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INHIBITION OF STRIATAL-ENRICHED TYROSINE PHOSPHATASE 61 IN THE DORSOMEDIAL STRIATUM IS SUFFICIENT TO INCREASED ETHANOL CONSUMPTION

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Abstract

The STriatal-Enriched protein tyrosine Phosphatase 61 ($STEP_{61}$) inhibits the activity of the tyrosine kinase Fyn and dephosphorylates the GluN2B subunit of the NMDA receptor, whereas PKA phosphorylation of $STEP_{61}$ inhibits the activity of the phosphatase (Goebel-Goody *et al.* 2012). Previously, we found that ethanol activates Fyn in the dorsomedial striatum (DMS) leading to GluN2B phosphorylation, which, in turn, underlies the development of ethanol intake (Wang *et al.* 2010). Here, we tested the hypothesis that inhibition of $STEP_{61}$ by ethanol is upstream of Fyn/GluN2B. We show that exposure of mice to ethanol increased $STEP_{61}$ phosphorylation in the DMS, which was maintained after withdrawal and was not observed in other striatal regions. Specific knockdown of $STEP_{61}$ in the DMS of mice enhanced ethanol-mediated Fyn activation and GluN2B phosphorylation, and increased ethanol intake without altering the level of water, saccharine, quinine consumption or spontaneous locomotor activity. Together, our data suggest that blockade of $STEP_{61}$ activity in response to ethanol is sufficient for the activation of the Fyn/GluN2B pathway in the DMS. Being upstream of Fyn and GluN2B, inactive $STEP_{61}$ in the DMS primes the induction of ethanol intake.

Keywords

Phosphatase; Ethanol; Striatum; Addiction; Alcohol; Phosphorylation

INTRODUCTION

Fyn is a tyrosine kinase that belongs to the Src family of protein tyrosine kinases (PTKs) (Ingley 2008). Fyn is highly expressed in the adult brain (Yagi *et al.* 1993), and plays an

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important role in the central nervous system (CNS) (Ohnishi *et al.* 2011). One of the best-characterized substrates of Fyn in the brain is the NR2B subunit of the N-methyl D-aspartate receptor (GluN2B) (Trepanier *et al.* 2012). Fyn associates with GluN2B by interacting with the scaffolding protein RACK1 (Yaka *et al.* 2002, Thornton *et al.* 2004), and the close proximity of Fyn to GluN2B allows the efficient phosphorylation of the subunit (Tezuka *et al.* 1999, Sato *et al.* 2008, Yaka *et al.* 2003). The consequence of Fyn-mediated phosphorylation of GluN2B is an enhancement of channel function (Yaka et al. 2002, Trepanier et al. 2012, Yaka et al. 2003), which is due, at least in part, to an increased retention of the channel containing the subunit in the membrane (Dunah *et al.* 2004, Nakazawa *et al.* 2001, Prybylowski *et al.* 2005).

Fyn is composed of a regulatory and a catalytic domain (Engen *et al.* 2008). The regulatory domain consists of a short unique region at the N-terminus that contains myristoylation and palmitoylation sites that anchor the kinase to membranes, a proline-rich SH3 binding domain and a phospho-tyrosine binding SH2 domain (Engen et al. 2008). In its inactive conformation, Fyn is phosphorylated on Tyrosine (Tyr) 527. Phospho-Tyr^{527} forms an intra-molecular bond with its SH2 domain that keeps the kinase in a closed inactive conformation (Engen et al. 2008). Dephosphorylation of this site results in a conformational change, allowing the kinase to undergo autophosphorylation at Tyr^{420}, which is the hallmark of the active kinase (Engen et al. 2008). Conversely, dephosphorylation of phospho-Tyr^{420} inhibits Fyn kinase activity (Engen et al. 2008).

In the CNS, STEP is the phosphatase responsible for the inactivation of Fyn via the dephosphorylation of phosphorTyr^{420} (Nguyen *et al.* 2002). STEP is a brain-specific tyrosine phosphatase (Lombroso *et al.* 1991) that is highly expressed in the striatum (Lombroso *et al.* 1993, Boulanger *et al.* 1995). *STEP* mRNA is alternatively spliced and a 46 kDa cytosolic form ($STEP_{46}$) and a 61 kDa membranal form ($STEP_{61}$) are produced (Lombroso et al. 1991, Sharma *et al.* 1995, Boulanger et al. 1995). Like Fyn (Yaka et al. 2002), $STEP_{61}$ is localized in the postsynaptic density (PSD) and associates with the NMDAR complex (Pelkey *et al.* 2002, Braithwaite *et al.* 2006). In addition to Fyn inactivation, $STEP_{61}$ dephosphorylates GluN2B at a regulatory Tyr^{1472} (Pelkey et al. 2002, Snyder *et al.* 2005), and the consequences of $STEP_{61}$ dephosphorylating GluN2B are the reduction of spontaneous activity of NMDARs, the inhibition of long-term potentiation (LTP) (Pelkey et al. 2002), as well as increased endocytosis of the channel (Snyder et al. 2005, Braithwaite et al. 2006).

We previously showed that acute *ex vivo* and *in vivo* exposure of rat dorsal striatum to ethanol leads to the activation of Fyn and the phosphorylation of GluN2B resulting in a long-term facilitation (LTF) of the activity of NMDARs containing GluN2B (Wang *et al.* 2007). We further localized the GluN2B-dependent LTF to the dorsomedial striatum (DMS) (Wang et al. 2010, Wang *et al.* 2011), and showed that repeated systemic administration of ethanol or repeated cycles of ethanol consumption and withdrawal produced a long-lasting activation of Fyn, leading to a sustained increase in the phosphorylation and synaptic retention of GluN2B in the DMS of rats (Wang et al. 2010, Wang et al. 2011, Gibb *et al.* 2011). Finally, we showed that intra-DMS infusion of the Src PTK inhibitor, PP2, or the GluN2B inhibitor, ifenprodil, decreased rat operant ethanol self-administration and

reinstatement of ethanol seeking in rats that consumed high levels of ethanol (Wang et al. 2010, Wang et al. 2011). As $STEP_{61}$ negatively regulates the phosphorylation state and activity of Fyn and GluN2B, we hypothesized that ethanol inactivates $STEP_{61}$ in the DMS, and that the inhibition of phosphatase activity is sufficient for the molecular and behavioral adaptations that lead to the induction of ethanol intake.

