STriatal Enriched Protein Tyrosine Phosphatase mediates Ethanol inhibition of NMDA Receptor Activity

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Abstract

We demonstrated previously that ethanol inhibition of NMDA receptor (NMDAR) function is accompanied by a reduction in tyrosine phosphorylation of Tyr^{1472} on the NR2B subunit, and this action of ethanol is attenuated by a broad spectrum tyrosine phosphatase inhibitor. Here we examined whether this ethanol inhibition of NMDAR activity was due to the actions of STriatal Enriched protein tyrosine Phosphatase (STEP) which has been shown to regulate NMDAR internalization by dephosphorylating Tyr^{1472} on the NR2B subunit. Using whole-cell recordings of pharmacologically isolated NMDAR-mediated excitatory post-synaptic currents (NMDA EPSCs) from hippocampal CA1 pyramidal neurons, we show that intracellular infusion of a substrate-trapping inactive form of STEP (TAT-STEP C/S) significantly blocks ethanol inhibition of NMDA EPSCs. Ethanol does not inhibit NMDA EPSCs or LTP in neurons from STEP knockout mice, but its effect is restored after acute intracellular delivery of wild type TAT-STEP, suggesting that STEP mediates ethanol inhibition of NMDAR function.

The majority of excitatory synaptic transmission in the mammalian CNS is mediated by the neurotransmitter glutamate, which activates postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxalone propionic acid (AMPA), kainate and N-methyl-D-aspartate (NMDA) subtypes of ionotropic glutamate receptors[1]. NMDA receptors (NMDARs) in the hippocampus consist of NR1/NR2A, NR1/NR2B, and NR1/NR2A/NR2B receptor subunit complexes[2,3]. While NR1 subunits are required to form an active ion channel, incorporation of the various NR2 subunits regulate NMDAR channel activity by altering the channel kinetics and/or mediating the differential effects of pharmacological agents including ethanol.

Acute ethanol application inhibits NMDAR channel activity[4]. In the hippocampus, this inhibitory effect of ethanol on NMDARs is widely thought to underlie both the acute amnestic effects of ethanol and also, in part, the addictive nature of ethanol[5]. However, the precise molecular mechanisms underlying ethanol's inhibition of NMDARs have not been well-understood. We previously demonstrated that ethanol inhibition of NMDAR function is associated with dephosphorylation of tyrosine residues on the NR2A and NR2B subunits[6]. In particular, ethanol reduced phosphorylation of a site, Tyr^{1472}, on the NR2B subunit which regulates endocytosis of NMDARs[7,8]. Moreover, ethanol-induced inhibition of NMDAR function was prevented by bath application of the protein tyrosine phosphatase (PTP) inhibitor, bpV(phen). Based on these and other findings that showed ethanol reduced the tyrosine phosphorylation of NR2 subunits in the cortex[9], we proposed that ethanol inhibition of NMDAR activity is mediated by a PTP.

PTPs are a large family of enzymes that are broadly divided into receptor-like PTPs and intracellular PTPs[10,11,12], and are implicated in a number of neuronal

functions[13,14,15,16]. STriatal Enriched protein tyrosine Phosphatase (STEP) is a brain-specific PTP expressed in the striatum, hippocampus and cortex among other brain regions[17,18]. Within neurons, STEP is localized to the endoplasmic reticulum[19] and in postsynaptic densities of glutamatergic synapses[20]. Of the four STEP isoforms, $STEP_{61}$ is the only one expressed in the hippocampus[18]. $STEP_{61}$ forms a complex with the NMDAR, reduces its activity, and opposes the induction of long-term potentiation (LTP)[21], a form of plasticity widely thought to play a role in learning and memory[22]. The current hypothesis for STEP function is that it blocks the development of synaptic strengthening[23,24]. Consistent with these findings, enhanced STEP activity is associated with dephosphorylation of the Tyr^{1472} residue on the NR2B subunit[25], a site that is dephosphorylated by ethanol[6]**.** In addition, NMDAR trafficking to synaptic membranes is increased after RNA interference of STEP[23]. Based on these data, we predicted that STEP mediates the inhibitory effects of ethanol on NMDARs in various brain regions, including the hippocampus. We utilized whole-cell recordings of pharmacologically isolated NMDA EPSCs in both rat and mouse hippocampal slices. Moreover, we utilized the recently generated STEP KO mice[26] to further test the hypothesis. Our results show that STEP is responsible, at least in part, for the inhibitory effects of ethanol on hippocampal NMDAR activity.

Results

Pretreatment of hippocampal slices with bpV(phen) attenuates the effects of ethanol on NMDA EPSCs.

Since the previous work with ethanol and the broad spectrum tyrosine phosphatase inhibitor bpV(phen) was determined using extracellular NMDA field EPSPs (fEPSPs)[6],

we first verified that bpV(phen) attenuates ethanol inhibition of NMDAR function in individual pyramidal neurons from rat hippocampal slices. Control or 30 min of 10 μM bpV(phen)-treated hippocampal slices were transferred to a submersion-type recording chamber, perfused with aCSF for 10 min, and monitored with whole-cell recordings in CA1 pyramidal neurons to examine the effects of ethanol. The NMDA EPSCs were evoked by electrical stimulation of synaptic inputs in the stratum pyramidale. Control NMDA EPSCs were inhibited by 35 ± 4% in response to bath application of ethanol (80 mM; Figure 1). In contrast, slices pre-treated with bpV(phen) did not show reduced NMDA EPSCs in response to ethanol. These results confirm earlier work by Alvestad et al.[6] and indicate that PTPs may be involved in mediating the inhibitory effects of ethanol on NMDAR function.

Postsynaptic administration of TAT-STEP (C/S) blocks ethanol inhibition of NMDA EPSCs

Intracellular injection of STEP (C/S) was previously found to increase NMDA currents[21] and to prevent STEP-mediated endocytosis of NMDARs[25]. To determine whether STEP mediates the inhibitory effect of ethanol on NMDA EPSCs, we administered TAT-STEP (C/S) intracellularly into postsynaptic neurons via the recording electrode. TAT-STEP (C/S) has a point mutation in its catalytic site that renders STEP catalytically inactive. TAT-STEP (C/S) binds to STEP substrates but does not dephosphorylate them, and consequently acts as a substrate-trapping protein[27].

TAT-STEP (C/S) was added to the recording microelectrode internal filling solution at a concentration of 30 nM. As described above and in other studies[28], ethanol (80 mM) inhibited NMDA EPSCs by 35 ± 4% in control neurons obtained from hippocampal

slices. However, in neurons that were pre-administered with TAT-STEP (C/S), ethanol inhibition of NMDA EPSCs was prevented (Figure 2). Unexpectedly, an ethanol-mediated enhancement of NMDA EPSCs (10.5 ± 4.9%) was observed in neurons pre-administered with TAT-STEP (C/S) when compared to EPSCs recorded during baseline (pre-ethanol) and washout (post-ethanol) periods [$t=2.143$, $p<0.05$, Student's t test]. A control TAT-Myc peptide (30 nM) did not affect the inhibitory actions of ethanol on NMDA EPSCs (36.2 ± 4.4% inhibition) and thus, the NMDA inhibition was similar to control EPSCs (Figure 2). Therefore, the effects of ethanol on NMDA EPSC amplitudes were significantly different between neurons pre-administered TAT-Myc and TAT-STEP (C/S) [$t=6.894$, $p<0.001$, Student's t test]. These findings demonstrate that TAT-STEP (C/S) prevents ethanol inhibition of synaptic NMDA EPSCs.

