Molecular Pharmacology Fast Forward. Published on June 16, 2011 as doi:10.1124/mol.110.068643
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MOL #68643

Functional Adaptation of the NMDA receptor to acute ethanol inhibition is modulated by striatal-enriched protein tyrosine phosphatase and p38 mitogen-activated protein kinase

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Running Title:

Acute functional ethanol tolerance of NMDA receptors

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Text pages: 34

Tables: 0

Figures: 6

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Number of words:

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List of Abbreviations:

aCSF, artificial cerebral spinal fluid; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; BMI, bicuculline methyl iodide; CGP-52432, 3[[[(3,4-dichlorophenyl)methyl]amino]propyl](diethoxymethyl) phosphinic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione disodium salt; D-APV, D-amino-phosphonovaleric acid; EGTA, ethylene glycol tetraacetic acid; PP2, 3-(4-chlorophenyl)1-(1, 1-dimethylethyl)-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine; EPSCs, excitatory postsynaptic currents; $GABA_A$ receptor, γ-amino butyric acid type A receptor; $GABA_B$ receptor, γ-amino butyric acid

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type B receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IPSCs, inhibitory postsynaptic currents; NMDA, N-methyl-D-aspartate

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Abstract

The hippocampal N-methyl-D-aspartate receptor (NMDAR) activity plays important roles in cognition and is a major substrate for ethanol-induced memory dysfunction. This receptor is a glutamate-gated ion channel, which composes of NR1 and NR2 subunits in various brain areas. While homomeric NR1 subunits form an active ion channel that conducts Na^+ and Ca^{2+} currents, the incorporation of NR2 subunits allows this channel to be modulated by the Src family of kinases, phosphatases, and by simple molecules such as ethanol. Recently, we found that acute ethanol inhibits the NMDAR activity via STriatal Enriched protein tyrosine Phosphatase (STEP)-regulated mechanisms. The genetic deletion of the active form of STEP, STEP61, leads to marked attenuation of acute ethanol inhibition of NMDAR currents. Also, STEP61 negatively regulates Fyn and p38 mitogen-activated protein kinase (p38 MAPK), and these proteins are members of the NMDAR super molecular complex. Here, we demonstrate, using whole-cell electrophysiological recording, Western blot analysis and pharmacological manipulations, that neurons exposed to a 3-hour, 45 mM ethanol treatment develop an adaptive attenuation of acute ethanol inhibition on NMDAR currents in brain slices. Our results suggest that this adaptation of NMDAR responses is associated with 1) a partial inactivation of STEP61, 2) an activation of p38 MAPK, and 3) a requirement for NR2B activity. Together, these data indicate that altered STEP61 and p38 MAPK signaling contribute to the modulation of ethanol inhibition of NMDARs in brain neurons.

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Introduction

Acute ethanol application inhibits N-methyl-D-aspartate receptor (NMDAR) activity (Lovinger et al., 1989), and subsequent studies have shown that the NMDAR in hippocampal brain slices develops resistance to the acute effects of ethanol during a 5 - 15 min ethanol (100 mM) exposure (Grover et al., 1994; Miyakawa et al., 1997). However, precise mechanisms that regulate the development of this resistance to the inhibitory effects of acute ethanol on the NMDAR are not fully understood. Since NMDARs can undergo both rapid and delayed activity-dependent changes in adult hippocampal neurons (Heynen et al., 2000), we hypothesized that time- and ethanol dose-dependent adaptive changes of the NMDAR also may occur during ethanol exposure under *ex vivo* conditions, and such changes may underlie the mechanisms necessary for the functional adaptation of these receptors to ethanol inhibition. Previous studies also showed that adaptive changes can occur as a result of increased NMDAR expression (Snell et al., 1996; Roberto et al., 2004; Lack et al., 2007) and/or by selective phosphorylation/dephosphorylation of NMDARs following chronic ethanol treatment (Clapp et al., 2010; Wu et al., 2010). Here, we demonstrate that the development of adaptation of the NMDAR to ethanol inhibition involves the STriatal Enriched protein tyrosine Phosphatase (STEP), the p38 mitogen-activated protein kinase (p38 MAPK), and the activity of the NMDA NR2B receptor subunit.

Functional activity of the NMDAR is increased by protein tyrosine kinases (Salter and Kalia, 2004), but its activity is also regulated by protein tyrosine phosphatases (Wang et al., 1996; Pelkey et al., 2002; Snyder et al., 2005; Paul et al., 2007). STEP61 is a brain-specific protein tyrosine phosphatase and is found in both membrane-bound

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and cytosolic fractions (Boulanger et al., 1995; Goebel-Goody et al., 2009). Moreover, it co-immunoprecipitates with NMDARs (Pelkey et al., 2002; Braithwaite et al., 2006), suggesting a strong physical association between these two molecules as a signaling unit (Xu et al., 2009). Inhibition of STEP61, the only active isoform of STEP expressed in the hippocampus, has been shown to enhance NMDAR function (Pelkey et al., 2002) and to attenuate ethanol inhibition of the NMDA receptor (Hicklin et al., 2011) in rodent hippocampus.

Previously, acute ethanol application was shown to decrease the level of the tyrosine (Y) 1472 phosphorylation site of the NR2B subunit, without altering the levels of this protein (Alvestad et al., 2003; Wu et al., 2010). Y-1472 is a site in the C-terminal tail of the NR2B where STEP61 has been shown to act (Paul et al., 2003; Braithwaite et al., 2006), suggesting that acute ethanol treatment activates STEP61, which is involved in dephosphorylating the Y-1472 site. In addition, we recently showed that ethanol inhibition of NMDA excitatory postsynaptic currents (NMDA EPSCs) was attenuated by microdialysis of STEP(C/S), a dominant negative STEP mutant, into CA1 pyramidal neurons, and also by animals having a STEP gene deletion (Hicklin et al., 2011). Here, we report that changes in STEP-regulated mechanisms may alter responses of the NMDAR to the acute inhibitory effects of ethanol.

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Materials and Methods

Materials. All drugs used to make up the artificial cerebrospinal fluid (aCSF) and internal recording solutions were purchased under the Fluka brand (Sigma Chemical Company, St. Louis, MO). Glutamatergic receptor antagonists, D-(-)-2-amino-5-phosphonopentanoic acid (D-APV) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and the $GABA_A$ receptor antagonist, bicuculline methiodide (BMI) also were purchased from Sigma. The $GABA_B$ antagonist, 3-[[[(3,4-Dichlorophenyl methyl] amino] propyl] diethoxymethyl) phosphinic acid (CGP-52432), the NMDA NR2B antagonists, ifenprodil, and the Fyn kinase inhibitor, PP2, were purchased from Tocris (Tocris Cookson Inc, Ellisville, MO). The mitogen-activated protein kinase (MAPK) inhibitor, SB2021980, and the protein synthesis inhibitors, anisomycin and cycloheximide, were also purchased from Tocris. The protein tyrosine phosphatase inhibitor, bpV(phen) was purchased from Calbiochem (EMD Biochemicals). An 8.0 M ethanol solution (in deionized water) was prepared immediately before each experiment from a 95% stock solution (Aaper, Shelbyville, KY) kept in a glass storage bottle at 4°C. Selective antibodies to NR2A and NR2B were produced in our laboratory (Snell et al., 1996) and the antibody to the NR1 subunit was purchased from BD Pharmingen (Franklin Lakes, NJ). Anti-phospho-p38 antibody was purchased from Affinity BioReagents (Boulder, CO), and the anti-STEP antibody was a gift from Dr. Lombroso (Yale University, New Haven, CT).

Animals. Young adult (6-9 week old; 160 to 220 g body weight) male Sprague-Dawley rats were purchased from Harlan Laboratories (Indianapolis, IN) and were housed three per cage with a 7 AM to 7 PM light cycle and with free access to food and water. The

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animals were maintained in an NIH accredited facility at University of Colorado Denver, and the animal procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All the experimental procedures were approved by the Institute for Animal Care and Use Committee at the University of Colorado Denver.

