lacking the threonine phosphorylation site (CKT286A). B) Traces show representative currents from cells expressing

NR1/2B subunits and either CaMKII-α or CaMKII-β in the absence and presence of 100 mM ethanol. C) Summary figure showing effects of 100 mM ethanol on currents from cells expressing NR1/2A or NR1/2B receptors in the absence and presence of CaMKII-α and CaMKII-β. Values represent percent inhibition by ethanol (mean±SEM; N=9–48 individual cells per group). D) Summary figure showing effects of 10 and 25 mM ethanol on currents from cells expressing NR1/2B receptors in the absence and presence of CaMKII-α and CaMKII-β. Values represent percent inhibition by ethanol (mean±SEM; N=6–7 individual cells per group).

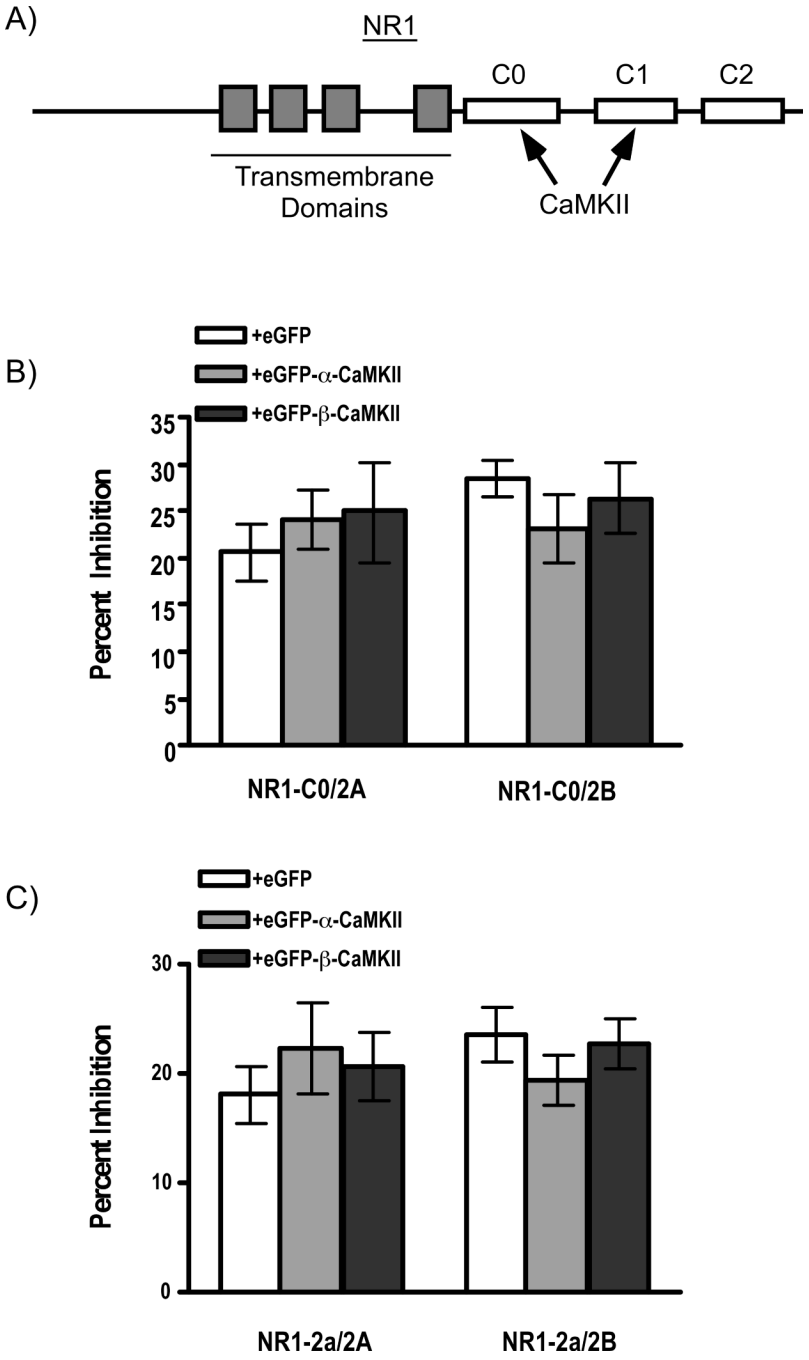


**Figure 2.**

CaMKII does not alter the acute ethanol inhibition of recombinant NMDA receptors lacking C-terminal CaMKII binding domains. HEK 293 cells were transfected with NR2B subunits, wild-type NR1 or C-terminal deletion mutants (NR1-C0, NR1-2a) subunits and either CaMKII-α or CaMKII-β and tested for sensitivity to ethanol using patch-clamp electrophysiology. Panels: A) Cartoon shows putative protein structure of the NR1 subunit with four transmembrane domains (TM; gray boxes) and C-terminal domains, B) Summary figure shows effects of 100 mM ethanol on currents from cells expressing NR1-C0/2B receptors in the absence and presence of CaMKII-α and CaMKII-β. Values represent percent inhibition by ethanol (mean±SEM; N=3–13 individual cells per group), C) Summary figure showing effects

of 100 mM ethanol on currents from cells expressing NR1-2a/2B receptors in the absence and presence of CaMKII-α and CaMKII-β. Values represent percent inhibition by ethanol (mean ±SEM; N=3–27 individual cells per group).