Ethanol fails to inhibit NMDA EPSCs in STEP KO mice

We next investigated the effects of ethanol on NMDA EPSCs using STEP null mutant (KO) mice[26]. Figure 3 indicates that pharmacologically-isolated synaptic NMDA EPSCs were readily evoked by electrical stimulation at the stratum pyramidale in hippocampal slices prepared from wild type (WT) and STEP KO mice. There was no significant difference in the resting membrane potential of CA1 pyramidal neurons in brain slices from WT and STEP KO mice (-72.6 ± 1.3 and -70.1 ± 1.5 mV, respectively; $t= 1.271$, $p=0.213$, Student's t test). In neurons from WT mice, bath application of ethanol (80 mM) produced a decrease in synaptic NMDA EPSCs [-25.4 ± 1.9%; $t= 13.368$, $p<0.001$, compared to the baseline values, Student's t test]. Ethanol inhibition of NMDA EPSPs in WT mice was reversed following washout of the ethanol. However, in neurons from STEP KO mice, bath application of ethanol (80 mM) produced a time-dependent

enhancement of NMDA EPSCs [24.2 ± 4.6%; t=5.261, p<0.001, Student's t test], compared to the baseline values. Enhancement of NMDA EPSCs by ethanol in STEP KO mice returned to baseline values upon washout of ethanol (Figure 3). This effect of ethanol was significantly different between WT and STEP KO mice [t=9.173, p<0.001, Student's t test] during ethanol treatment (Figure 4). The results demonstrate that ethanol inhibits synaptic NMDA EPSCs in WT neurons, whereas ethanol actually enhances synaptic NMDA EPSCs in STEP KO neurons.

To determine whether the changes in NMDA EPSCs were due to presynaptic alterations in glutamate release, we next measured paired-pulse facilitation (PPF) in hippocampal slices from WT and STEP KO mice in the presence and absence of ethanol. Paired-pulse stimulation with an inter-pulse interval of 50 ms (an interval that gave optimal facilitation) produced control PPFs with a paired-pulse ratio (PPR, peak2/peak1; P2/P1) of 1.75 ± 0.16 for WT and 1.82 ± 0.17 for STEP KO mice (Figure 4). Ethanol did not significantly alter presynaptic glutamate release in STEP KO mice [F(1,22)=0.0391, p>0.845, two-way ANOVA] or in WT mice [F(1,22)=0.593, p>0.449, two-way ANOVA]. In addition, there was no significant interaction between genotype and ethanol treatment [F(1,22)=0.0396, p>0.844, two-way ANOVA]. Therefore, deletion of the STEP gene does not alter presynaptic glutamate release, and ethanol has no significant effect on presynaptic glutamatergic transmission. Therefore, these data indicate that ethanol inhibition of synaptic NMDA EPSCs is mediated by the STEP molecules that are localized in the postsynaptic neuron.

Intracellular administration of wildtype TAT-STEP restores the inhibitory effects of ethanol on NMDA EPSCs in STEP KO neurons

To conclusively demonstrate that STEP mediates the inhibitory effects of ethanol on NMDA EPSCs, we restore STEP activity by adding WT TAT-STEP back into neurons from STEP KO mice. WT TAT-STEP (30 nM) was pre-administered intracellularly to CA1 pyramidal neurons in slices prepared from WT and STEP KO mice. As described previously (Figure 3), ethanol (80 mM) inhibited NMDA EPSCs in neurons from WT mice by 35 ± 4% (Figure 5). Pre-administration of WT TAT-STEP to WT neurons did not significantly alter the inhibitory effect of ethanol on NMDA EPSCs (Figure 5**a**). We again observed that ethanol (80 mM) significantly potentiated NMDA EPSCs in neurons from STEP KO mice (Figure 5**c**). Importantly, pre-administration of WT TAT-STEP to neurons from STEP KO mice showed an inhibitory effect of ethanol on NMDA EPSCs of 31.1 ± 4.4% [$F(3,27)=51.134$, $p<0.001$, one-way ANOVA] (Figure 5**e**). These results indicate that the introduction of WT TAT-STEP into neurons from STEP KO mice restored the inhibitory effect of ethanol on NMDA EPSCs.

Ethanol effects on GABAergic transmission do not differ between wild type and STEP KO mice.

We have previously shown that ethanol potentiates synaptic $GABA_A$ receptor-mediated inhibitory postsynaptic currents ($GABA_A$ IPSCs) in rodent hippocampal slices[28]. Therefore, we next examined whether $GABA_A$ IPSCs in STEP KO mice were altered by ethanol. Resting membrane potentials were not significantly different between neurons from WT (-72.5 ± 2.7 mV) and STEP KO (-69.5± 2.0 mV) mice for these experiments. Previous work has shown that electrical stimulation of the stratum pyramidale in several mouse and rat strains readily evokes synaptic $GABA_A$ IPSCs that are potentiated by 80 mM ethanol[29, 30]. Here we found that bath application of ethanol (80 mM) enhanced

$GABA_A$ IPSCs in neurons from both WT and STEP KO mice (+29.8 ± 5.2% and +35.6 ± 1.8%, respectively) [t=5.780, p=0.321, Student's t test]. Paired-pulse determinations of $GABA_A$ IPSCs also are not different in the KO compared to the WT mice with respect to genotype [F(1,16)=3.691, p<0.075, two-way ANOVA] and to the effects of ethanol [F(1,16)=0.578, p>0.458, two-way ANOVA] (Figure 6). Therefore, we conclude that the potentiating effects of ethanol on $GABA_A$ IPSCs were not altered in the KO mice, so STEP does not seem to be involved in the ethanol action on GABAergic function.

Ethanol fails to impair high frequency stimulus-induced LTP in STEP KO mice

A number of previous studies have demonstrated that ethanol prevents induction of LTP[31,32]. NMDARs have been shown to be required for LTP induction in the CA1 region of the hippocampus[33], and therefore, a widely accepted hypothesis underlying ethanol's blockade of LTP induction involves ethanol's inhibition of NMDAR function. Since ethanol fails to inhibit NMDAR function in STEP KO mice (Figure 3), we predicted that LTP would be observed in STEP KO mice even in the presence of ethanol. To test this assumption, high frequency stimulation (HFS) was applied to the Schaeffer-collateral commissural fiber pathway, and LTP was measured extracellularly in the CA1 region of hippocampal slices obtained from WT and STEP KO mice. In slices from WT mice, HFS elicited robust LTP as measured by both the amplitude and slope of the fEPSP. Bath application of ethanol (80 mM) for 10 min prior to as well as during the HFS period blocked the induction of LTP in slices from WT mice (Figure 7). In contrast, ethanol was unable to block the induction of LTP in slices from STEP KO mice [F(3,26)=5.443, p<0.005, one-way ANOVA]. Post-hoc pair-wise comparison shows that there was no significant difference in the slope of LTP in control slices from WT and STEP KO mice,

and that ethanol only blocked the LTP in slices from WT mice (Figure 7**e**). Similar results were seen when the LTP amplitudes were measured (Figure 7**f**). In conclusion, ethanol inhibition of LTP induction was not prevented in STEP KO mice.

Discussion

Previous work from our laboratory demonstrated that acute ethanol reduced NMDAR function and decreased tyrosine phosphorylation of NR2A and NR2B[6]. Given that the PTP inhibitor bpV(phen) prevented ethanol-induced inhibition of NMDAR function[6], we concluded that the inhibitory effects of ethanol on NMDARs were mediated by PTPs. However, the identity of the PTP(s) mediating this inhibitory effect on NMDARs was unknown. In the present study, we examined whether STEP was the PTP responsible for ethanol's inhibition of NMDAR activity.