Brain slice preparation, storage and whole cell recordings. Rats were quickly decapitated and the brains were rapidly removed and immersed in ice-cold, sucrose-containing slicing buffer for 40-60 s to cool the interior of the brain. This sucrose-containing slicing buffer consisted of (in mM): 87 NaCl, 2.5, KCl, 7 $MgCl_2$, 0.5 $CaCl_2$, 1.25 NaH_2PO_4, 25 D-glucose, 35 sucrose, and 25 $NaHCO_3$. After removing one or both hippocampi from the brain, transverse slices 400 µm-thick were made using a tissue chopper/slicer (TC-2 Tissue Sectioner, Sorvall) and the slices were transferred to individual compartments in a storage unit (Proctor et al., 2006), where they were kept at 33°C in an aCSF solution that was gassed with 95% O_2 and 5% CO_2. This aCSF contains the following chemicals (in mM): 126 NaCl, 3.0 KCl, 1.5 $MgCl_2$, 2.4 $CaCl_2$, 1.2 NaH_2PO_4, 11 D-glucose, and 25.9 $NaHCO_3$. The low Mg^{2+} aCSF contained 0.2 mM $MgCl_2$ instead of 1.5 mM.

Ex vivo ethanol exposure. Freshly prepared hippocampal slices were stored in aCSF for 1.5 h for recovery from the preparation procedure in a storage chamber made of individualized compartments with a bottom netting that was suspended in a 500 ml glass beaker. Following the recovery, some of the brain slices were transferred to a drug-treatment unit which is a miniature version of the storage chamber (100 ml glass beaker) that contained control aCSF or either ethanol (45 mM), ifenprodil (5 µM),

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anisomycin (20 µM), cycloheximide (60 µM), or SB202190 (0.5 µM) and was aerated with 95% O_2 and 5% CO_2. After an additional 3 hours of exposure to control aCSF or drug treatment, whole-cell recordings were made from individual hippocampal CA1 pyramidal neurons while perfusing the slices with gassed aCSF. The baseline period, an acute ethanol challenge (80 mM or 120 mM ethanol) application, and washout period were monitored for evoked NMDA EPSC responses. In a separate group of slices, the brain slices were perfused with low Mg^{2+} aCSF during the recording of NMDA EPSCs. In these *ex vivo* ethanol exposure experiments, 100 µl of the solution in the storage unit was removed and stored at -20°C for analysis of ethanol concentrations using the enzymatic method as described by Smolen and Smolen (1989). The ethanol concentration in the storage chamber was maintained at approximately 200 mg% (45 mM) during the 3-hour exposure. Slices were then individually transferred from either the control or the drug-treatment storage unit to a recording chamber where they were perfused with aCSF or low Mg^{2+} aCSF at 33°C for 20 min to allow for washout of the residual ethanol and/or excess Mg^{2+} before whole cell recording began. After a baseline recording period (10-15 min), acute ethanol (80 or 120 mM) was applied for 10 min, followed by a 15-30 min washout period.

Electrophysiological recordings. Whole-cell recordings were made at 33°C while the slices were constantly superfused with oxygenated aCSF at 2 ml/min bulk flow rate. A Flaming/Brown electrode puller (Sutter Instruments, Novato, CA) was used to fabricate 6-9 MΩ whole-cell micro-electrodes when filled with a K^+-gluconate internal solution containing (in mM): 130 K^+-gluconate, 1 EGTA, 2 $MgCl_2$, 0.5 $CaCl_2$, 2.54 disodium ATP, 0.3 NaGTP, and 10 HEPES adjusted to pH 7.3 with KOH, and

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280 - 290 mOs. CA1 pyramidal neurons were recorded within the stratum pyramidale layer. Evoked NMDA synaptic responses (EPSCs) were obtained by stimulation in the stratum pyramidale layer with a twisted bipolar stimulating electrode made from 0.0010-in Formvar-coated nichrome wire. This stimulating electrode was positioned to activate a few proximal presynaptic fibers that innervate on or near the soma of the recorded cell using brief electrical current pulses (200 µs). This stimulation paradigm routinely evokes a current responses of 1-3 nA and a pharmacologically isolated NMDAR current response of approximately 100 to 200 pA (average of 121.5 ± 6.3 pA, n=101). Drugs were applied at 100-fold concentrations by bath superfusion at 1/100 bulk flow into the aCSF/low Mg^{2+} aCSF flow line via calibrated syringe-pumps (Razel Scientific Instruments Inc, Stamford, CT).

Measurement of NMDA EPSCs. CA1 pyramidal neurons were voltage-clamped at -60 mV (corrected for the liquid-junction potential) from the normal resting membrane potential (-65 to -74 mV; average: -67.6 ± 0.63 mV) to enhance the size of the current responses. The NMDA receptor-mediated EPSCs (NMDAR currents) were pharmacologically isolated using 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 µM), bicuculline methiodide (BMI, 30 µM), and CGP-52432 (1 µM) to block α-amino-3-hydroxy-5-methyl-4-isoxzole propionate (AMPA), γ-aminobutyric acid-A ($GABA_A$), and γ-aminobutyric acid-B ($GABA_B$) receptor-mediated PSCs, respectively. These isolated NMDA EPSCs were completely blocked by the NMDAR antagonist, D-amino-phosphonovaleric acid (D-APV, 25 µM).

Western blot determinations. A separate group of hippocampal slices, CA1 mini-slices (Coultrap et al., 2005) were prepared and exposed to aCSF or ethanol

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(45 mM) for 3-hour under conditions identical to those used for the electrophysiological experiments. These slices were harvested and sonicated in buffer containing 10 mM Tris, 1 mM EDTA, and 1% SDS. Following SDS-polyacrylamide gel electrophoresis (PAGE), Western blotting was performed as described by Coultrap et al. (2005). A 5-point dilution series of the CA1 hippocampal homogenate was included on each gel to increase the probability that samples were within the linear range of detection for each antibody. For the measurement of STEP33 and pp38 MAPK levels, the immune-reactivity was determined without a full 5-point dilution standard because of the low levels of STEP33 and pp38MAPK in control samples. Imaging of the blots was performed using the SuperSignal chemiluminescent substrate (Pierce, Rockford, IL), and an Alpha Innotech imaging system (Alpha Innotech, San Leandro, CA). The digital images were quantified using AlphaEase software (Alpha Innotech) and only values falling within the standard curve generated from the dilution series on each gel were incorporated into the final analysis.

Statistical analysis. Drug effects were quantified and presented as the percent change of the NMDA EPSC amplitude following drug application, relative to the average of baseline values. Statistical analyses were carried out with the use of SigmaStat (Systat software Inc. Point Richmond, CA) for Student's t-test and ANOVA analyses. A Mann-Whitney Rank Sum test was used for nonparametric analysis of the STEP33 and pp38 MAPK data. The minimal significance level was set at $p < 0.05$.

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Results

NMDARs develop resistance to the acute effects of ethanol in brain slices. In control rodent brain slices, application of 80 mM ethanol typically inhibits synaptic NMDA EPSCs by 25-35% in CA1 hippocampal pyramidal neurons, and this inhibition by acute ethanol was ethanol concentration-dependent with an estimated EC_{50} of 50 mM in rodent brains (Proctor et al., 2006). In routine experiments, we applied ethanol for a period of 10 min which was followed by 10-30 min of washout, during which NMDA EPSCs usually returned to baseline levels (Figure 1A). However, in brain slices which were exposed to 3 hours of 45 mM ethanol, we found that the acute ethanol application resulted in very little inhibition, but showed a time-dependent delayed facilitation of the NMDA EPSCs. During the first 2-5 min of acute ethanol application, the inhibition of NMDA currents showed attenuation (resistance). After 5-10 min, the averaged NMDAR current responses show 13-17% enhancement. We found that significant attenuation of ethanol inhibition occurred after 1 hour of 45 mM ethanol exposure, and a maximum attenuation was reached by 3 hours of the 45 mM ethanol treatment (Figure 1B). To determine whether this attenuated ethanol effect was reversible after removal of ethanol, we superfused the brain slices with normal aCSF for 20 to 80 min following their 3 hours of 45 mM ethanol treatment, and the cells did not re-gain ethanol inhibition of the NMDA responses during that time (Figure 1C). To demonstrate that the reduced acute ethanol effect was not due to a substantial desensitization of NMDARs during the 45 mM ethanol exposure, we applied a higher acute ethanol concentration (120 mM) challenge application. This increased concentration of ethanol still produced a significant inhibition of the NMDA EPSCs (Figure 1D and 1E). However, the ethanol

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inhibition was significantly reduced. Thus, the adaptive resistance of the NMDAR to the acute effects of ethanol (NMDAR resistance) was not due to complete desensitization of NMDARs to the action of ethanol in these neurons. To further determine that these ethanol-insensitive currents were indeed NMDA currents, we applied 20 μM D-APV to these neurons and found that D-APV completely blocked the current response.