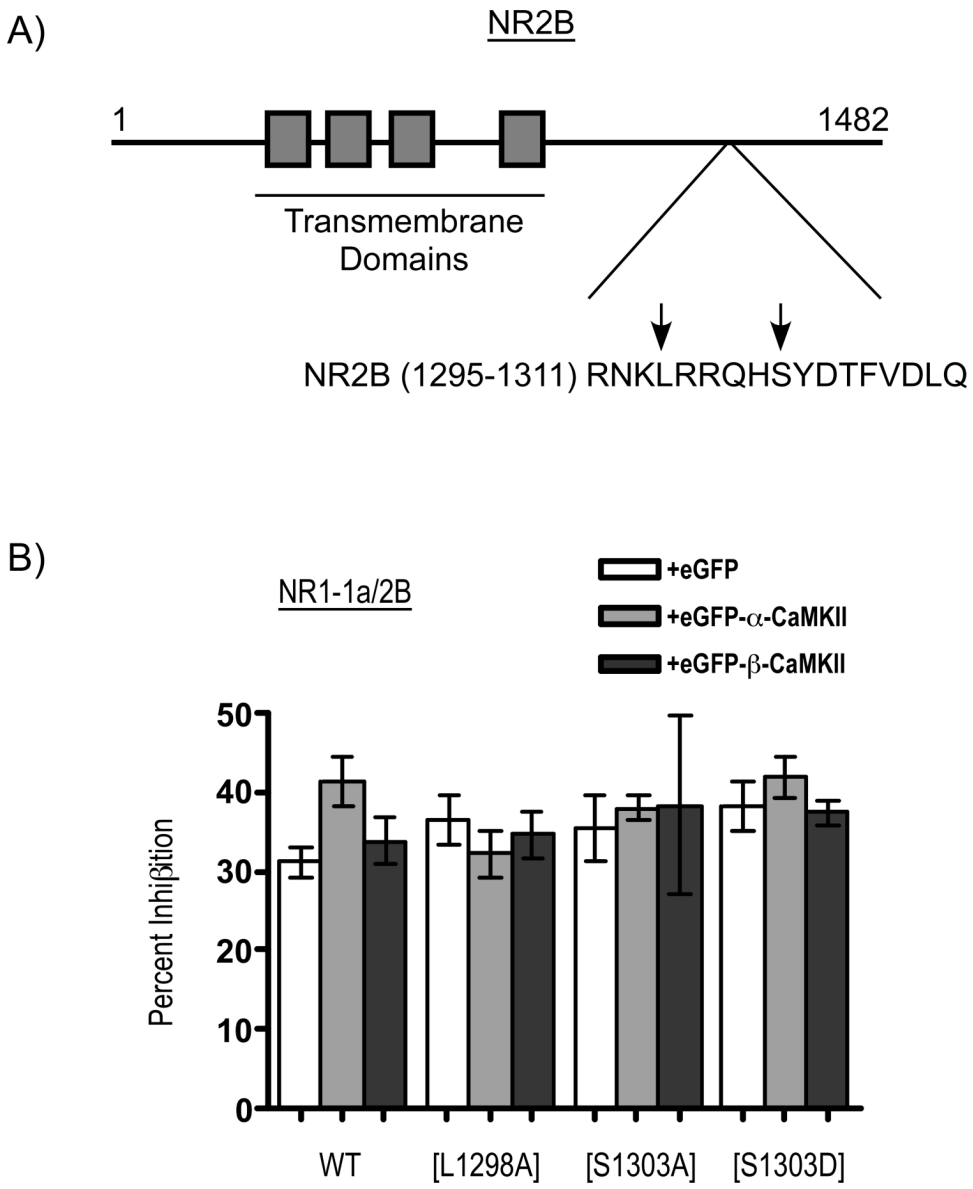


**Figure 3.**

Ethanol inhibition of CaMKII phospho-site NMDA receptor mutants. HEK 293 cells were transfected with NR1, wild-type or mutant NR2B subunits, and either CaMKII-α or CaMKII-β and tested for sensitivity to ethanol using patch-clamp electrophysiology. Panels: A) Cartoon shows putative protein structure of the NR2 subunit with four transmembrane domains (TM; gray boxes) and C-terminal sequence of the CaMKII binding domain. Arrows indicate residues chosen for mutagenesis, B) Summary figure shows effects of 100 mM ethanol on currents from cells expressing NR1-1a and wild-type and mutant NR2B subunits in the absence and presence of CaMKII-α and CaMKII-β. Values represent percent inhibition by ethanol (mean±SEM; N=3–13 individual cells per group), C) Summary figure showing effects of 100 ethanol on currents from cells expressing NR1-2a/2B receptors in the absence and presence of CaMKII-α and CaMKII-β. Values represent percent inhibition by ethanol (mean±SEM; N=3–18 individual cells per group).