NMDAR activity is regulated by protein phosphorylation[34, 35]. Specifically, tyrosine phosphorylation of NMDARs by the Src family of protein kinases enhances receptor function[36,37,38], whereas PTPs reduce NMDAR channel activity[36, 21]. In the CNS, several PTPs have been identified to influence NMDAR activity[39, 40] ; however, these particular PTPs indirectly modulate NMDAR activity by dephosphorylating an inhibitory site on Src-family tyrosine kinases and consequently increase NMDAR function. We were in search of a PTP that directly dephosphorylates NMDAR subunits and would regulate the inhibitory effects of ethanol on NMDAR function. One likely candidate is the PTP STEP.

Two relevant studies by Pelkey et al.[21] and by Braithwaite et al.[24] demonstrated that the PTP STEP and NMDARs co-immunoprecipitate together, suggesting an interaction of STEP with NMDAR complexes. STEP reduces NMDAR function and

negatively influences LTP[21]. In addition, STEP is required for internalization of both AMPARs and NMDARs. Specifically, beta amyloid activation of STEP leads to dephosphorylation of the regulatory Tyr^{1472} on the NR2B subunit and promotes endocytosis of NMDARs[25]. Moreover, (RS)-3, 5-dihydroxyphenylglycine (DHPG) stimulation of hippocampal slices leads to STEP-mediated internalization of the AMPAR subunits GluR1/GluR2[41]. Based on these results, we predicted that STEP is the PTP which regulates the inhibitory effects of ethanol on NMDAR activity.

If ethanol inhibits NMDAR activity via STEP, we hypothesized that inhibition of STEP activity should attenuate ethanol-induced inhibition of NMDAR function. To test this possibility, we first investigated whether postsynaptic micro-injection of the substrate-trapping TAT-STEP (C/S) peptide[27] prevented ethanol inhibition of NMDA EPSCs. Indeed, we found that TAT-STEP (C/S) attenuated the effects of ethanol on NMDA EPSCs, suggesting that competition for available endogenous STEP substrates sufficiently blocks the ability of ethanol to reduce NMDAR activity.

Since TAT-STEP (C/S) binds to several STEP substrates[12, 27, 26, 41] , we utilized STEP KO mice which were recently generated [26] . We tested the effects of ethanol on hippocampal synaptic NMDA EPSCs in WT and STEP KO mice. Synaptically evoked NMDA EPSCs were resistant to the inhibitory effects of ethanol in STEP KO mice, suggesting that STEP is necessary for ethanol's inhibition of NMDA EPSCs. An important consideration is that compensatory mechanisms may occur in the STEP KO mice which contribute to the failure of ethanol to inhibit NMDA EPSCs in these mice. For example, previous studies in mice created with other gene deletions report that compensatory mechanisms develop and contribute to the behavioral effects of ethanol as a means of homeostasis[42]. To explore this possibility, we acutely restored WT TAT-

STEP to neurons from STEP KO slices and found that the ability of ethanol to inhibit NMDA EPSCs was rescued. This finding strongly supports the hypothesis that STEP is directly involved in mediating the ethanol inhibition of NMDA EPSCs.

During ethanol application to STEP KO slices, we observed facilitation of NMDA EPSCs that continued during the early period of washout (Figure 3). This facilitation was not observed in neurons administered with TAT-STEP (C/S) or in WT mice. As discussed previously, one possible explanation could be that compensatory mechanisms arise during development of STEP KO mice. The hypothesis we favor is that the absence of STEP leads to increased tyrosine phosphorylation of STEP substrates under basal conditions. In support of this hypothesis, recent evidence demonstrates that STEP KO mice have elevated levels of pY^{1472}-NR2B (Venkitaramani and Lombroso, unpublished observations) and pY^{204}-ERK[26]. A consequence of this elevated tyrosine phosphorylation is increased surface expression of NR1/NR2B (Venkitaramani and Lombroso, unpublished observations) and GluR1/2 in STEP KO mice[41]. Perhaps ethanol enhances NMDAR activity during ethanol application and early washout in STEP KO mice by aberrantly increasing the surface expression of NMDARs.

We also investigated the effects of ethanol on LTP induction in STEP KO and WT mice. Pelkey et al[21] previously showed that endogenous STEP functions as a "brake" to regulate NMDAR activity and LTP. We reasoned that removal of STEP might enhance the expression of LTP. To our surprise, we found that hippocampal LTP elicited by HFS does not differ between STEP KO and WT mice. One possible explanation for this result may be that STEP KO mice exhibit similar degrees of HFS-induced LTP but that their threshold for induction is lower than WT mice. Importantly, STEP KO mice are *resistant* to the inhibitory effects of ethanol on LTP induction in the

CA1 region of the hippocampus, a brain region where LTP induction is NMDAR dependent[31, 32]. These results are consistent with the involvement of STEP in mediating the effects of ethanol on NMDAR activity in the hippocampus.

Several reports have shown that PTPs, and in particular STEP, are critically involved in NMDAR and AMPAR internalization[25, 41]. For example, inhibition of PTPs enhances NMDAR surface expression[43, 44, 45], and knock-down of STEP by RNA interference markedly increases the surface expression of functional NMDARs[23]. Phosphorylation of Y**1472**-NR2B is highest in synaptic membranes[44], and dephosphorylation of Y**1472**-NR2B is required for endocytosis of NR2B-containing NMDARs[7, 8]. Additionally, beta amyloid-induced NMDAR endocytosis requires activity of STEP for dephosphorylation of Tyr**1472** -NR2B[25]. We predict that increased surface expression of NMDARs after RNAi of STEP[23] is due to impaired STEP-dependent dephosphorylation of pY**1472**-NR2B, as well as increased activity of Fyn[46], the Src-family member which phosphorylates pY**1472**-NR2B[35]. Based on these studies, we propose that ethanol inhibition of NMDAR function is due to STEP-dependent endocytosis of NMDARs from neuronal surface membranes.

In conclusion, we demonstrate that STEP plays a crucial role in regulating the inhibitory effects of ethanol on NMDARs in the hippocampus. STEP KO mice are resistant to the disrupting effects of ethanol on NMDAR function and LTP induction, and the effects of ethanol on NMDARs are strongly implicated in ethanol tolerance and dependence[5]. As a result, STEP may be an important new target for the development of therapeutic strategies for treating alcoholism.

METHODS

Reagents and animals. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Colorado Denver and Health Sciences Center. STEP KO mice were generated by classical homologous recombination and back-crossed for 7 generations onto C57/B6 mice[26].

We used a catalytically inactive mutant of STEP, in which an essential cysteine in the catalytic domain was converted to a serine (C/S).[21,27] STEP is inactivated by this mutation, but still binds to its substrates and acts as a substrate-trapping protein[47]. We fused the human immunodeficiency virus-type 1, to the N-terminus of TAT-STEP (C/S) to make it cell-permeable[48]. A similar TAT-Myc fusion peptide was made and used as a control in these experiments.