Reduced ethanol inhibition of NMDAR currents correlates with NR2B inhibition. The NMDA NR2B subunit is known to be expressed in adult hippocampal neurons (Goebel-Goody et al., 2009). To determine the involvement of NR2B-containing NMDA receptors in these evoked synaptic NMDA EPSCs, we tested ifenprodil, a potent NR2B selective antagonist, to inhibit NMDAR currents. The extent of inhibition by ifenprodil was used to estimate the contribution of active NR2B subunits in the evoked synaptic NMDA EPSC responses. In control brain slices, ifenprodil (5 μM or 50 μM) did not significantly inhibit NMDA EPSCs, but in 45 mM ethanol-treated brain slices, both 5 μM and 50 μM ifenprodil inhibited the NMDA EPSC amplitude by about 25% (Figure 2). Both the acute ethanol inhibition of the NMDA EPSCs, as well as the size of NMDA EPSC amplitude, varied among CA1 neurons. To determine whether the potency of ethanol inhibition of NMDA EPSCs is influenced by the size of the NMDA EPSC, we tested this possibility by examining the correlation between NMDA EPSC amplitude and the percent inhibition by acute 80 mM ethanol application from 101 pyramidal neuronal recordings. The result showed no correlation between these parameters (Correlation Coefficient: 0.0681, $p>0.5$, Pearson Product Moment Correlation) (Figure 2). In addition, these data showed that the overall baseline NMDA EPSC amplitude from

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control neurons was 121.5 ± 6.33 pA (n=101), and that the neurons incubated in 45 mM ethanol was 119.9 ± 14.33 pA (n=18; t=0.092, p>0.9). We also determined whether the NMDA EPSC amplitude was correlated with the resting membrane potential of each recorded neuron; the results indicated that there was no correlation between these two parameters (Correlation Coefficient: 0.165, p>0.09, Pearson Product Moment Correlation). Since AMPA receptors are blocked for these determinations, it is possible that Mg^{2+} blockade of the NMDA receptors could be altered due to prolonged ethanol exposure. Thus, we also determined whether 45 mM ethanol exposure of brain slices could enhance NMDA EPSCs under reduced extracellular Mg^{2+} concentration. When we measured ethanol inhibition of NMDAR currents in low (0.2 mM) and normal (1.5 mM) Mg^{2+}, acute ethanol (80 mM) inhibited NMDAR currents by 34.8 ± 2.9% in normal aCSF and 28.8 ± 2.6% in low Mg^{2+} aCSF (t=1.481, p>0.16). In 45 mM ethanol-treated brain slices, acute 80 mM ethanol did not significantly inhibit NMDAR currents in low Mg^{2+} aCSF (+5.6 ± 3.5%) and in normal Mg^{2+} aCSF (-1.4 ± 1.9%) [t=1.830, p>0.08] (Figure 2E).

NMDAR resistance to the acute inhibitory effects of ethanol is associated with STEP inactivation. To determine if the NMDA receptor subunit expression was increased during the 3-hour *ex vivo* ethanol exposure, we used Western blot methods to measure protein levels of NR1, NR2A, and NR2B in CA1 mini slices (Coultrap et al., 2005) from 3-hour control or 45 mM ethanol-exposed hippocampal slices. These data showed that the levels of these subunit proteins in the CA1 region of the hippocampus did not differ significantly between control and 45 mM ethanol-exposed slices (Figure 3A-3C).

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Previously we found that acute ethanol inhibition of NMDAR activity was attenuated by inhibition of STEP activity (Alvestad et al., 2003; Hicklin et al., 2011). Also, STEP61 was shown to be degraded to the inactive form of STEP33 (Xu et al., 2009). Therefore, we measured the levels of STEP33 following the 45 mM ethanol treatment. We also analyzed for changes in the levels of phospho-p38 mitogen-activated protein kinase (pp38 MAPK), since this protein kinase has been shown to be regulated by STEP61 (Xu et al., 2009). The data showed that there were significant increases in the levels of STEP33 [$p<0.05$, Mann-Whitney Rank Sum Test] (Figure 3D) and pp38 MAPK [$p<0.05$, Mann-Whitney Rank Sum Test] (Figure 3E) in the CA1 mini slices that were exposed to 45 mM ethanol *ex vivo* for 3 hours compared to slices in control aCSF. These data suggest that there is an inactivation of STEP61 (to STEP33) and an activation of p38 MAPK, which are involved in the adaptive resistance of the NMDAR to the inhibitory effects of acute ethanol.

bpV(phen) attenuates acute ethanol inhibition of NMDA EPSCs. We next examined whether inhibition of protein tyrosine phosphatases, including STEP61, would mimic the adaptive resistance of NMDARs to the acute effects of prolonged ethanol. Brain slices were exposed to 10 µM bpV(phen), a non-selective protein tyrosine phosphatase inhibitor, for 30 min before whole-cell recording of the NMDA EPSCs. While acute 80 mM ethanol application (10 min) produced a 32.3 ± 1.87% inhibition of synaptic NMDA EPSCs from control brain slices, this concentration of ethanol now enhanced synaptic NMDA EPSCs from bpV(phen)-treated brain slices (13.7 ± 7.70%). The effect of ethanol on NMDA EPSCs in bpV(phen)-treated neurons was significantly different

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from that of control neurons [t=5.808, p<0.001, Student's t-test] (Figure 4), so these results also indicate that inhibition of protein tyrosine phosphatase activity is involved in the adaptive resistance of NMDARs during prolonged exposure to ethanol in these neurons. However, in bpV(phen)-treated slices, acute ethanol application enhanced NMDA EPSCs that persisted into the washout period, a finding similar to what we found in STEP KO mice (Hicklin et al., 2011), and that of Yaka (2003) and Wang (2007) during ethanol washout. To assess whether bpV(phen) treatment alone can increase the baseline NMDA EPSC amplitude, we measured the baseline NMDA current amplitude before (121.5 ± 6.33 pA) and after (121.6 ± 14.78 pA) [t=0.008, p>0.9] bpV(phen) exposure. These data indicated that bpV(phen) treatment alone did not significantly modify the basal NMDA current amplitudes.

Prolonged Ifenprodil treatment blocks the development of the NMDAR resistance to ethanol inhibition. We found that although ifenprodil (5 µM) had no effect on NMDA EPSCs from control slices, it inhibited NMDAR currents by approximately 25% in neurons from brain slices exposed to 45 mM ethanol for 3 hours (Figure 2). Therefore, we wanted to test whether NR2B subunit activity is necessary for the development of the adaptive resistance of NMDARs during the 3 hours of ethanol exposure. For this study, brain slices were pre-incubated with or without ifenprodil (5 µM) for 30 min before and continuing throughout the 45 mM ethanol treatment. Incubation of brain slices with ifenprodil (5 µM) alone showed no change in the effect of 80 mM ethanol (an inhibition of NMDA EPSCs by 29.8 ± 4.7%) that was no different from control data (p>0.4; Figure 5). However, treatment with ifenprodil and 45 mM ethanol for 3 hours,

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significantly blocked the development of the adaptive resistance of NMDARs to the inhibitory effects of the acute ethanol challenge (80 mM), resulting in a 27.3 ± 4.2% inhibition, compared to a 17.3 ± 5.2% enhancement of ethanol-exposed slices without ifenprodil [$F(3,28)=28.765$, $p<0.001$; one-way ANOVA] (Figure 5). These data indicate that NR2B subunit function is required for the adaptive resistance of NMDARs during the prolonged ethanol treatment and tested by the acute ethanol challenge.

Activation of p38 MAPK leads to diminished acute ethanol inhibition of NMDA EPSCs.