Materials and Methods

Materials

The generation and characterization of rabbit anti-[pS^{49}/pS^{221}]STEP antibodies are described (Paul *et al.* 2003). Mouse anti-STEP and mouse anti-NeuN antibodies were purchased from EMD Millipore (Billerica, MA). Rabbit anti-GAPDH, goat anti-GluN2B, mouse anti-Src, rabbit anti-Fyn antibodies and the horseradish peroxi-conjugated (HRP) secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-[pY^{1472}] GluN2B and rabbit anti-[$pY^{418/420}$] Src/Fyn antibodies were purchased from Cell Signaling Technology (Beverly, MA). Phosphatase inhibitor cocktails 1 and 2, mouse anti-GFAP antibodies and primers for PCR were purchased from Sigma (St Louis, MO). Rabbit anti-GFP (green fluorescence protein) antibodies were purchase from Abcam (Cambridge, MA). Enhanced Chemiluminescence (ECL) Plus was purchased from GE Healthcare (Pittsburg, PA) and BioMax MR Film was purchased from Kodak (Rochester, NY). Bicinchoninic acid (BCA)™ protein assay kit was obtained from Pierce (Rockford, IL). p24 antigen ELISA kit was purchased from Zeptometrix (Buffalo, NY). NuPAGE 10% Bis-Tris gels, Alexa Fluor 488-labeled donkey anti-rabbit and Alexa Fluor 594-labeled donkey anti-mouse were purchased from Invitrogen (Carlsbad, CA). Vectashield mounting medium was purchased from Vector Laboratories (Burlingame, CA). Complete™ mini, EDTA-free protease inhibitor cocktail was purchased from Roche (Indianapolis, IN). Reverse transcription system and 2X PCR Master Mix were purchased from Promega (Madison, WI). The Paris Kit was purchased from Ambion (Austin, TX).

Animals

Male C57BL/6J mice (The Jackson Laboratory; 22–25 g, 3 months old) were housed in a temperature- and humidity-controlled room under a 12 hr light/dark cycle, with food and water available *ad libitum*. All animal procedures in this report were approved by the Gallo Center Institutional Animal Care and Use Committee and were conducted in agreement with the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996.

Drugs and treatments

Ethanol solution for the drinking experiments was prepared from absolute anhydrous ethanol (190 proof) diluted to 10% or 20% ethanol (v/v) in tap water. For systemic administration, ethanol was diluted to 20% ethanol (v/v) in saline.

Production of lentivirus expressing shRNA targeting STEP

The 21 nucleotide short hairpin RNA (shRNA sequence targeting STEP (shSTEP)) 5′-AAA CAT GCG AAC AGT ATC AGT-3′ was chosen based on a previous study (Braithwaite et al. 2006). A scrambled sequence (shSCR), 5′-AGT CAG TAC GTA TAA CAA GCA-3′

was designed using a public siDesign website (http://www.sirnawizard.com/scrambled.php) and was used as a non-targeting shRNA control. Both sequences were incorporated into a previously described stem-loop structure (Rubinson *et al.* 2003). Synthesized oligonucleotides were annealed and subcloned into the HpaI and XhoI restriction sites in the recombinant lentiviral vector pLL3.7, which also expresses enhanced GFP (EGFP). The shRNA is driven by the U6 promoter and the GFP by the CMV promoter. The pLVX-shSTEP or pLVX-shSCR plasmid was transfected into HEK293T (Clonetech, Mountain View, CA) cells along with the packaging plasmids, psPAX2 and pMD2.G using Lipofectamine 2000. Forty-eight hours after transfection, the supernatant was collected and lentiviral particles were purified by ultra-centrifugation (26000 rpm, 90 min at 4°C) as described previously (Lasek *et al.* 2007). Titers were determined using HIV-1 p24 antigen ELISA Kit (ZeptoMetrix) as per the manufacturer's instructions. Viral titer for *in vivo* administration was 1.8×10^7 pg/ml.

Collection of brain samples for molecular analyses

Mice were sacrificed 4 weeks after virus infusion or after 6 weeks of intermittent access to 20% ethanol. Brains were quickly removed and placed on an ice-cold platform. Striatal tissues were collected and immediately homogenized in 300 µl of RadioImmuno Precipitation Assay (RIPA) buffer (in mM: 50 Tris-HCl, pH 7.6, 150 NaCl, 2 EDTA, and 1% NP-40, 0.1% SDS and 0.5% sodium deoxycholate). Protease and phosphatase inhibitor cocktails were added to the lysate.

Western Blot Analysis

Equal amounts of homogenates (30 µg) were resolved on NuPAGE 10% Bis-Tris gels and transferred onto nitrocellulose membranes. Blots were blocked with 5% milk-PBST and then probed with antibodies overnight at 4°C. Membranes were washed and probed with HRP-conjugated secondary antibodies for 2 hrs at room temperature. Phosphorylation level of proteins was determined by probing membranes with the anti-phospho-specific and anti-GAPDH antibodies. Membranes were then stripped for 30 min at room temperature in a buffer containing 25 mM Glycine-HCL, 1% (w/v) SDS, pH 3.0, and reprobed with the appropriate total antibody. Membranes were visualized using ECL. Band intensities were quantified by ImageJ.

Immunochemistry

Animals were deeply anesthetized with an overdose of Euthasol® and perfused with 0.9% NaCl, followed by 4% paraformaldehyde (PFA) in PBS, pH 7.4. Brains were removed, post-fixed in the same fixative for 2 hrs, and transferred to PBS at 4°C. On the following day, brains were transferred into 30% sucrose and stored for 3 days at 4°C. Frozen, 50 µm-thick coronal sections were cut on a cryostat (Microm, Thermo Fisher Scientific), collected in 24-well plates, and stored in PBS at 4°C. Free-floating sections containing the infusion site in the striatum were selected, permeabilized with 50% ethanol for 10 min, and rinsed in PBS. Sections were then blocked with 10% normal donkey serum in PBS for 30 min and incubated for 48 hrs at 4°C on an orbital shaker with antibodies for either the neuronal marker (anti-NeuN antibodies) or the glial marker (anti-GFAP antibodies) in combination

with anti-GFP antibodies diluted in PBS/0.05% Triton X-100. Next, sections were washed in PBS, incubated with 2% normal donkey serum for 10 min and incubated for 4 hrs with the secondary antibodies: Alexa Fluor 488-labeled donkey anti-rabbit and Alexa Fluor 594-labeled donkey anti-mouse (both 1:300) antibodies. After staining, sections were rinsed in PBS and cover slipped using Vectashield mounting medium. Images were acquired using Zeiss LSM 510 META laser confocal microscope (Zeiss MicroImaging) using manufacture recommended filter configurations.

Virus infusion

Mice were anesthetized using a mixture of ketamine (120 mg/kg) and xylazine (8 mg/kg). Bilateral microinfusions were made using stainless steel injectors (33 gauge, Small Parts) into the DMS (4 infusion sites per hemisphere; the stereotaxic coordinates were anterioposterior +1.6 mm and +1.1 mm from bregma; mediolateral ±1.15mm from bregma and dorsoventral −3.0 and −3.5 mm from the skull surface). Animals were infused with Ltv-shSTEP or Ltv-SCR (1.2 μl/injection site with 4 sites of injection per hemisphere) at a concentration of 1.8×10^7 pg/ml at an injection rate of 0.1 μl/min. After each infusion, the injectors were left in place for an additional 12 min to allow the virus to diffuse. Mice recovered for 4 weeks before the ethanol-drinking experiment was initiated.

Voluntary ethanol consumption after viral infusion

Mice underwent continuous access to ethanol (10% or 20%) in a two-bottle choice drinking paradigm. Drinking sessions were conducted 24 hrs a day, 7 days a week, with one bottle containing tap water and the other containing 10% ethanol solution for 6 days and 20% ethanol solution for 6 days. The bottles were weighed daily, and the mice were weighed once a week. The position (left or right) of each solution was alternated as a control for side preference. Mice underwent an intermittent voluntary access to 20% ethanol in a two-bottle choice procedure as described below for additional 2 weeks (6 sessions).