Hippocampal Slice Recordings. Acute hippocampal slices were prepared from 6-8 weeks old male wild type mice, STEP null mutant mice, or Sprague-Dawley rats and placed in a storage chamber for at least 1.5 hr prior to recording[30, 28]. For whole-cell recording of NMDA EPSCs or $GABA_A$ IPSCs, a single slice was transferred to a recording chamber and superfused with artificial cerebrospinal fluid (aCSF) at a bulk flow rate of 2 ml/min. The aCSF consisted of (in mM): 126 NaCl, 3 KCl, 1.5 $MgCl_2$, 2.4 $CaCl_2$, 1.2 $NaHPO_4$, 11 D-glucose, 25.9 $NaHCO_3$, saturated with 95% O_2 and 5% CO_2 at 32.5 ± 1.0 °C. A Flaming/Brown electrode puller (Sutter Instruments, Novato, CA) was used to fabricate whole-cell microelectrodes with resistances of 6-9 MΩ when filled with a K^+-gluconate internal solution. The K^+-gluconate internal solution contained (in mM): 130 K^+-gluconate, 1 EGTA, 2 $MgCl_2$, 0.5 $CaCl_2$, 2.54 disodium ATP, and

10 HEPES; adjusted to pH 7.3 with KOH and 280 mOsm. CA1 pyramidal neurons were recorded within the stratum pyramidale layer and electrically evoked synaptic responses were obtained by stimulation at the stratum pyramidale layer with twisted bipolar stimulating electrodes made from 0.0026-in diameter Formvar-coated nichrome wire[49, 50] to activate presynaptic fibers on or near the pyramidal cell soma (proximal stimulation). Drugs were applied at 100-fold concentration by bath superfusion at 1/100 of the aCSF bulk flow rate of 2 ml/min via calibrated syringe-pumps (Razel Scientific Instruments Inc, Stamford, CT) to obtain the desired concentrations in the bath perfusate.

Measurement of $GABA_A$ IPSCs: CA1 pyramidal neurons were voltage-clamped to -55 mV (corrected for the liquid-junction potential) from the normal resting membrane potential of -65 to -70 mV. $GABA_A$ receptor-mediated IPSC ($GABA_A$ IPSCs) responses were evoked (200 μs, 4-10 V pulses) with a bipolar stimulating electrode at 60 s intervals placed in the stratum pyramidale approximately 200-300 μm from the recorded cell. 6-cyano-7-nitroquinoxaline-2,3-dione disodium salt (CNQX, 20 μM) and D (-)-2-amino-5-phosphonovaleric acid (APV, 25 μM), were added to the superfused aCSF to block α-amino-3-hydroxy-5-methyl-4-isoxalone propionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptor-mediated EPSCs, respectively. This stimulation-recording paradigm evokes synaptic GABAergic responses predominantly from proximal inputs (i.e., $GABA_A$ responses from interneurons that synapse on or near the soma of the recorded pyramidal cell in the stratum pyramidale). Since $GABA_B$ activity can reduce the effects of ethanol on the $GABA_A$ response, pretreatment with the $GABA_B$ antagonist, 3-[[(3,4-dichlorophenyl) methyl]amino] propyl] diethoxymethyl) phosphinic acid (CGP-52432, 0.5 μM) was added to the bath perfusate.

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 of -65 to -70 mV. The NMDA receptor-mediated EPSCs (NMDA EPSCs) were isolated pharmacologically using CNQX (20 μM) and bicuculline methiodide (BMI, 30 μM) to block AMPA and $GABA_A$ receptor-mediated currents, respectively. NMDA EPSC responses were evoked at proximal positions as described for recording $GABA_A$ responses (i.e., stimulation of glutamatergic neurons that synapse on or near the soma of the recorded pyramidal cell). Also, the $GABA_B$ receptor activity was inhibited by the $GABA_B$ antagonist, CGP-52432 (0.5 μM).

Measurement of Long Term Potentiation. Synaptic responses were evoked with bipolar tungsten electrodes placed in the CA1 pyramidal cell layer. Test stimuli were delivered at 0.033 Hz with the stimulus intensity set to 40-50% of that which produced maximum synaptic responses. Tetanic stimulation consisted of two trains of 100 Hz stimuli lasting for 1 s each, with an inter-train interval of 15 s. Field potential recordings were made with glass micropipettes filled with aCSF and placed in the stratum radiatum approximately 200-300 μm from the cell body layer. In control wild type and STEP knockout mice, this stimulation caused a potentiated response (LTP) that persisted at an elevated level (> 20% above baseline) for more than 40 min. Field EPSP (fEPSP) slopes were calculated as the initial slope measured between 10-30% from the origin of the negative deflection.

Statistical Analysis. All data were expressed as mean ± S.E.M. Significant differences between two groups were determined by unpaired t-test while significance

among multiple groups were evaluated either by one-way or two-way ANOVA with Tukey's post hoc pairwise comparisons. P values (α) less than 0.05 were set as significance throughout the experiments. Computer-assisted software Sigma Stat Program (SYSTAT SOFTWARE INC, San Jose CA) was used in statistical analysis.

Author contributions. P.H.W. designed, performed, analyzed the experiments and prepared the manuscript; R.K.F. designed, performed and analyzed data for the LTP experiments shown in figure 7; W.R.P. and S.M.G. helped coordinating the experiments and editing the manuscript; M.D.B. and P.J.L. were responsible for helping with designing the project, and editing the manuscript.

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Figure Legend

Figure 1. The protein tyrosine phosphatase inhibitor bpV(phen) blocks ethanol inhibition of synaptic NMDA EPSCs. (**a**) Representative NMDA current traces of a neuron from a rat hippocampal slice shows that acute ethanol application inhibits synaptic NMDA EPSCs from control (Con) brain slices. (**b**) Representative NMDA EPSC traces of a CA1 neuron from a bpV(phen)-treated brain slice (bpV) shows that the effects of acute ethanol on NMDA EPSCs are blocked by 10 μM bpV(phen) treatment. (**c**) Representative traces show input-output (I/O) relationship of NMDA EPSCs. The stimulus strength (input) is shown as 1X, 2X, or 4X times of the threshold stimulus strength that can evoke an NMDA EPSC response. (**d**) The composite data show that bpV(phen) blocks the ethanol inhibition of NMDA EPSCs. Control cells (n=14); bpV(phen)-treated cells (n=8); *** $p<0.001$

Scale bar represents 50 ms and 50 pA

Figure 2. Microinjection of TAT-STEP (C/S) into postsynaptic neurons blocks the inhibitory effects of ethanol on NMDA EPSCs. (**a**) Current traces show that microinjection of Tat-Myc (Myc) does not alter the inhibitory effects of ethanol on NMDA EPSCs. (**b**) Administration of TAT-STEP (C/S) (STEP C/S) into the postsynaptic pyramidal neuron blocks the inhibitory effects of ethanol on NMDA EPSCs. (**c**) The composite data show that Tat-Myc (n=4) does not alter the effects of ethanol while TAT-STEP (C/S) (n=5) blocks the effects of ethanol on the NMDA EPSCs. *** $p<0.001$

Scale bar represents 50 ms and 50 pA

Figure 3. Ethanol fails to inhibit NMDA EPSCs in STEP KO mice. (**a**) Whole-cell current traces of a hippocampal CA1 neuron from a wild type (WT) mouse are shown. The NMDA EPSC trace that is measured at baseline (*A*), during ethanol application (*B*), an early period during ethanol washout (*C*), and a late period of ethanol washout (*D*) of a WT mouse (WT) at the times indicated in panel c. (**b**) Whole-cell current traces from a hippocampal CA1 neuron of the STEP KO mouse (KO) are shown. An NMDA EPSC that is measured at the time indicated in part **c**. (**c**) The time course of the effects of ethanol on NMDA EPSC amplitude from WT (● , n=8 cells) or STEP KO (○ , n=10 cells) mice. These data indicate that acute ethanol application inhibits NMDA EPSCs from WT mice, whereas acute ethanol has an enhancing effect on NMDA EPSCs from KO mice.