In 45 mM ethanol- treated brain slices, there was an increased level of phospho-p38 MAPK (Figure 3), suggesting an activation of this kinase. We tested whether a direct activation of p38 MAPK could produce similar adaptive changes of the NMDAR to the inhibitory effects of acute ethanol in brain slices following the 3-hour 45 mM ethanol exposure. Brain slices were exposed to 20 µM anisomycin, a p38 MAPK activator, for 30 min to 3 hours, and then we performed whole-cell recordings to measure the acute effect of an 80 mM ethanol challenge on synaptic NMDA EPSCs. The results showed that anisomycin treatment alone significantly increased the basal NMDA current amplitude (154.4 ± 11.59 pA, $t=2.134$, $p<0.05$, compared to control) and also produced an acute ethanol-induced enhancement of NMDA EPSCs (14.3 ± 4.80% increase, $p<0.001$, Tukey test) (Figure 6A), a result similar to that of the 3-hour, 45 mM ethanol exposure. To test if the activation of p38 MAPK was selectively involved, we added SB202190, a potent p38 MAPK inhibitor, to determine if it blocked the anisomycin-induced acute ethanol enhancement of the NMDAR current responses. Indeed, the SB202190 (0.5 µM) pre-treatment completely blocked the anisomycin-induced

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enhancement of NMDA EPSCs by acute ethanol application (36.4 ± 5.0% inhibition, $p<0.001$, Tukey test) (Figure 6B). In addition to being a protein synthesis inhibitor, anisomycin also acts as a p38 MARK activator (Shifrin and Anderson, 1999). Therefore, we wanted to determine whether non-selective inhibition of protein synthesis may have a significant effect on the development of adaptive resistance of NMDARs to the inhibitory effects of acute ethanol application. To separate the effect of anisomycin on p38 MAPK activation from protein synthesis inhibition, we tested the effects of pretreatment with cycloheximide (60 μM), a protein synthesis inhibitor with only weak stimulation of p38 MAPK (Iordanov et al., 1997), on the development of NMDAR resistance to the acute effects of ethanol. Cycloheximide enhanced the basal NMDA current amplitude (152.1 ± 9.51 pA, $t=2.187$, $p<0.05$ compared to that of control neurons), but it had no direct effect on the 80 mM ethanol inhibition of NMDA EPSCs, as these responses were similar to those from control slices: control, 34.3 ± 4.1% inhibition and cycloheximide-treated, 28.4 ± 1.7% inhibition [$t=1.906$, $p>0.08$] (Figure 6). Therefore, inhibition of protein synthesis cannot account for the anisomycin-induced acute ethanol enhancement of NMDA EPSCs. If the activation of p38 MAPK is involved in adaptive changes of the NMDA receptors, the inhibition of p38 MAPK should block the development of adaptation of NMDA receptors by 45 mM ethanol incubation. Our results show that the NMDA current amplitude was not affected by SB202190 treatment alone (148.1 ± 10.19 pA, $t=1.706$, $p>0.05$ compared to control), but SB202190 blocked the development of NMDA receptor adaptation ($p<0.001$, post hoc Tukey analysis) (Figure 6). To determine the involvement of the Src family of kinases (SFKs) in the adaptive changes of the NMDA receptor responses by the 3- hour 45 mM ethanol

exposure, we pretreated the brain slices with PP2, a Src family kinase (SFK) inhibitor, before 45 mM ethanol exposure. The results show that while PP2 did not alter the acute ethanol inhibition of NMDA EPSCs in control slices, it also did not block the adaptive resistance of NMDA EPSCs to the 3-hour 45 mM ethanol treated brain slices (Figure 6). Although PP2 during the 45 mM ethanol treatment did not block the development of resistance of NMDAR currents to the acute 80 mM ethanol inhibition, it did block the late enhancement of NMDAR currents. These data indicate that SFKs are involved in late ethanol-induced increases in NMDAR currents. p38 MAPK, on the other hand, prevented the development of adaptive changes of the NMDA receptor.

Discussion

This study, using the *ex vivo* brain slice model for a 3-hour 45 mM ethanol exposure, provides new evidence for the molecular mechanisms regulating the functional adaptive changes of the NMDAR activity due to ethanol treatment. Previous studies (Grover et al., 1994; Miyakawa et al., 1997) showed that electrophysiological NMDA responses became partially resistant (tolerant) to the effects of 100 mM ethanol during 5-15 min of ethanol application in hippocampal brain slices. We show here that, in whole-cell recordings from CA1 pyramidal neurons, NMDARs also become resistant to inhibitory effects of acute ethanol (80 mM) following 45 mM ethanol exposure *ex vivo* for 3 hours. With this model system, we demonstrated that NMDA EPSCs become partially resistant to the acute inhibitory effects of ethanol within 1 hr and reached a maximum by 3 hrs during the 45 mM ethanol exposure (Figure 1). This resistance was not caused by an overall NMDAR desensitization or by an upregulation of NMDA receptors in these CA1 hippocampal pyramidal neurons; the adaptive changes that occurred may be a result of subunit phosphorylation changes, as shown by STEP inactivation, p38 MAPK and NR2B subunit activation.

The individual data points for the acute ethanol inhibition of NMDA EPSCs in the pyramidal neurons contain substantial scatter, and thus, we wanted to test whether the percent inhibition correlates with the neuronal properties of each individual cell, including the size of the NMDAR current and/or the resting membrane potential. These results show that the magnitude of the ethanol inhibition was not significantly influenced by the size of the NMDAR current or the resting membrane potential. Therefore, our

Molecular Pharmacology Fast Forward. Published on June 16, 2011 as DOI: 10.1124/mol.110.068643
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data are consistent with an earlier report showing that ethanol inhibition of NMDAR currents was non-competitive (Peoples et al., 1997).

Previous studies (Alvestad et al., 2003; Wu et al., 2010; Hicklin et al., 2011) showed that ethanol inhibition of NMDAR currents required STEP, since in the absence of STEP activity, ethanol not only failed to inhibit NMDAR currents, but it produced a late enhancement of the NMDAR current. This late enhancement was found to be mediated by SFKs, because it can be blocked by PP2, a SFK inhibitor (Yaka et al., 2003; Wang et al., 2007; Hicklin et al., 2011). These observations prompted the hypothesis that ethanol inhibition of NMDAR currents may be mediated both by ethanol-induced inhibitory and facilitatory processes, and the net effect of acute ethanol on these currents depends on the balance of these processes. However, we did not observe that PP2 reversed or enhanced the acute inhibitory effect of acute 80 mM ethanol, suggesting that if there is an ethanol-induced facilitation, it is a minor component under these control conditions. Furthermore, if the adaptive resistance of NMDAR currents to the acute ethanol inhibition is due to a selective increase of ethanol-induced facilitation processes, then PP2, which blocks the ethanol-induced facilitation, should be able to reverse the ethanol-induced late-phase enhancement of the NMDAR currents. Instead, PP2 and 45 mM ethanol treatment of the brain slices resulted in only blocking the facilitation of NMDAR currents by acute ethanol application (Figure 6). Therefore, the adaptive resistance of NMDAR currents to acute ethanol application, after prolonged ethanol treatment, is not likely due to a selective increase in acute ethanol-induced facilitation. In addition, the late ethanol-induced facilitation was reversible during a 15-20 min washout of ethanol, but acute ethanol still failed to inhibit

NMDAR currents following an 80-min washout of ethanol after the 45 mM ethanol treatment (Figure 1). Thus, it is unlikely that adaptive resistance of NMDAR currents is due to increased ethanol-induced facilitation which masks the ethanol-induced inhibition.

We also tested the hypothesis that the adaptive changes of NMDAR current responses to acute ethanol inhibition was due to alteration of the Mg^{2+} blockade following a 3-hour 45 mM ethanol exposure. The data show that acute ethanol inhibition of NMDAR currents did not significantly differ in low Mg^{2+} and control Mg^{2+} recording conditions, consistent with results reported by Peoples et al. (1997). Therefore, altered Mg^{2+} blockade of NMDAR currents is not likely to explain the adaptive changes of NMDAR currents by 45 mM ethanol exposure.