Intermittent access to 20% ethanol two-bottle choice paradigm

Mice were given 24 hrs concurrent access to one bottle containing 20% ethanol and one bottle containing tap water (Warnault *et al.* 2013). Drinking sessions started at 12:00 h on Monday, Wednesday, and Friday, with 24 or 48 hrs (weekend) ethanol-deprivation periods between the drinking sessions. The placement (left or right) of each solution was alternated between each session to control for side preference. All the mice that were assessed for binge drinking consumed 5–6g/kg/4 hrs. The mice that consumed less were not used for biochemical analysis.

Quinine and saccharin consumption

One week after the end of the ethanol-drinking study, mice were tested for saccharin (0.066%) or quinine hemisulfate (0.06 mM) intake. Each solution was offered for 3 days and the amount of fluid intake was recorded every day.

Assessment of spontaneous locomotor activity

One week after the end of the fluid intake experiment, spontaneous locomotor activity was measured in activity-monitoring chambers (43 cm × 43 cm) with horizontal photo beams (Med Associates, St Albans, VT). Horizontal locomotor activity was monitored and the distance traveled (cm) by the animals was recorded for 60 min.

Histology

Mice infused with Ltv-shSTEP or Ltv-shSCR received an intraperitoneal injection of pentobarbital followed by transcardial perfusion with 4% PFA. For each subject, the infected area was verified in 50 μm coronal sections using Zeiss LSM 510 META laser confocal microscope. Animals showing localized infection in the DMS were included in the studies.

Statistical analysis

Biochemical data were analyzed using an unpaired *t* test or two-way ANOVA. Significant main effects and interactions of ANOVAs were further investigated with the Student-Newman-Keuls (SNK) *post hoc* test or method of contrast analysis. Behavioral data were analyzed using one- or two-way ANOVA with or without repeated measures (RM-ANOVA). Significant main effects and interactions of the ANOVAs were further investigated with the Student-Newman-Keuls (SNK) *post hoc* test or by the method of contrast analysis. Statistical significance was set at $p < 0.05$.

Results

Ethanol exposure increases $STEP_{61}$ phosphorylation in the DMS

We previously found that *in vivo* exposure to ethanol results in the activation of Fyn, which, in turn, phosphorylates GluN2B in the dorsal striatum (Wang et al. 2007) and specifically in the DMS (Wang et al. 2010, Gibb et al. 2011). Both Fyn (Nguyen et al. 2002) and GluN2B (Snyder et al. 2005) are substrates of the membranal associated form of STEP, e.g., $STEP_{61}$, and the phosphatase regulates the phosphorylation-dependent enhancement of channel activity (Pelkey et al. 2002). We therefore reasoned that in order for the Fyn/GluN2B pathway to be activated in response to ethanol, $STEP_{61}$ activity should decrease. Both the cytosolic $STEP_{46}$ and the membranal $STEP_{61}$ isoforms are phosphorylated by PKA, on serine 49 and serine 221, respectively (Paul *et al.* 2000). PKA phosphorylation prevents the association of the phosphatase with its substrates, thus preventing tyrosine dephosphorylation (Paul et al. 2000). As ethanol-mediated activation of PKA is well-documented (reviewed in (Ron & Messing 2013), we hypothesized that DMS exposure to ethanol leads to PKA phosphorylation (and thus inhibition) of $STEP_{61}$ and possibly also $STEP_{46}$. To test this possibility, C57BL/6 mice underwent 6 weeks of intermittent access to 20% ethanol using a two-bottle choice drinking paradigm (Warnault et al. 2013) and the levels of PKA phosphorylation of $STEP_{46/61}$ were measured after 4 hrs of ethanol intake (binge drinking) or after 24 hrs of withdrawal (see timeline in Suppl. Fig. 1A). Using specific phospho-$STEP_{46/61}$ antibodies that recognize the PKA phosphorylation sites (Paul et al. 2003), we observed that repeated cycles of binge drinking of ethanol produced a robust

phosphorylation of $STEP_{61}$ in the DMS (Figure 1A; $t_{(10)} = -5{,}79$, $p<0.001$), which corresponded with an increase in Fyn activity (Figure 1B; Fyn $t_{(10),} = -8{,}93$, $p<0.001$), and GluN2B phosphorylation (Figure 1C, $t_{(10)} = -4{,}07$, $p=0.002$). We also observed that ethanol-mediated phosphorylation of $STEP_{61}$ was long-lasting as it was still detected after 24 hrs of withdrawal (Fig. 2A; $t_{(10)} = -6{,}62$, $p<0.001$). However, the increase in $STEP_{61}$ phosphorylation was not due to an alteration of the total protein levels of the phosphatase in response to ethanol (Fig. 1A, $STEP_{61}$/GAPDH (% of water) after binge: 115.11% ± 14.11; $t_{(10)} = -0.91$, $p=0.38$; Fig. 2A, $STEP_{61}$/GAPDH (% of water) after withdrawal: 93.38% ± 2.54; $t_{(10)} = 1.09$, $p=0.30$). We did not observe changes in $STEP_{46}$ phosphorylation or levels after ethanol binge drinking or withdrawal or expression in the DMS (data not shown). Interestingly, the increase in $STEP_{61}$ phosphorylation in response to a drinking session was specific for the DMS and was not detected in the dorsolateral striatum (DLS) (Fig. 2B; $\boldsymbol{t_{(10)} = -1{,}17}$, $p=0.11$) or in the nucleus accumbens (NAc) (Fig. 2C; $t_{(10)}=0{,}81$; $p=0.44$).

Finally, the specificity of the anti-phospho[$Ser^{49/221}$] $STEP_{46/61}$ antibodies was confirmed by testing the level of STEP phosphorylation in the DMS of wild-type (WT) mice vs. STEP KO littermates (Venkitaramani *et al.* 2009). Mice were systemically administered with saline or ethanol (2.5 g/kg), and the DMS was dissected 15 min later, a time point in which Fyn is activated and GluN2B is phosphorylated (Wang et al. 2007). STEP was then immunoprecipitated and the level of phosphorylation was measured as described above. As shown in Supplemental Figure 2, systemic administration of ethanol led to an increase in the phosphorylation of $STEP_{61}$ but not $STEP_{46}$ in the DMS of WT mice but not in STEP KO littermates.