Figure 4. Presynaptic glutamate release is not influenced by ethanol or genotype. (**a**) Representative NMDA EPSC traces from WT mice (n=10 cells) demonstrate that acute ethanol application inhibits the amplitude of NMDA EPSCs. (**b**) Representative NMDA EPSC traces from STEP KO neurons (n=10 cells) show that ethanol potentiates the amplitude of NMDA EPSCs. (**c**) The composite data show that acute ethanol application produces approximately a 28% reduction in the amplitude of synaptic NMDA EPSCs in WT mice, but it stimulates the NMDA EPSCs by 24% in STEP KO mice. Current traces show the paired pulse facilitation of synaptic NMDA EPSCs in WT (**d**) and STEP KO (**e**) mice. Although ethanol inhibits these responses in WT neurons and enhances these responses in STEP KO neurons, the paired pulse ratios remain unchanged in both genotypes. (**f**) The composite data show that the STEP gene

deletion does not alter presynaptic events or ethanol effects on these events on NMDAR neurotransmission (n=5 for WT and n=7-8 for STEP KO neurons). *** p<0.001 Scale bar represents 50 ms and 50 pA

Figure 5. Microinjection of WT TAT-STEP restores the inhibitory effects of ethanol on NMDA EPSCs in neurons from STEP KO mice. (**a**) Whole-cell recordings show that microinjection of vehicle (Con) does not alter the inhibitory effects of ethanol on NMDA EPSCs in neurons from WT mice (n=8 cells). (**b**) Representative NMDA EPSC traces show that intracellular injection of wild type TAT-STEP (STEP) into a neuron does not alter the effects of ethanol on NMDA EPSCs in WT mice (n=8 cells). (**c**) Representative NMDA EPSC traces show that intracellular injection of vehicle (Con) does not alter the stimulatory effects of ethanol on NMDA EPSCs from STEP KO mice (n=10 cells), but it restores the effects of ethanol on NMDA EPSCs in STEP KO neurons (n=5 cells) (**d**). The composite data (**e**) show that there is sufficient STEP activity in WT neurons to mediate the action of ethanol on NMDARs, so that additional STEP does not further enhance the effects of ethanol. However, replacement of WT TAT-STEP can restore the effects of ethanol in STEP KO neurons. *** p<0.001 Scale bar represents 50 ms and 50 pA

Figure 6. $GABA_A$ IPSCs are potentiated in both WT and STEP KO mice.

Whole-cell current responses show synaptic $GABA_A$ IPSCs and the ethanol enhancement of these $GABA_A$ IPSCs from WT (**a**) and STEP KO (**b**) neurons. The composite data (**c**) show that ethanol stimulates $GABA_A$ IPSCs to a similar extent in both WT (n=5) and STEP KO (n=5) neurons. Paired-pulse responses and the effects of

ethanol on these $GABA_A$ IPSCs in WT (**d**) and STEP KO (**e**) neurons are shown. The composite data (**f**) show that neither the control (Con) paired pulse ratio (PPR) nor the effects of ethanol (EtOH) on the PPR was altered in WT (n=5 cells) or in STEP KO (n=5 cells). Scale bar represents 50 ms and 200 pA

Figure 7. Ethanol fails to inhibit the induction of LTP in STEP KO mice. Field excitatory postsynaptic potential (fEPSP) traces show hippocampal LTP induction in brain slices treated with control aCSF (Con) or acute ethanol (80 mM) (EtOH) in WT (**a**) and STEP KO (**b**) mice. High frequency stimulation (HFS, two trains of 100 Hz stimulation, separated by 15 s) of the stratum radiatum produces a robust LTP of the fEPSP slope in both WT (**c**) and STEP KO (**d**) mouse hippocampal slices under control conditions (●) or following 10 min of EtOH administration (○). However, after acute ethanol application, the same stimulation fails to induce LTP in WT mice, while LTP can still be obtained from STEP knockout mice. Mean changes in the slope (**e**) and amplitude (**f**) of the fEPSPs by ethanol from WT (n=8) and STEP KO (n=7) mice are shown. ** $p<0.005$