Since ethanol failed to inhibit NMDAR currents when STEP was impaired (Alvestad et al., 2003; Hicklin et al., 2011), additional experiments were conducted to further our understanding of the underlying molecular mechanisms of prolonged ethanol effects. We found that increased levels of STEP33 and phospho-p38 mitogen-activate protein kinase (pp38 MAPK) were correlated with the failure of acute ethanol application to inhibit NMDAR currents (Wu et al., 2010). Here we find that adaptive resistance of NMDAR currents to acute ethanol inhibition correlated with the impairment of STEP-mediated mechanisms, because inhibition of STEP activity with bpV(phen) prevented the adaptation of the NMDAR currents to the inhibitory effects of acute ethanol. STEP has been shown to regulate endocytosis of NMDA receptors by dephosphorylation of the phospho-NR2B Y1472 site (Paul et al., 2007) and acute ethanol enhanced dephosphorylation of Y1472 pNR2B (Wu et al., 2010; Hicklin et al., 2011). We

reasoned that the adaptive resistance of NMDAR currents to the acute ethanol inhibition likely involves NR2B subunit activity. Our results show that although a 3-hour 45 mM ethanol exposure did not alter the total NR2B protein expression, this treatment resulted in a 25% increase in ifenprodil inhibition of the NMDAR currents, suggesting an increased NR2B functional contribution to NMDAR currents by activation of NR2B subunit proteins. It is unlikely that an increase in NR2B function directly altered the acute ethanol sensitivity of these NMDAR currents, because ifenprodil did not alter the ethanol inhibition of recombinant NR2A or NR2B subunit-containing NMDAR currents (Lovinger, 1995). Moreover, pretreatment of brain slices with ifenprodil could block the development of adaptive resistance of the NMDAR currents to ethanol, suggesting that the functional activity of the NR2B subunit is involved in the adaptive resistance, but ifenprodil does not directly alter ethanol sensitivity of the NMDAR currents. In another study, our preliminary data indicate that changes in NR2B-mediated synaptic Ca^{2+} concentration and CaMKII activity may be necessary for the adaptive resistance.

Following the 3-hour 45 mM ethanol exposure, there was a 30-40% increase in the level of phospho-p38 MAPK, indicating an increase in p38 MAPK activity (Xu et al., 2009). If activation of p38 MAPK is involved in the adaptive resistance of NMDAR currents, then the p38 MAPK activator, anisomycin, also should be able to produce resistance of NMDAR currents to the inhibitory effects of ethanol. Our results show that anisomycin treatment did indeed result in the development of resistance of the NMDAR currents to the acute ethanol inhibition, and this effect of anisomycin was effectively blocked by pretreatment of brain slices with the p38 MAPK inhibitor, SB202190. Finally, we also showed that pretreatment of brain slices with SB202190 could block the

development of adaptive resistance of NMDAR currents by the 3-hour 45 mM ethanol treatment. Xu et al. (2009) reported that the increase in the levels of STEP33 and pp38 MAPK are related to the excitotoxcity of these neurons via activation of extrasynaptic NMDARs. Our data show that adaptive resistance of NMDAR currents can be mimicked by STEP inhibition and p38 MAPK activation, suggesting that adaptive resistance of NMDAR currents could be associated with the ethanol-induced excitotoxicity. Future studies will be focused on delineating the mechanisms of ethanol exposure-induced neuronal excitotoxicity.

Studies in humans have shown that individuals with low initial sensitivity (high resistance) to acute ethanol effects on cognition are also at higher risk for becoming alcohol dependent (Schuckit and Smith, 2001). However, Newlin and Renton (2010) showed those individuals that develop greater acute ethanol tolerance (greater adaptive changes to the effects of ethanol) also have a greater risk for alcohol dependence. Our findings that STEP61 and p38 MAPK activities can modulate ethanol inhibitory sensitivity of the NMDAR activity, indicating that these proteins are important modulators for acute and chronic actions of ethanol in the brain. Moreover, this work suggests that NR2B subunit antagonists are likely to be effective in regulating the acquisition of functional tolerance to the acute inhibitory effects of ethanol.

Molecular Pharmacology Fast Forward. Published on June 16, 2011 as DOI: 10.1124/mol.110.068643
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Acknowledgements

We would like to thank Dr. Richard Deitrich for his comments on this manuscript.

Authorship Contribution

Participated in research design – Wu, Proctor

Participated in conducting the research and analyzing data – Wu, Coultrap

Participated in writing the manuscript – Wu, Proctor

Participated in editing the manuscript – Proctor, Wu, Coultrap, Browning

Provided reagents, materials and/or equipment – Proctor, Browning

Molecular Pharmacology Fast Forward. Published on June 16, 2011 as DOI: 10.1124/mol.110.068643
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Molecular Pharmacology Fast Forward. Published on June 16, 2011 as DOI: 10.1124/mol.110.068643
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Footnotes

This work was supported by NIH National Institute on Alcohol Abuse and Alcoholism [Grants AA015086, AA018328] and VA Merit Review grants.

Disclosure/Conflicts of Interest

The author(s) declare that no financial support or compensation has been received from any individual or for profit corporate entity over the past 3 years for research or professional service, and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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Figure Legends

Figure 1. Adaptive change of the NMDAR response to the acute effects of 80 and 120 mM ethanol application in hippocampal brain slices. **A**. Time course of NMDAR current responses during whole-cell recordings from control (Con, n=8) or 3-hour 45 mM ethanol treated (E45, n=7) brain slices. To demonstrate the stability of the recordings, whole-cell patched CA1 neurons were superfused with normal aCSF for these baseline recordings (BSL, n=6), without application of the 80 mM acute ethanol. **B**. Brain slices were exposed to 45 mM ethanol *ex vivo* for 0, 1, or 3 hours. The NMDAR current responses to acute 80 mM ethanol application were recorded. Some resistance of NMDAR currents to 80 mM ethanol inhibition already occurred following 1 hour of the 45 mM ethanol exposure, and maximum resistance was seen following 3 hours of exposure. The number of cells recorded at 0, 1 and 3 hours: 18, 7, and 16, respectively. **C**. Following the 3-hour 45 mM ethanol exposure, brain slices were superfused with normal aCSF for 0, 20, 40, or 80 min. The acute 80 mM ethanol inhibition of NMDAR currents was measured in these slices. Acute ethanol application failed to significantly inhibit NMDAR currents even after 80 min of washout following the 3-hour 45 mM ethanol exposure. The number of cells recorded at the 0, 20, 40 and 80 min time periods: 18, 8, 8, and 6, respectively. **D**. Representative traces of NMDAR current responses in neurons from control or 3-hour 45 mM ethanol-treated brain slices that were recorded during the application of 80 mM (E80) or 120 mM (E120) ethanol. **E**. Composite graph showing the average acute ethanol (80 or 120 mM) inhibition of NMDA EPSC amplitude responses from control or 45 mM ethanol-treated brain slices. The number of cells for control neurons: 80 mM ethanol (n=8); 120 mM (n=6) and 45

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mM ethanol-treated neurons: 80 mM ethanol (n=7); 120 mM ethanol (n=6). One-way ANOVA shows significant differences between groups [$F(3,23)=39.328$, $p<0.001$]. Scale bars indicate 50 pA and 50 msec. Significance of the statistical analyses: *$p<0.01$, ** $p<0.005$, ***$p<0.001$

Figure 2. The adaptive change in functional NMDAR evoked responses to the effects of 45 mM ethanol treatment is accompanied by ifenprodil inhibition of NMDA EPSCs. Representative traces of NMDA EPSC amplitude during baseline (BSL), a 10-min application of ifenprodil (5 µM, Ifen 5; or 50 µM, Ifen 50), and washout of ifenprodil (Wash) from control **(A)** and 45 mM ethanol treated **(B)** brain slices. The dashed lines indicated the relative sizes of the NMDA EPSC amplitude. **C**. The composite data show that ifenprodil only alters the cells in slices exposed to the 45 mM ethanol treatment. **D**. A scatter-plot was utilized to determine if there is a correlation between the NMDAR current inhibition by 80 mM acute ethanol and the NMDA EPSC size (n=101). The line indicates the linear regression of this plot ($r^2=0.00464$, $p>0.49$). The overall NMDA current amplitude was 121.5 ± 6.33 pA and the ethanol caused an inhibition of 25.0 ± 1.17%. **E**. The effect of 80 mM ethanol on NMDA EPSCs recorded under low (0.2 mM Mg^{2+}) or normal (1.5 mM Mg^{2+}) conditions in neurons from control or 45 mM ethanol-treated brain slices. Scale bars indicate 25 pA and 50 msec. ***$p<0.001$