Infection of DMS neurons with a lenti-virus expressing shRNA targeting $STEP_{61}$ produces a specific knockdown of the phosphatase

Next, we hypothesized that if $STEP_{61}$ inhibition is sufficient for ethanol-mediated activation of Fyn/GluN2B, then down-regulation of the phosphatase will result in a further activation of this pathway in response to ethanol. To test this possibility, we generated a lentivirus expressing EGFP and a short hairpin RNA (shRNA) sequence targeting $STEP_{61}$ (Ltv-shSTEP) (Braithwaite et al. 2006), as well as a virus expressing a scrambled STEP shRNA sequence (Ltv-shSCR). Infusion of Ltv-shSTEP or Ltv-shSCR into the DMS of mice led to a high level of virus infection in DMS neurons but not in glia (Fig. 3A), and the levels of $STEP_{61}$ protein were significantly reduced in the DMS of mice infected with the Ltv-shSTEP virus as compared with mice infected with Ltv-shSCR control (Fig. 3B; ($t_{(13)} = 8.06$, $p<0.001$)). Infection of virus was localized to the DMS as knockdown of $STEP_{61}$ was not detected in the neighboring DLS (Fig. 3C; ($t_{(4)} = 1.07$, $p=0.17$)), and $STEP_{61}$ knockdown in the DMS did not alter the levels of Fyn (Fig. 3D; ($t_{(13)} = 0.74$, $p=0.48$)) or GluN2B (Fig. 3D; ($t_{(13)} = -0.27$, $p=0.79$)).

Next, the activation state of Fyn and the phosphorylation of GluN2B were determined in the DMS of mice infected with Ltv-shSTEP or Ltv-shSCR after acute systemic administration of ethanol. Importantly, the lentivirus is known to induce permanent expression of the gene and shRNA of interest in the targeted neuron (Warnock *et al.* 2011). Four weeks after virus infusion into the DMS, animals were administered systemically with ethanol (2 g/kg) or

saline, and 15 min later, the total amount of the phosphatase was measured. As in Fig. 3B, we found a decrease of the total amount of $STEP_{61}$ protein in the DMS of mice infected with the Ltv-shSTEP in comparison with mice infected with Ltv-shSCR control (Fig. 4A: SNK test, $q = 7.09$, $p < 0.001$). Then, Fyn activation was determined using antibodies that recognize the active form of the kinase. As shown in Fig. 4B, and consistent with our previous findings (Wang et al. 2007), acute systemic administration of ethanol increased Fyn activation in the DMS of mice infected with Ltv-shSCR (Fig. 4B: two-way ANOVA analysis showed a significant effect of ethanol treatment ($F_{(1,13)} = 43.61$, $p < 0.001$), no significant main effect of the virus infusion ($F_{(1,13)} = 0.88$, $p = 0.365$), but a significant interaction between virus infusion X ethanol treatment ($F_{(1,13)} = 4.829$, $p = 0.0047$) and *post hoc* SNK test, $q = 4.29$, $p = 0.01$). Importantly, the increase in kinase activity in response to ethanol was significantly enhanced in the DMS of mice infected with Ltv-shSTEP (Fig. 4B: SNK test, $q = 3.06$, $p = 0.05$).

As Fyn activation by ethanol results in GluN2B phosphorylation (Wang et al. 2007), we tested whether DMS knockdown of $STEP_{61}$ increases the phosphorylation of the NMDAR subunit. As shown in Fig. 4C, GluN2B phosphorylation was increased by acute administration of ethanol in the DMS of mice infected with Ltv-shSCR (Fig. 4C: two-way ANOVA showed a significant main effect of the virus infusion ($F_{(1,12)} = 4.84$, $p = 0.048$), a significant effect of ethanol treatment ($F_{(1,12)} = 38.1$, $p < 0.001$) and a significant interaction between virus infusion X ethanol treatment ($F_{(1,12)} = 9.80$, $p = 0.009$) and *post hoc* SNK test, $q = 3.13$, $p = 0.047$). In line with our hypothesis, ethanol-mediated GluN2B phosphorylation in the DMS was further enhanced in mice infected with Ltv-shSTEP as compared with mice infected with Ltv-shSCR (Fig. 4C: SNK test, $q = 5.26$, $p = 0.003$). Together, these results suggest that the down-regulation of $STEP_{61}$ levels (and thus activity) enhances ethanol-mediated activation of Fyn and GluN2B phosphorylation.

Down-regulation of $STEP_{61}$ in the DMS increases ethanol intake and preference

DMS specific inhibition of Fyn and GluN2B activity or knockdown of protein tyrosine phosphatase alpha (PTPalpha), a positive regulator of Fyn, decreases rodents' intake of ethanol (Wang et al. 2010, Ben Hamida *et al.* 2013). Thus, we hypothesized that the down-regulation of $STEP_{61}$ in the DMS will result in an opposite effect on ethanol consumption, e.g., a further increase in intake. To test this possibility, mice had daily access to 10% or 20% ethanol and the level of ethanol and water intake was compared in mice infected with Ltv-shSCR vs. mice infected with Ltv-shSTEP (see timeline in Suppl. Fig. 1B). As shown in Fig. 5A–F, knockdown of $STEP_{61}$ in the DMS produced a significant increase in the consumption and preference of both a 10% and 20% ethanol solutions as compared to mice infected in the DMS with Ltv-shSCR control (Fig. 5A: continuous 10% ethanol. $t_{(20)} = -2.10$, p=0.048; Fig. 5B: water, $t_{(20)} = 1.24$, p=0.23; Fig. 5C: preference, $t_{(20)} = -2.13$, p=0.046; Fig. 5D: continuous 20% ethanol, $t_{(19)} = -2.35$, p=0.029; Fig. 5E: water, $t_{(19)} = 0.84$, p=0.41; Fig. 5F: preference, $t_{(19)} = -2.57$, p=0.019).

Next, we tested whether down-regulation of $STEP_{61}$ levels in the DMS also produce an increase in ethanol intake in the 20% intermittent access two-bottle choice protocol in which mice had access to ethanol every other day. As shown in Fig. 5G–I, knockdown of $STEP_{61}$

in the DMS produced a robust increase of 20% ethanol intake as compared with mice infected with Ltv-shSCR and preference over water (Fig. 5G: intermittent 20% ethanol, $t_{(20)} = -4.54$, $p<0.001$; Fig. 5H: water, $t_{(20)} = 1.81$, $p > 0.08$; Fig. 5I: preference, $t_{(20)} = -2.1$, $p=0.04$).

Finally, we determined whether the increase in ethanol intake and preference in response to STEP knockdown was due to an alteration in consumatory behaviors and/or spontaneous locomotor activity. As shown in Fig. 6A, mice infected with Ltv-shSTEP or Ltv-shSCR consumed similar amounts of saccharin (sweet) ($t_{(20)} = 0.14$, $p > 0.05$) and quinine (bitter) ($t_{(20)} = -0.99$, $p > 0.05$) solutions. Furthermore, no differences in spontaneous locomotor activities were observed between the two groups of mice (Fig. 6B: two-way RM-ANOVA, no effect of Ltv-shSTEP infusion ($F_{(1,220)} = 0.15$, $p > 0.05$), a significant effect of time ($F_{(11,220)} = 31.4$, $p < 0.001$) and no interaction between Ltv-shSTEP infusion X time ($F_{(11,220)} = 0.49$, $p > 0.05$). Together, these results indicate that $STEP_{61}$ knockdown in the DMS of mice produces a specific increase in ethanol intake, which is not due to a general behavioral impairment.