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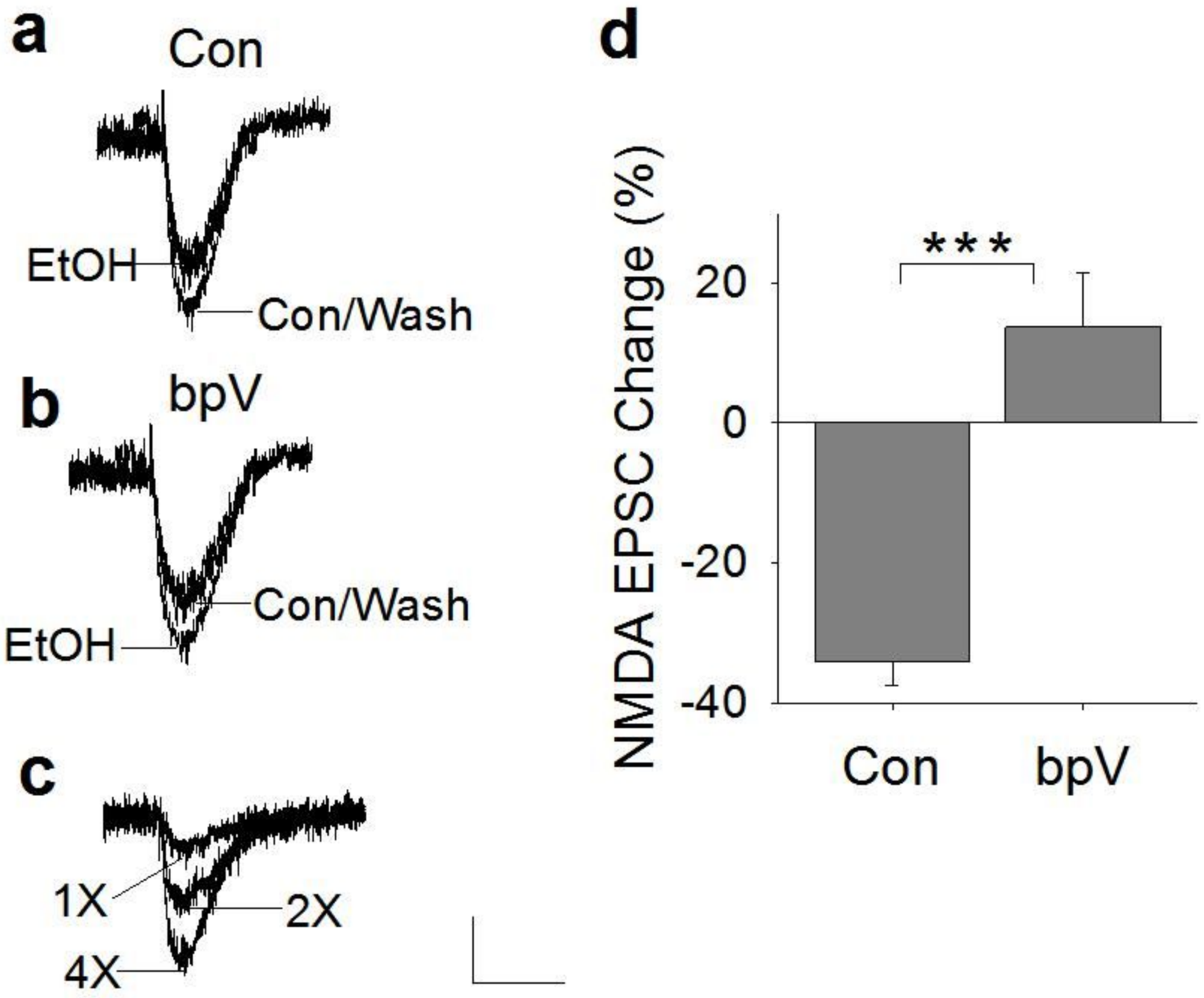
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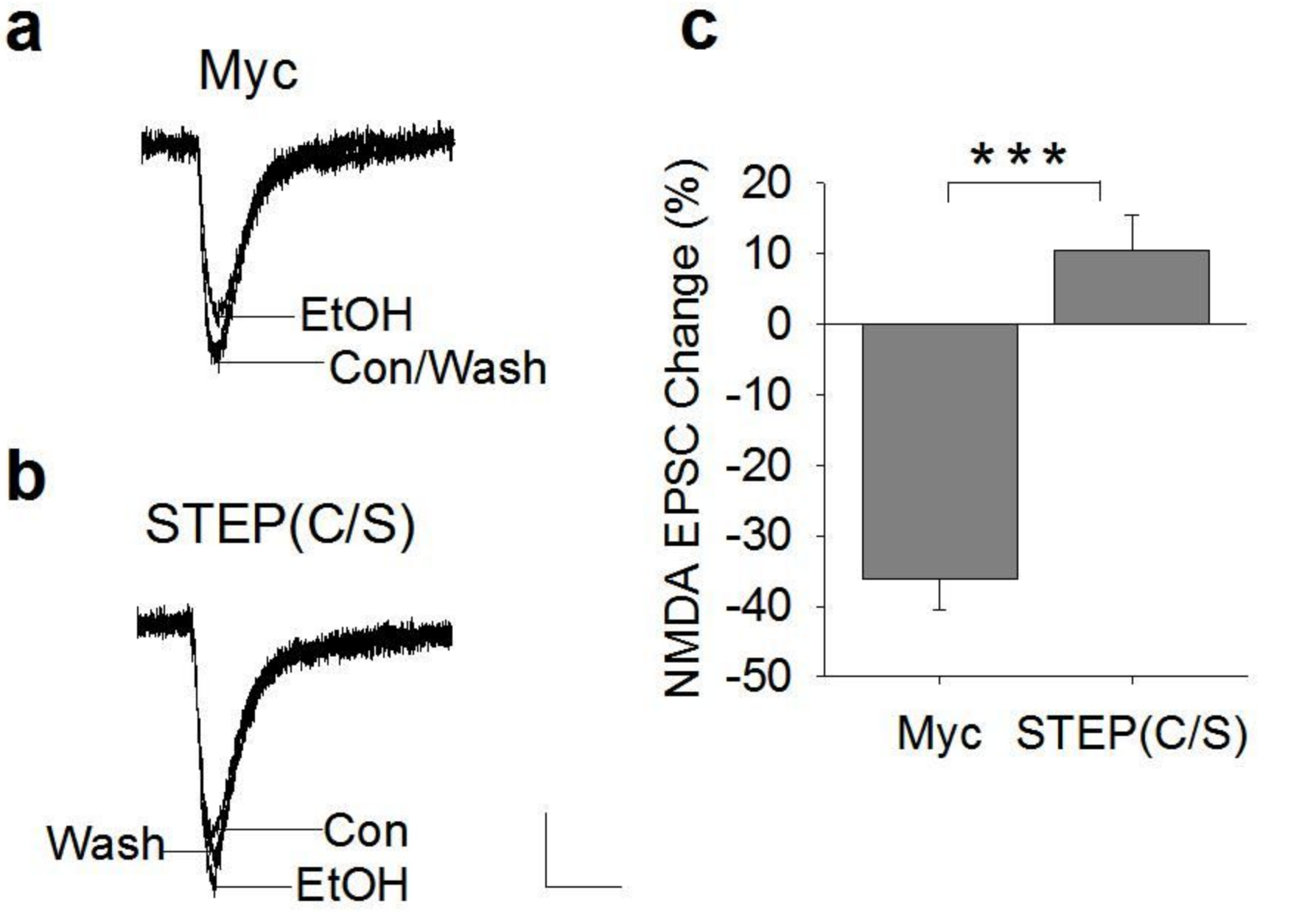
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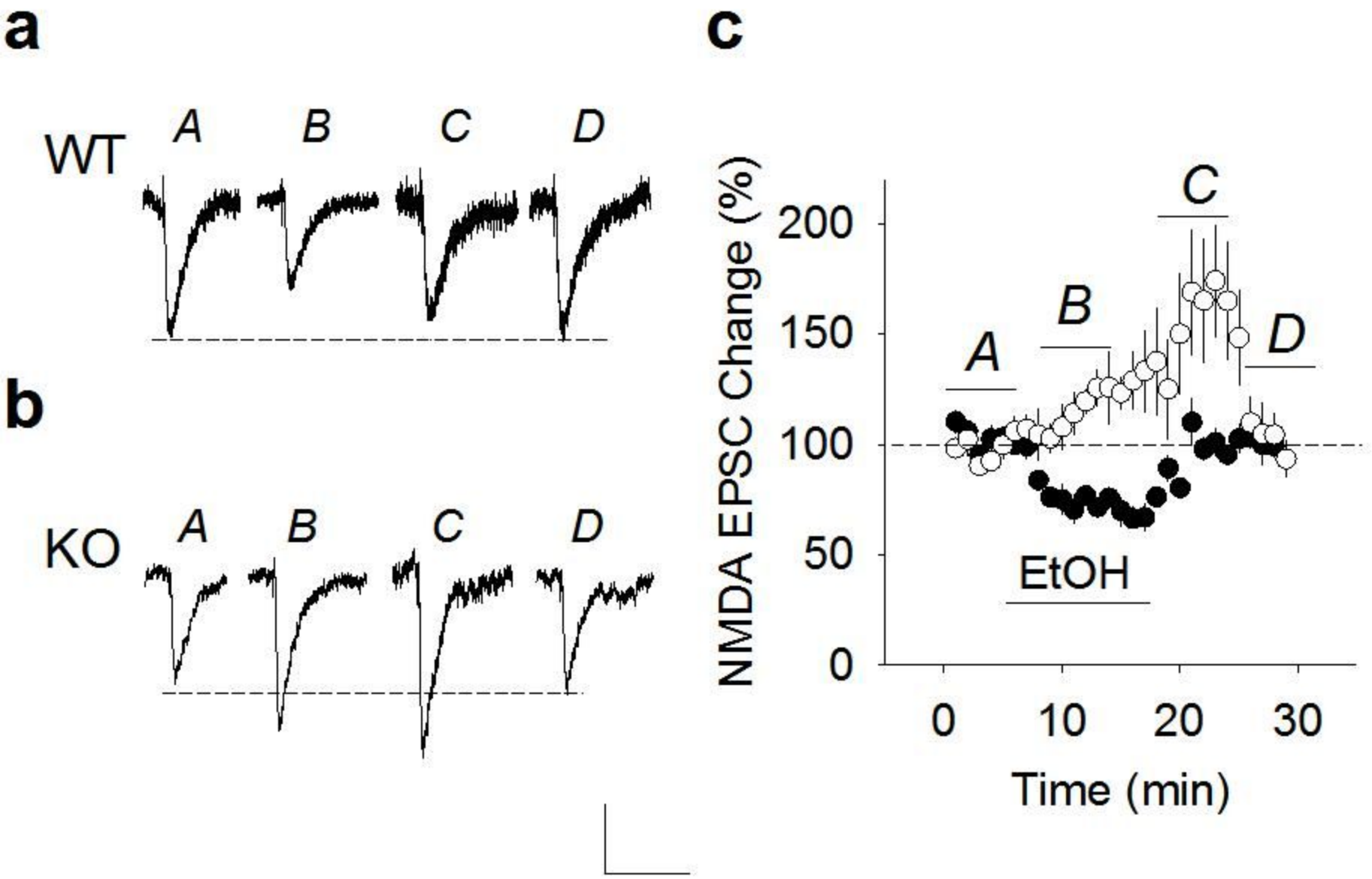
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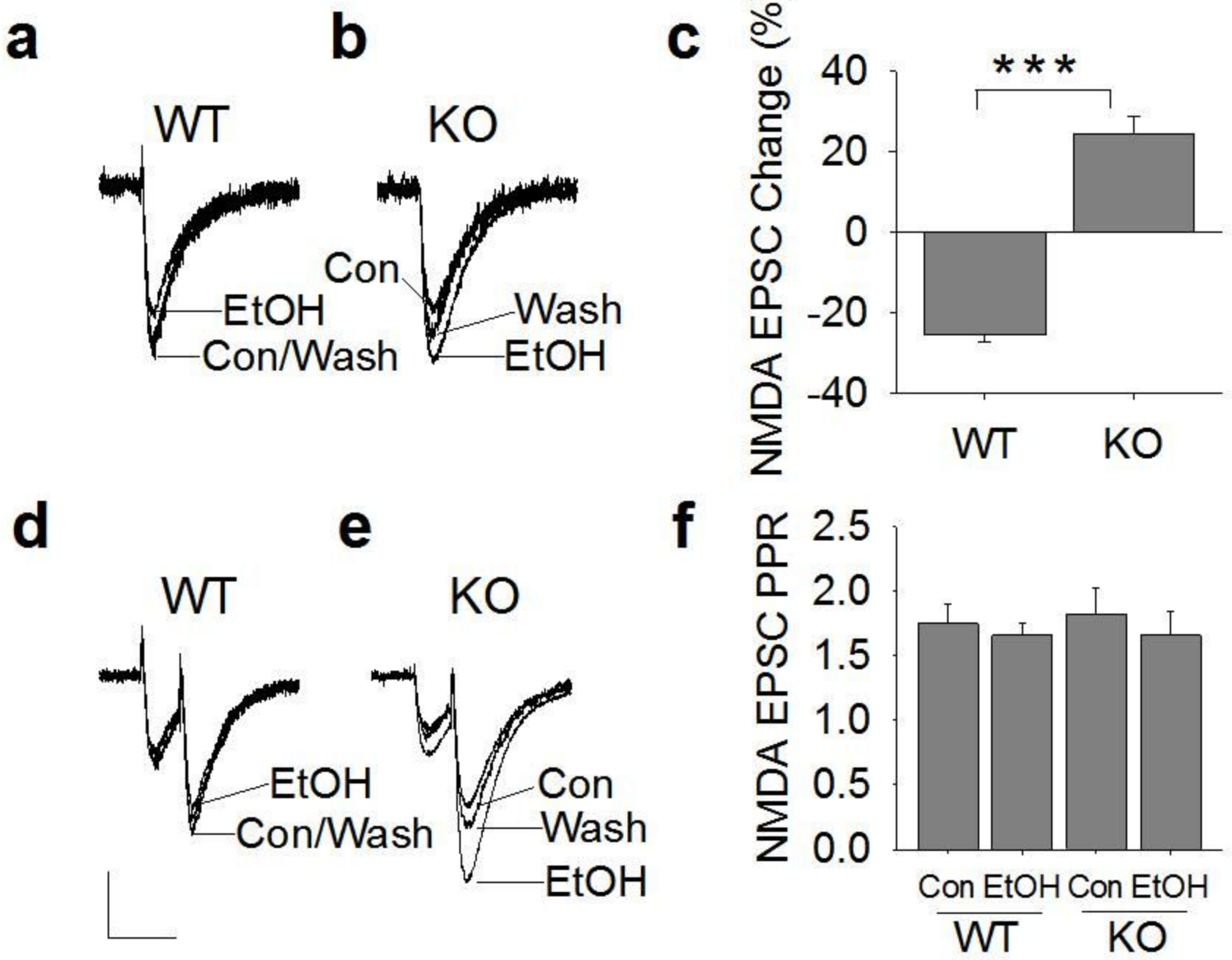