Figure 3. The adaptive change of the NMDAR response to the acute effects of ethanol is associated with increases in the levels of STEP33 and pp38 MAPK. **A**. The expression of NR1 subunit is not different between control (Con) and 45 mM ethanol-

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exposed (E45) CA1 mini slices. **B**. The expression of the NR2A subunit also is not altered by the 45 mM ethanol-exposure (E45) in CA1 mini slices compared to control (Con) slices. **C**. Similarly, the expression of the NR2B subunit is not changed by 45 mM ethanol-exposure (E45). These data are the average results of 8 animals (n=8). **D**. However, the level of STEP33 is increased in the 45 mM ethanol-treated (E45) hippocampal slices (n=6) compared to those of control brain slices (n=6). **E**. Also, the level of phospho-p38 MAPK (pp38) also is significantly increased in 45 mM ethanol-exposed hippocampal slices (n=6) compared to control treated values (n=6). **p<0.01

Figure 4. Inhibition of protein tyrosine phosphatases can block acute ethanol inhibition of the NMDAR currents. Brain slices were treated with 10 μM bpV(phen) for 30 min to 3 hrs and were tested for the effects of an acute 80 mM ethanol challenge. Representative traces show that ethanol (80 mM, EtOH) inhibits NMDA EPSCs in control brain slices **(A)** compared to bpV(phen)-treated brain slices **(B)**. Dotted lines represent baseline and the extent of ethanol inhibition. BSL, baseline; Wash, washout. **C**. Time course of the average responses for synaptic NMDA EPSC amplitudes from control (Con) (n=8) or bpV-treated brain slice preparations (n=8). **D**. Bar graphs show the averaged effects of 80 mM ethanol on NMDA EPSCs from control (Con, -35.4 ± 3.9%) or from bpV(phen)-treated (12.4 ± 7.3%) slices. There is a significant difference between these two groups (t=5.808, p<0.001, Student's t-test). Scale bars indicate 15 pA and 25 msec. ***p<0.001

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Figure 5. Ifenprodil blocks the adaptive change of the NMDAR response to the acute effects of 80 mM ethanol. Brain slices were pre-treated with ifenprodil (5 μM, Ifen) for 30 min and then continued in the presence (Ifen+E45) or the absence (Ifen) of 45 mM ethanol for 3 hours. Representative traces show NMDA EPSC responses during baseline (BSL), 80 mM ethanol application (EtOH), and during washout (Wash) from ifenprodil-treated brain slices only **(A)** or in ifenprodil+45 mM ethanol-treated brain slices **(B)**. Dotted lines show the baseline and the extent of ethanol inhibition. **C**. The time course of the NMDA EPSC responses during the experiment. The duration of the 80 mM ethanol application is shown by the solid line. **D**. Plots of the average response change from a) control, b) 45 mM ethanol (E45, 17.3 ± 5.2%), c) ifenprodil-treated (Ifen, -27.3 ± 4.2%, n=10) and d) ifenprodil+E45-treated (Ifen+E45, -29.8 ± 4.7%, n=7) slices. Scale bars in **A** and **B** indicate 25 pA and 25 msec. ***p<0.001

Figure 6. Activation of p38 MAPK results in the development of resistance to the inhibition of the NMDAR response to acute ethanol application. Brain slices were incubated with anisomycin (Anis; 20 μM), anisomycin (20 μM) + SB202190 (0.5 μM; SB+Anis), cycloheximide (Cyhx; 60 μM), SB202190 alone (SB), or SB202190+E45 (SB+E45). The effects of an 80 mM ethanol challenge on NMDA EPSCs were measured. Representative traces show NMDA EPSC responses during baseline (BSL), 80 mM ethanol challenge (EtOH), or during washout (Wash) from anisomycin-treated brain slices **(A),** from anisomycin+SB-treated brain slices **(B)**, from SB-treated brain slices **(C)**, and from a 45 mM ethanol +SB-treated neuron **(D)**. Dotted lines indicate the baseline and the extent of acute 80 mM ethanol inhibition. **E**. The average values

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show the effects of an 80 mM ethanol challenge on NMDA EPSCs in cells from the control (Con, -34.3 ± 4.1%, n=9), anisomycin-treated (Anis, 14.3 ± 4.8%, n=5), anisomycin+SB-treated (SB+Anis, -36.4 ± 5.0%, n=6) brain slices, and from a sub-group of slices treated with cycloheximide (Cyhx, -23.4 ± 1.7%, n=5). There are significant statistical differences among groups [$F(3,21)=28.087$, $p<0.001$, One-way ANOVA]. Post hoc Holm-Sidak analysis shows that the cycloheximide treatment does not differ significantly from control slices. **F**. Time course of the NMDAR current responses during baseline, 80 mM ethanol application, and washout periods from brain slices treated with 45 mM ethanol (E45), PP2 (2 μM), or PP2 plus 45 mM ethanol (E45+PP2) for 3 hours. **G**. Mean values for the effects of acute ethanol (80 mM) on NMDA currents recorded from control neurons (Con, -34.8±2.9%, n=8), E45-treated neurons (E45, 5.0±3.4%, n=18), SB202190-treated neurons (SB, -30.0 ± 3.2%, n=12), neurons treated with SB and 45 mM ethanol (SB+E45, -27.8 ± 3.4%, n=8), PP2-treated neurons (-28.8 ± 5.1%, n=7), and PP2+E45 treated neurons (-0.7 ± 5.5%, n=6). Scale bar: 15 pA and 25 msec. ***$p<0.001$