Discussion

We report here that *in vivo* exposure of mice to ethanol results in phosphorylation of $STEP_{61}$ specifically in the DMS. We further show that DMS-specific knockdown of $STEP_{61}$ enhances ethanol-mediated activation of Fyn and GluN2B phosphorylation. Finally, we demonstrate that $STEP_{61}$ knockdown in the DMS increases ethanol intake and preference without altering consumption of saccharin or quinine, or changes in locomotor activity. Together, our results put forward the possibility that ethanol-mediated phosphorylation of $STEP_{61}$ that presumably leads to the inhibition of kinase activity in the DMS, is sufficient for the activation of Fyn/GluN2B that, in turn, facilitates the induction of ethanol-drinking behaviors (model, Fig. 7).

We found that ethanol increases $STEP_{61}$ phosphorylation using specific antibodies that recognize the PKA phosphorylation (Paul *et al*. 2000). Numerous studies showed that PKA is activated by ethanol (reviewed in (Ron & Messing 2013). Thus, the increase of $STEP_{61}$ phosphorylation in response to ethanol exposure is likely to be mediated by PKA.

Interestingly, the increase of phosphorylation of $STEP_{61}$ in response to ethanol was specifically observed in the DMS but not in the other striatal regions, the DLS and the NAc, although all 3 striatal regions are composed of over 90% of GABAergic medium spiny neurons (MSNs) (Meredith *et al.* 1993). This observation is intriguing and the possible mechanism underlying the sub-brain region specificity of ethanol's actions on this pathway is an area of active investigation. One possibility is that the proximity of $STEP_{61}$ to its substrates is different in the DMS vs. DLS or NAc. This possibility stems from previous studies in which we observed that Fyn is compartmentalized via the scaffolding protein RACK1 to GluN2B in the dorsal striatum and hippocampus but not in the NAc or cortex (Wang et al. 2007, Yaka et al. 2003). We further observed that this region specific compartmentalization determines whether or not the Fyn/GluN2B pathway is responsive to ethanol (Wang et al. 2007, Yaka et al. 2003). PKA and its substrates are compartmentalized

in close proximity to each other via the interaction with A-kinase anchoring proteins (AKAPs) (Welch *et al.* 2010). Several AKAPs are localized to the membrane and AKAP75 is found in the PSD (Welch et al. 2010). Thus, it is plausible that differences in the distribution of AKAPs in the subregions of the striatum determine whether or not $STEP_{61}$ is phosphorylated in response to ethanol.

We previously showed that one consequence of Fyn-mediated phosphorylation of GluN2B by ethanol is a long-lasting activation of the channel (Wang et al. 2010). Activation of NMDAR results in the activation of calcium calmodulin protein kinase II (CaMKII), which, in turn, activates the phosphatase calcineurin that dephosphorylates the regulatory serine residue within the kim domain and thus activates STEP (Paul *et* al. 2003). Thus, it is curious that the ethanol-mediated phosphorylation of $STEP_{61}$ is long-lasting and is maintained even after 24 hrs of ethanol withdrawal. One possibility is that calcineurin is inhibited in response to ethanol exposure. Alternatively, it is plausible that $STEP_{61}$ is further inactivated by ubiquitination and degradation (Kurup *et al.* 2010). These possibilities need to be further explored.

We show that drinking of ethanol or withdrawal resulted in a parallel activation of Fyn in the DMS of mice, and that the specific knockdown of $STEP_{61}$ leads to an upregulation of ethanol-mediated activation of Fyn. It should be noted though that the anti-phospho antibodies that recognize the active conformation of Fyn also detect active Src. However, as shown in Supplemental Fig. 3, Src is not activated in the DMS in response to systemic administration or binge drinking of ethanol and these results are in line with previous data showing that *ex vivo* exposure of dorsal striatal or hippocampal slices to ethanol result in Fyn but not Src activation (Yaka et al. 2003, Suvarna *et al.* 2005, Wang et al. 2007). Thus, our results suggest that the consequence of $STEP_{61}$ in the DMS is the specific activation of Fyn. Other $STEP_{61}$ substrates are Pyk2 (Xu *et al.* 2012), and the mitogen activated protein kinase family members (MAPK), extracellular signal-regulated kinase 1/2 (ERK1/2) and stress-activated protein kinase, p38 (Paul et al. 2003). It is therefore plausible that ethanol-mediated inhibition of $STEP_{61}$ in the DMS leads to alteration of function of these $STEP_{61}$ substrates and/or others, and this intriguing possibility will also be examined in future studies.

The specific knockdown of $STEP_{61}$ in the DMS parallels with increased levels of GluN2B phosphorylation. In contrast, using hippocampal slices from STEP KO and WT mice, Hicklin *et al.* reported that a hypnotic dose of ethanol led to a STEP-mediated dephosphorylation of GluN2B, which was associated with the inhibitory actions of ethanol on the NMDAR function (Hicklin *et al.* 2011). The contradicting results could be due to different brain regions, hippocampus vs. DMS, different concentrations of ethanol, as well as the global deletion of STEP during development and adulthood of the mice (Hicklin et al. 2011) vs. a DMS specific knockdown of the phosphatase in adult mice herein.

Importantly, we present evidence suggesting that the activity of $STEP_{61}$ in the DMS is **sufficient** for the **expression** of ethanol intake. Specifically, we show that knockdown of STEP in the DMS robustly and specifically increased ethanol consumption, whereas saccharin, quinine and water intake were unaltered. These findings are in line with previous

data showing that alteration of the level and/or activity of other components of the Fyn/GluN2B pathway in the DMS produce changes in ethanol intake but do not alter consumatory behavior *per se* (Wang et al. 2007, Wang et al. 2010, Ben Hamida et al. 2013). In contrast to the potential role of STEP inhibition in ethanol intake, upregulation of STEP expression is associated with Alzheimer's disease, schizophrenia, Fragile X syndrome (Goebel-Goody et al. 2012) and stress (Paul *et al.* 2007, Yang *et al.* 2012). As such, STEP has been gaining interest as a novel drug target for the treatment of these as well as others (Goebel-Goody et al. 2012). Thus, the possibility that high levels of STEP inhibition could result in potentially undesirable side effects of increased ethanol drinking merits further investigation.

In summary, we present data to suggest that the inhibition of $STEP_{61}$ activity is sufficient for molecular and behavioral adaptations that are centered around the Fyn/GluN2B pathway in the DMS and is sufficient for the expression of ethanol-drinking behaviors. Addiction is characterized by compulsive ethanol or drug taking and seeking and the DMS has been implicated in maladaptive persistent goal-directed behaviors (Everitt *et al.* 2008, Balleine *et al.* 2009). It would therefore be of great interest to determine whether loss of function mutations within the STEP gene are associated with increased propensity to develop alcohol abuse disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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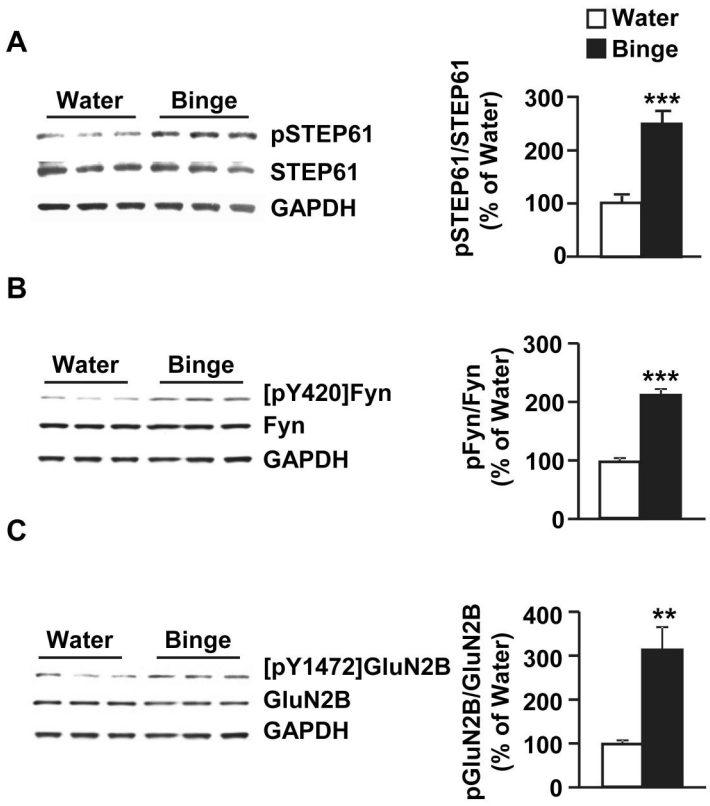