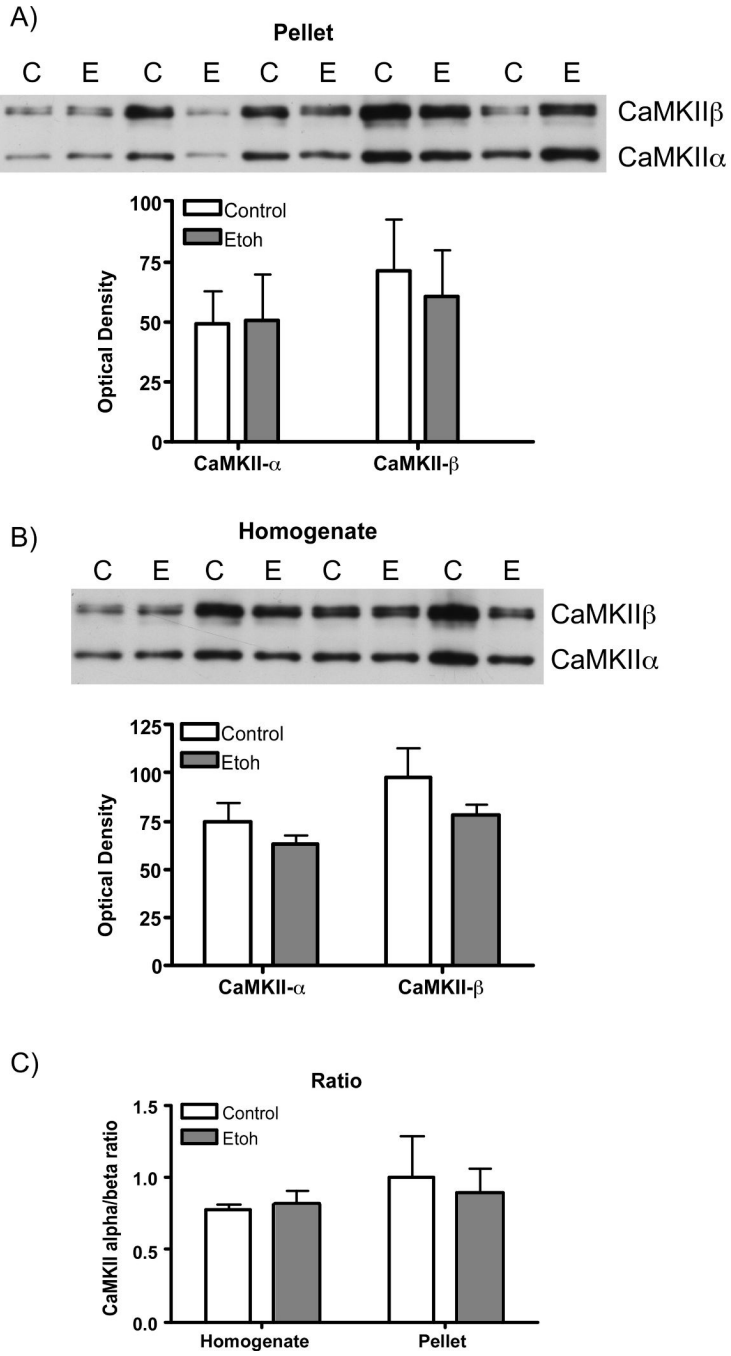


**Figure 4.**

Chronic treatment of cortical neurons with ethanol does not alter the expression of CaMKII. Primary cultures of cortical neurons were treated for 4 days with 100 mM ethanol beginning at day 10 in vitro. Cells were collected and analyzed for CaMKII expression using western blotting. Panels: A) Immunoblot of CaMKII-α and CaMKII-β from the pellet fraction collected from control (C) and ethanol treated (E) cultures. Summary figure shows optical density of CaMKII-α and CaMKII-β from control and ethanol treated cultures. Values are mean (±SEM) from 5 separate cultures, B) Immunoblot of CaMKII-α and CaMKII-β from the homogenate fraction of control (C) and ethanol treated (E) cultures. Summary figure shows optical density of CaMKII-α and CaMKII-β from control and ethanol treated cultures. Values are mean

(±SEM) from 4 separate cultures, C) Summary figure showing average ratio of CaMKII-α to CaMKII-β from control and ethanol treated cultures. Values are mean (±SEM) from 4 separate cultures.