Figure 1

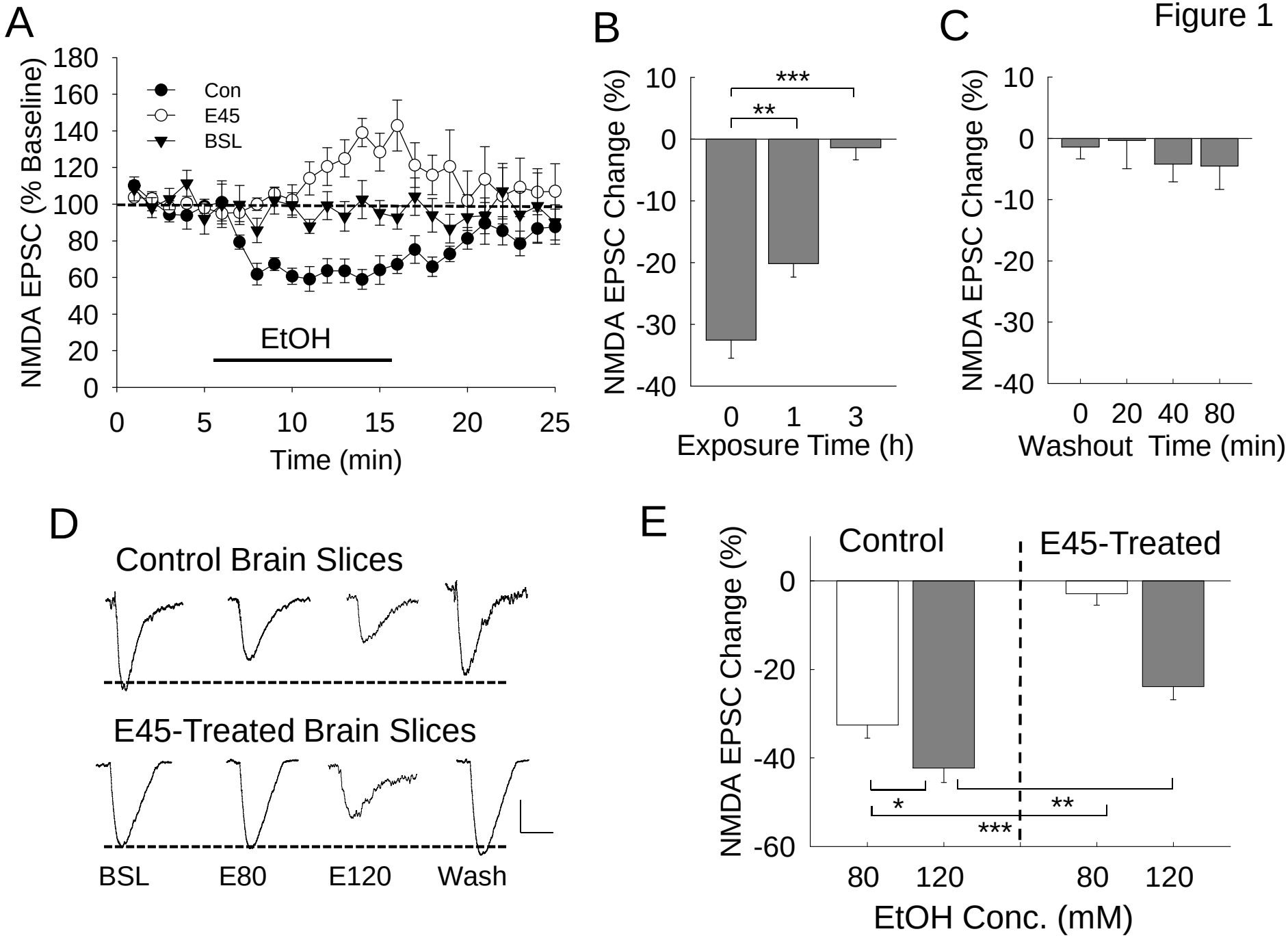

Figure 2

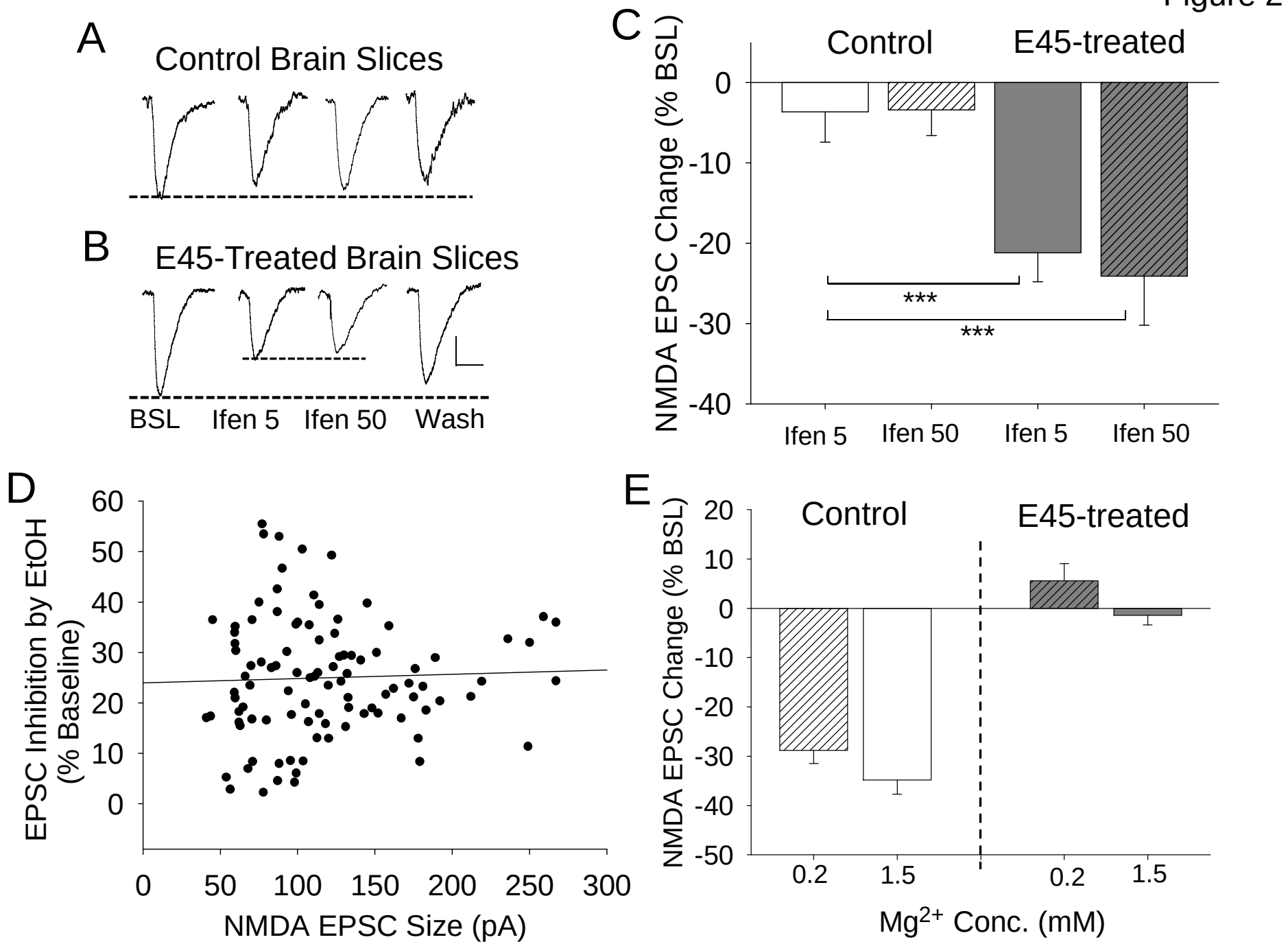

Figure 3