Figure 1. Ethanol consumption increases $STEP_{61}$ inhibition, Fyn activation and GluN2B phosphorylation in the DMS

Mice underwent 6 weeks of intermittent-access 20% ethanol in a two-bottle choice drinking procedure. Control animals underwent the same paradigm but had access to water only. The DMS was removed 4 hrs after the initiation of the last ethanol drinking session (binge). (**A**) The level of $STEP_{61}$ phosphorylation was determined by western blot analysis using anti-[$pSer^{221}$]STEP antibodies. The nitrocellulose membrane was stripped and re-probed with anti-STEP to measure the total level of the phosphatase, and anti-GAPDH antibodies were used as loading control. (**B–C**) Anti-[$pY^{418/420}$]Src/Fyn, and anti-Fyn antibodies (**B**) and anti-[pY^{1472}]GluN2B and anti-GluN2B antibodies (**C**) were used to detect the activated form and the total amount of Fyn (**B**), and the phosphorylated and total amount of GluN2B (**C**), respectively. Optical density of immunoreactivity of phosphorylated-protein bands were normalized to total protein and plotted as percentage of the water only control group. Two-tailed unpaired *t*-test; **p < 0.01 and ***p < 0.001. (**A–C**) n = 6.

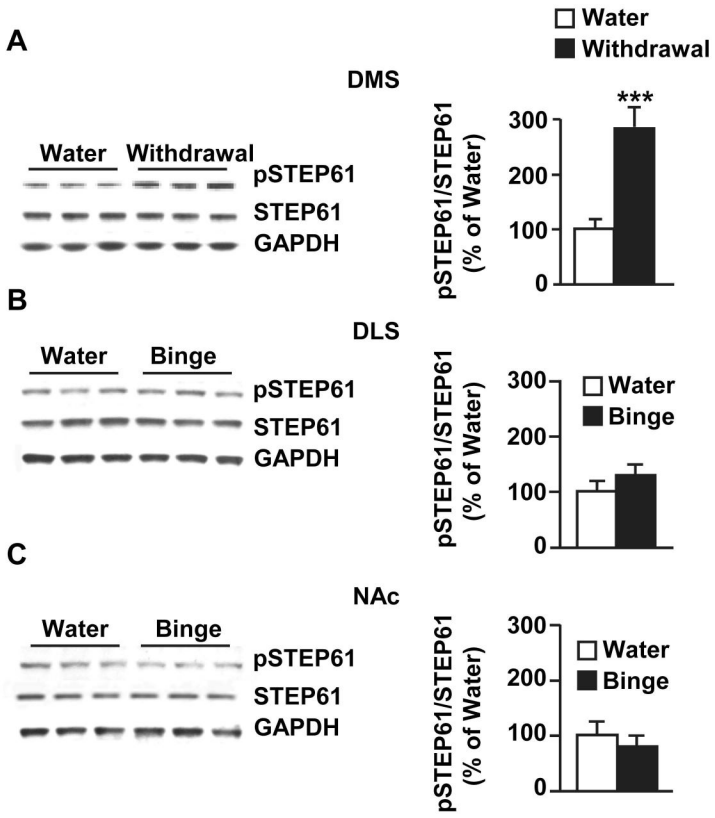

Figure 2. STEP$_{61}$ phosphorylation in the DMS is long-lasting and is not observed in the DLS or NAc

The drinking paradigm was conducted as described in Fig. 1. (**A)** The DMS was dissected 24 hrs after the end of the last ethanol drinking session (withdrawal). **(B–C)** The DLS (**B**), and NAc (**C**) were removed 4 hrs after the initiation of the last ethanol drinking session (binge). **(A–C)** The level of STEP$_{61}$ phosphorylation was determined as described in Fig. 1A. Optical density of immunoreactivity of phosphorylated-protein bands were normalized to total protein and plotted as percentage of the water only control group. Two-tailed unpaired *t*-test; ***p < 0.001. n=6.