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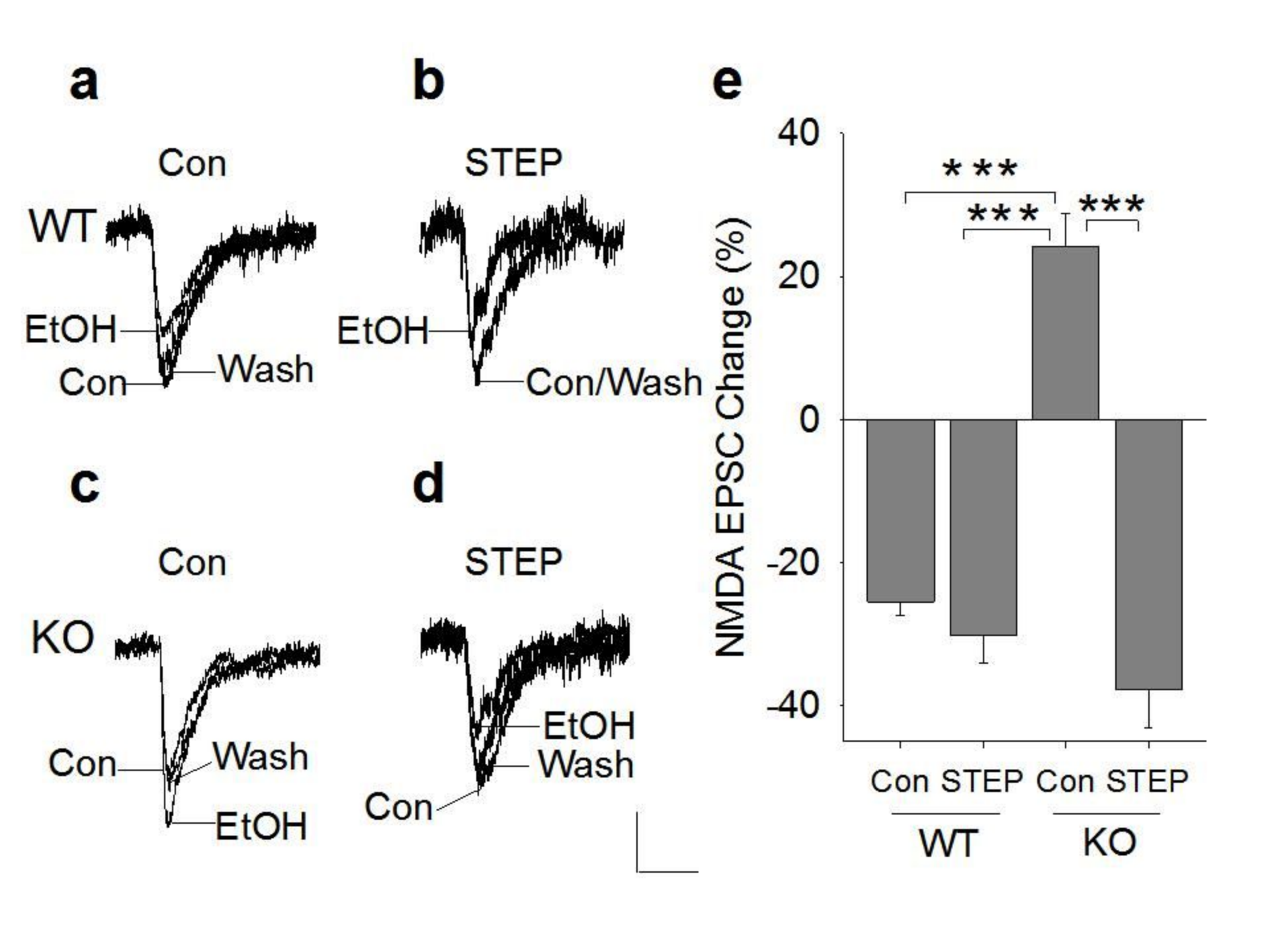
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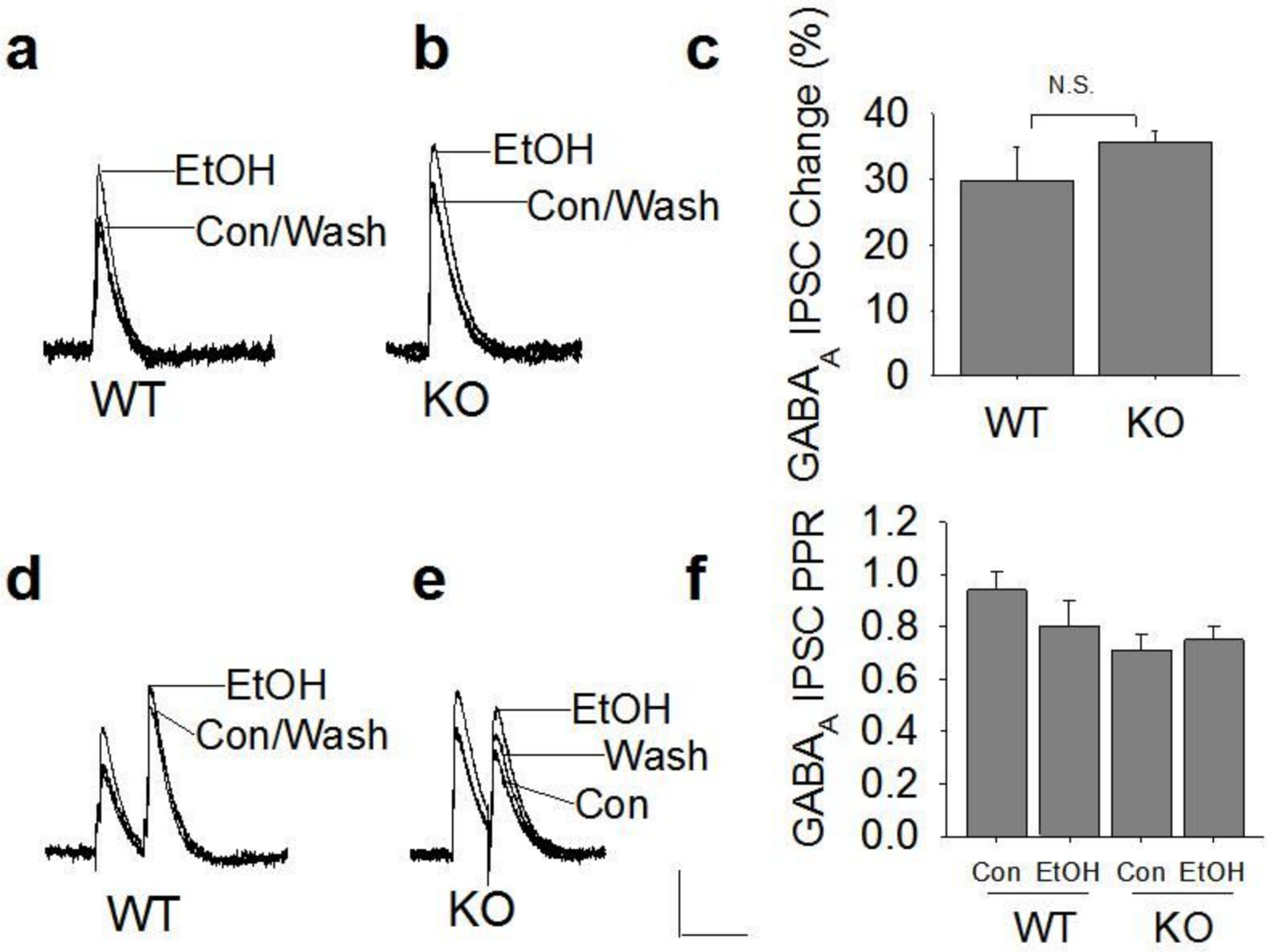
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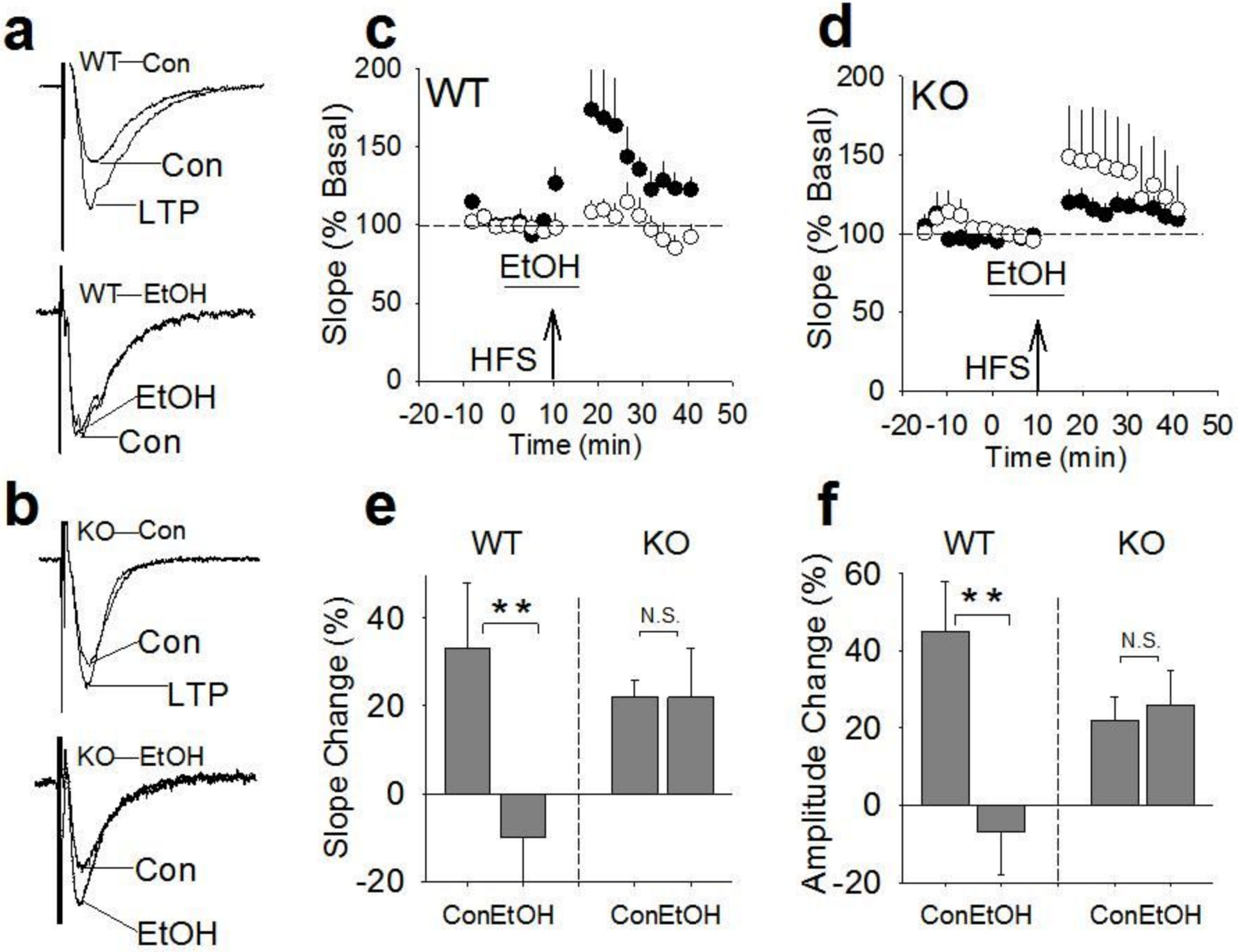
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Slope (% Basal)
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-20 -10 0 10 20 30 40 50
Time (min)
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Slope (% Basal)
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-20 -10 0 10 20 30 40 50
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Slope Change (%)
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