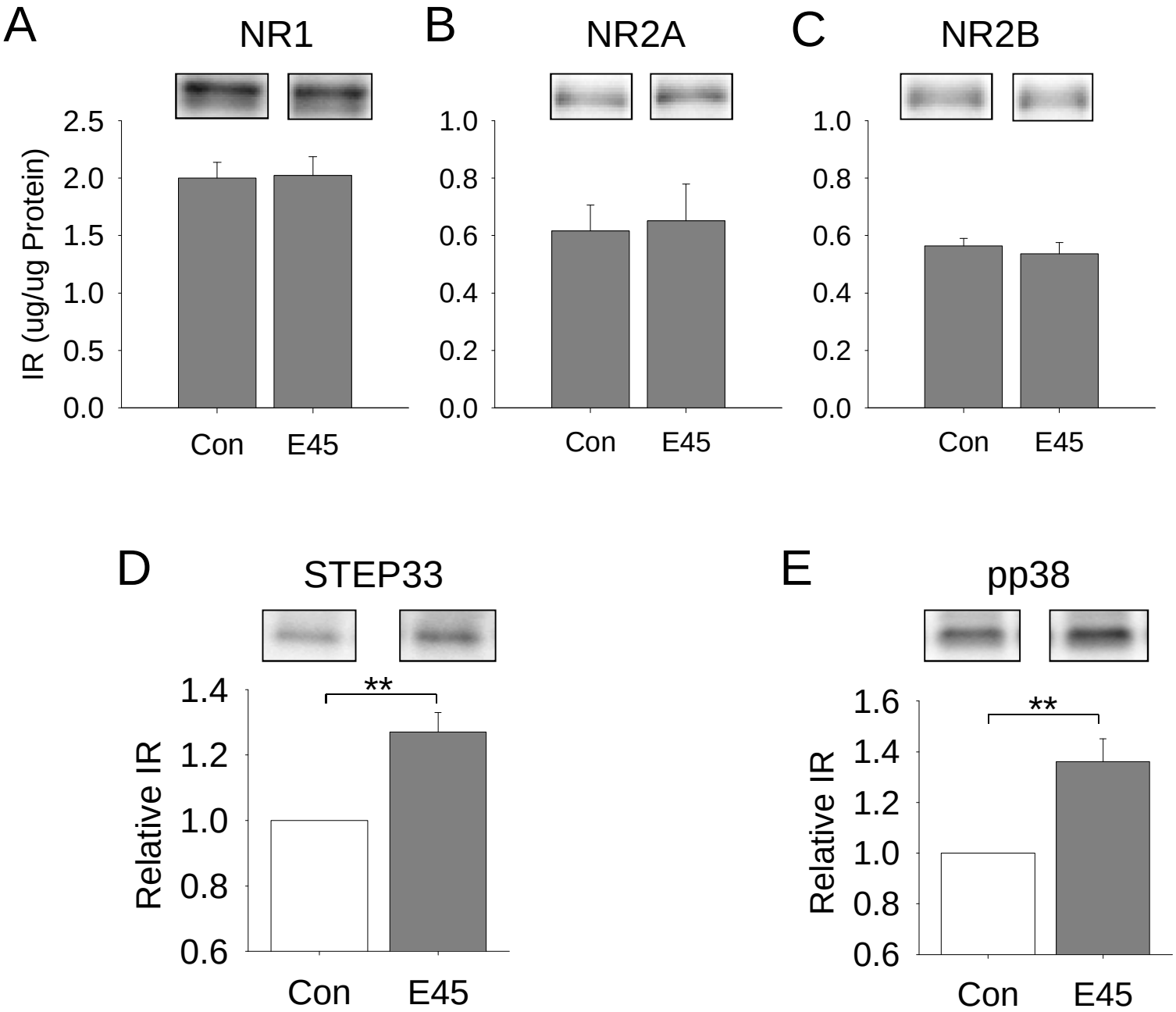

Figure 4

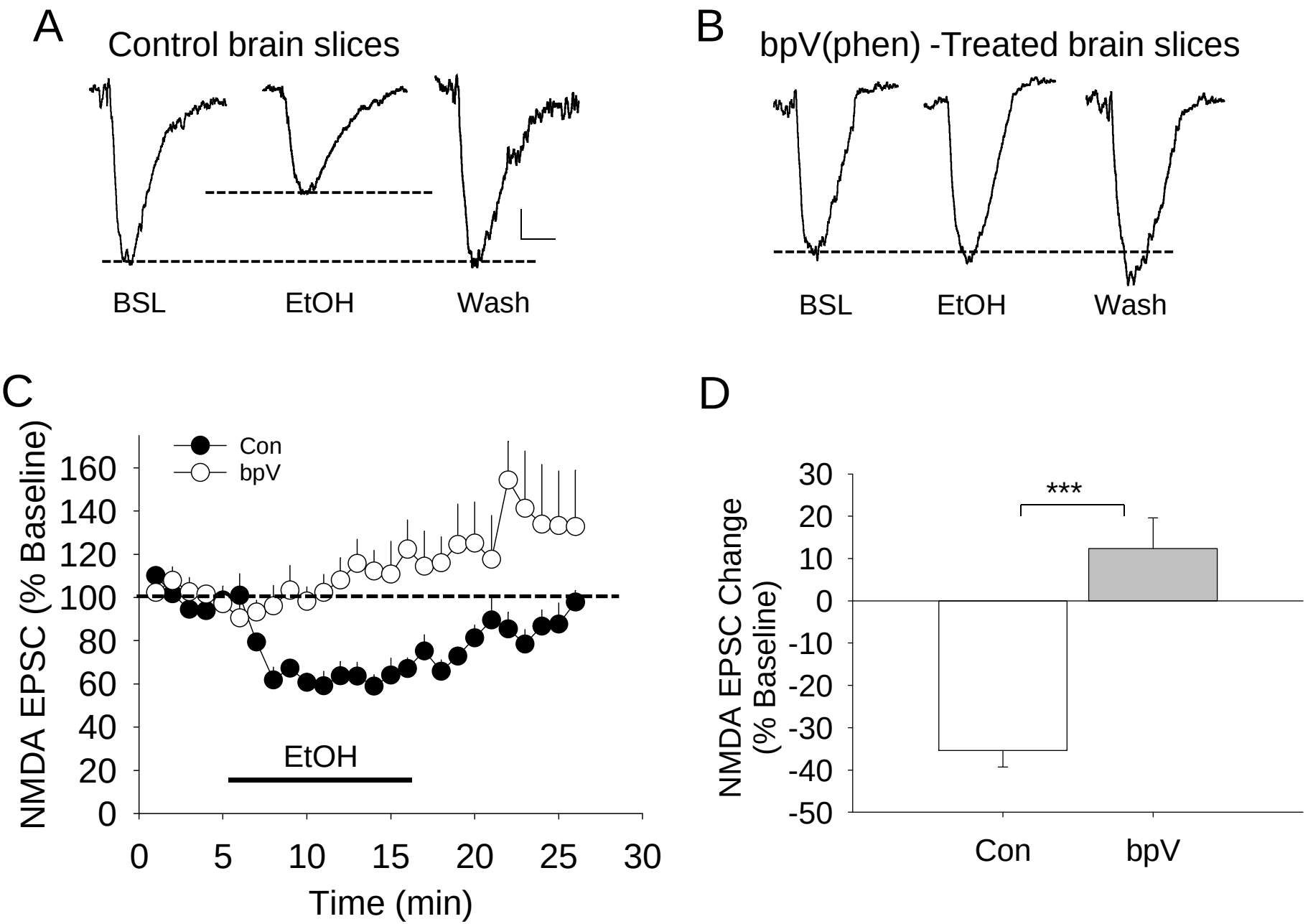

Figure 5

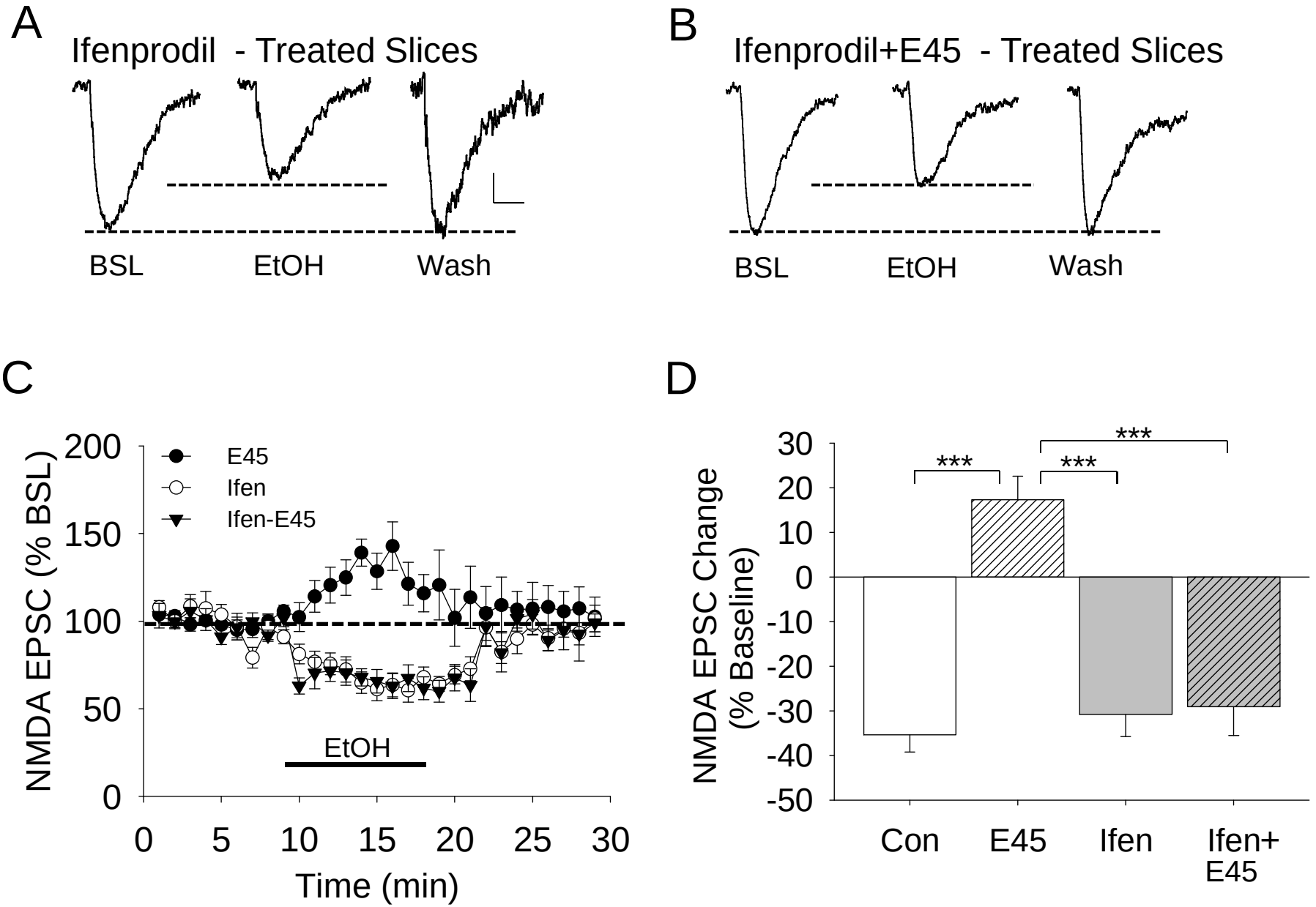

Figure 6