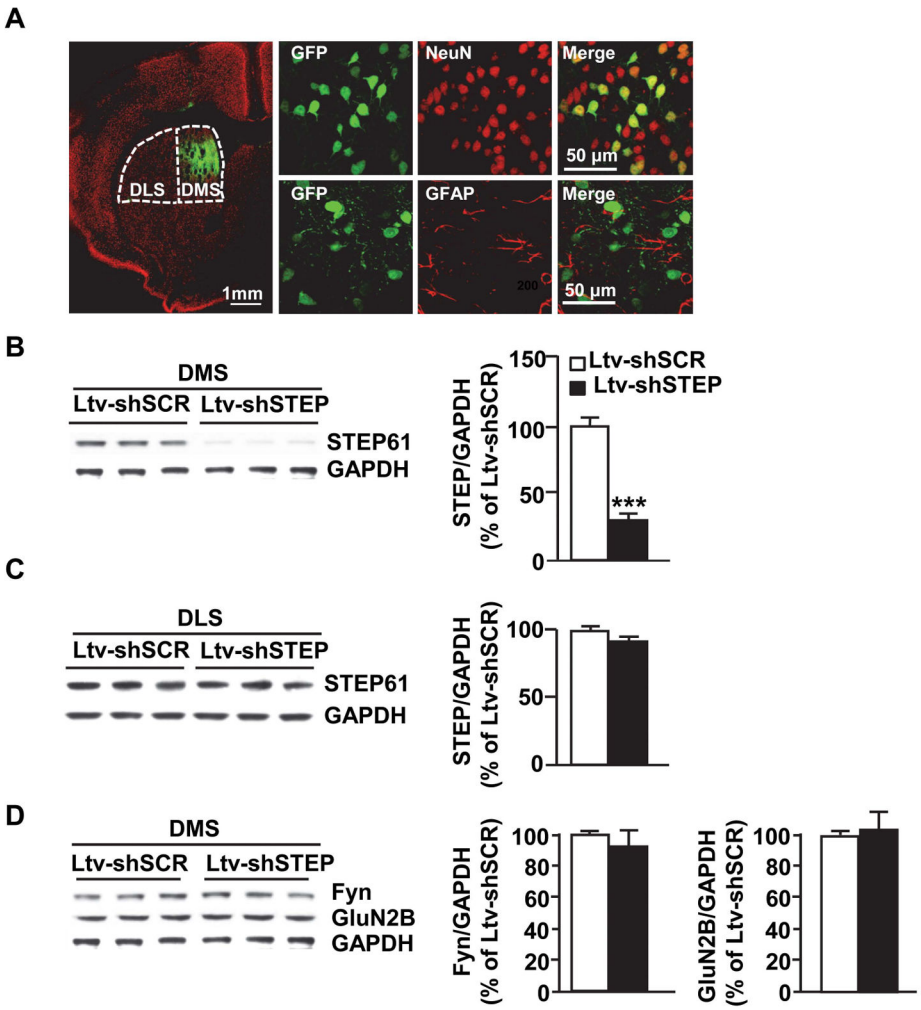

Figure 3. Infection of the DMS of mice with lentivirus expressing short hairpin RNA that targets $STEP_{61}$ produces a knockdown of the protein

Ltv-shSTEP or Ltv-shSCR control were bilaterally infused at a titer of 1.8×10^7 pg/ml into the DMS of mice. Striatal tissues were collected 4 weeks after virus infusions and used for immunohistochemistry (**A**), and western blot analysis (**B–D**). **A**, Ltv-shSTEP infects DMS neurons. Left, depicts the specificity of the site of infection. Slices were co-stained with anti-GFP and anti-NeuN antibodies. Scale bar, 1 mm. Right image depicts Ltv-shSTEP infection of neurons but not glia. Slices were co-stained with anti-GFP and anti-NeuN (top) or anti-GFAP (bottom) antibodies. Scale bar, 50 µm. **B**, Ltv-shSTEP infection decreases $STEP_{61}$ expression in the DMS. The protein level of $STEP_{61}$ was determined by western blot analysis (top panel) and GAPDH immunoreactivity was used as an internal loading control (bottom panel). **C**, Ltv-shSTEP in the DMS does not change the protein level of the phosphatase in the DLS. $STEP_{61}$ protein levels were determined as described in B. **D**, Ltv-shSTEP does not change the protein level of Fyn and GluN2B in the DMS. The level of Fyn (top panel) and GluN2B (middle panel) were determined by western blot analysis, and GAPDH immunoreactivity was used as an internal loading control (bottom panel). The histograms depict the mean ratio of $STEP_{61}$, Fyn or GluN2B/GAPDH ± SEM and data are

expressed as the percentage of control (Ltv-shSCR infected mice). Two-tailed unpaired *t*-test; ***$p < 0.001$. n = 7–8 (**B and D**), n = 3 (**C**).

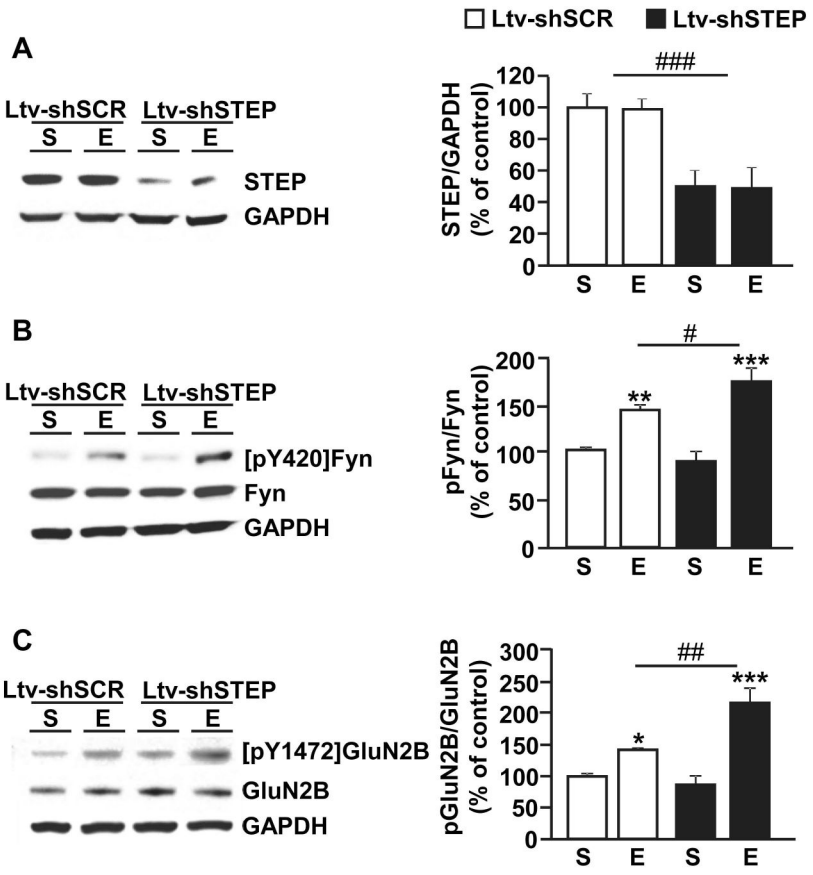

Figure 4. STEP$_{61}$ knockdown in the DMS increases ethanol-mediated Fyn activation and GluN2B phosphorylation

Mice were infused with Ltv-shSCR or Ltv-shSTEP at a titer of 1.8×10^7 pg/ml in the DMS of mice. Four weeks after the virus infusion, animals were treated with an acute administration of saline (S) or ethanol (E, 2.0 g/kg, i.p.) and the DMS was collected 15 min later. Anti-STEP antibodies (**A**), Anti-[pY^{420}]Fyn, and anti-Fyn antibodies (**B**) and anti-[pY^{1472}] GluN2B and anti-GluN2B antibodies (**C**) were used to detect the total amount of STEP (**A**), the activated form and the total amount of Fyn (**B**), and the phosphorylated and total amount of GluN2B (**C**), respectively. Optical density of immunoreactivity of phosphorylated-protein bands were normalized to total protein and plotted as percentage of Ltv-shSCR+Saline treatment. Two-way ANOVA with SNK *post hoc* test (**A**) ###$p < 0.001$ versus Ltv-shSCR (**B**) **$p < 0.01$, ***$p < 0.001$ versus Ltv-shSCR+S and #$p<0.05$ versus Ltv-shSCR+E. (**C**) * $p<0.05$, ***$p < 0.001$ versus Ltv-shSCR+S and ##$p < 0.01$ versus Ltv-shSCR+E. n = 4–5.

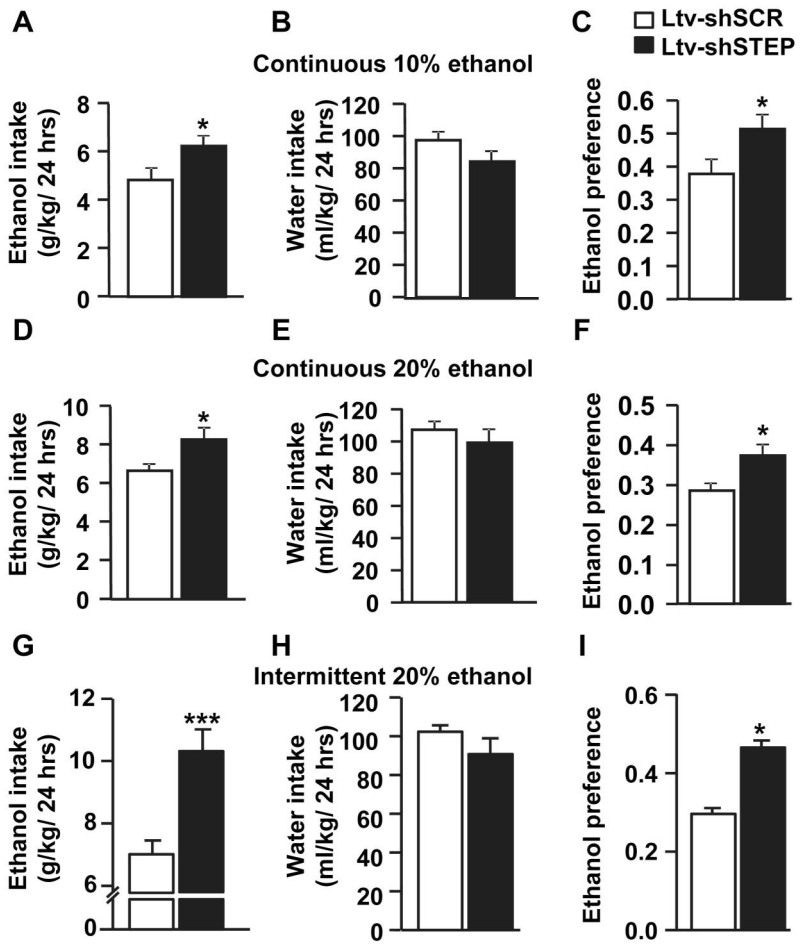

Figure 5. Knockdown of $STEP_{61}$ in the DMS of mice increases ethanol intake and preference

Mice were bilaterally infused in the DMS with Ltv-shSTEP or Ltv-shSCR at a titer of 1.8×10^7 pg/ml, and ethanol intake was determined using a continuous-access 10% ethanol for 6 days (**A–C**), continuous-access 20% ethanol for 6 days (**D–F**), and intermittent-access 20% ethanol for 6 sessions (**G–H**). Intake was averaged for each mouse across all days of access and data are presented as mean ± SEM of ethanol intake per 24 hrs (**A, D and G**), water intake per 24 hrs (**B, E and H**) and ethanol preference (**C, F and I**). Ethanol preference was calculated as the percentage of ethanol solution consumed relative to total fluid intake (ethanol + water). n=10–12

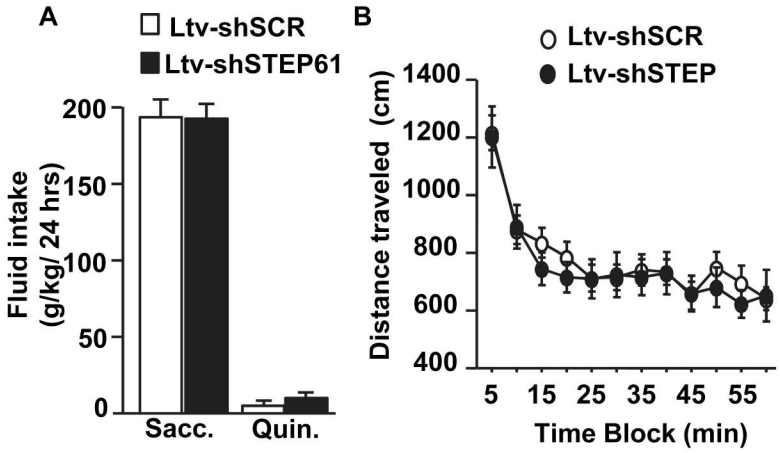

Figure 6. Knockdown of $STEP_{61}$ in the DMS of mice does not alter saccharin or quinine intake or alter spontaneous locomotion

DMS neurons were infected with Ltv-shSTEP or Ltv-shSCR as described in the legends of Fig. 5 (**A**) $STEP_{61}$ knockdown in the DMS does not alter saccharin or quinine intake. Experiments were conducted one week after the last ethanol drinking session. Saccharin (0.066%) or quinine (0.06 mM) solution was each provided for 3 successive days. Data are presented as average of daily saccharine or quinine intake. (**B**) $STEP_{61}$ knockdown in the DMS does not alter spontaneous locomotor activity. Experiments were conducted one week after the last quinine drinking session. Mice were placed in locomotor activity chambers and the distance traveled was recorded for 60 min. Data are presented as cumulative locomotor activity (cm) during the testing period. Data are expressed mean ± SEM. n=10–12.

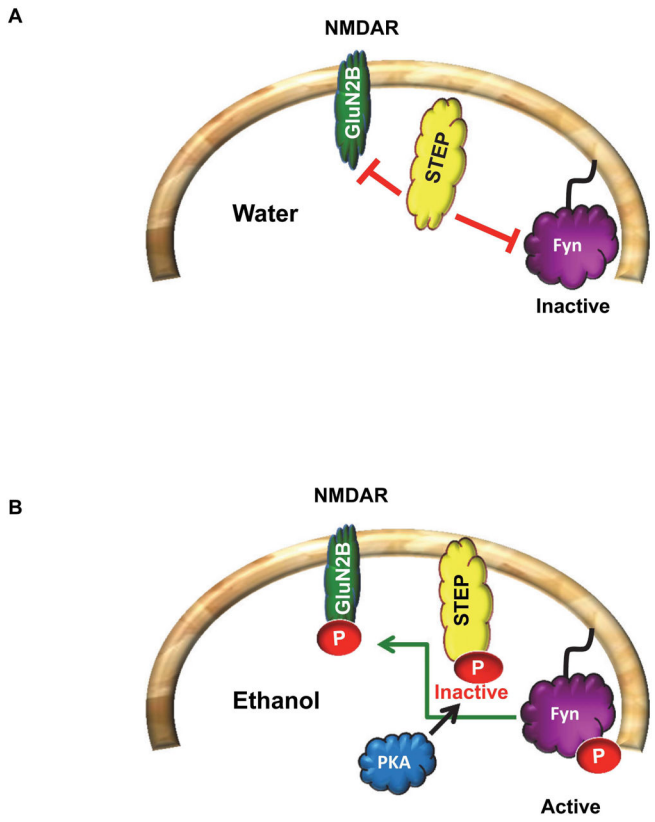

Figure 7. Ethanol-mediated inhibition of $STEP_{61}$ in the DMS leads to Fyn activation and GluN2B phosphorylation

(**A**) Under basal conditions, active $STEP_{61}$ inhibits Fyn activity and dephosphorylates GluN2B. (**B**) Ethanol leads to the phosphorylation of $STEP_{61}$ on a specific inhibitory site. The inhibition of $STEP_{61}$ activity contributes to the activation of Fyn in response to ethanol, which, in turn, phosphorylates GluN2B. These molecular adaptations in the DMS promote ethanol drinking.