Ethanol Alters Trafficking and Functional N-Methyl-d-aspartate Receptor NR2 Subunit Ratio via H-Ras*

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The N-methyl-d-aspartate receptor (NMDAR) plays a critical role in synaptic plasticity and is one of the main targets for alcohol (ethanol) in the brain. Trafficking of the NMDAR is emerging as a key regulatory mechanism that underlies channel activity and synaptic plasticity. Here we show that exposure of hippocampal neurons to ethanol increases the internalization of the NR2A but not NR2B subunit of the NMDAR via the endocytic pathway. We further observed that ethanol exposure results in NR2A endocytosis through the activation of H-Ras and the inhibition of the tyrosine kinase Src. Importantly, ethanol treatment alters functional subunit composition from NR2A/NR2B to mainly NR2B-containing NMDARs. Our results suggest that addictive drugs such as ethanol alter NMDAR trafficking and subunit composition. This may be an important mechanism by which ethanol exerts its effects on NMDARs to produce alcohol-induced aberrant plasticity.

NMDARs are major mediators of processes such as synaptic plasticity and learning and memory (1) and of pathological states such as schizophrenia, seizures, pain, drug and alcohol addiction (2–5), and alcohol intoxication (6).

NMDARs are heteromeric ligand-gated ion channels composed of an obligatory NR1 subunit and regulatory NR2A-D and NR3 subunits (2). The NMDAR subunits can undergo lateral movement between synaptic and extrasynaptic sites, as well as internalization from, or forward trafficking to, the plasma membrane (7, 8). For example, prolonged inhibition of NMDAR activity results in the redistribution of the subunits from extrasynaptic to synaptic sites (9). Synaptic activity and long-term potentiation increase the forward trafficking of NMDAR subunits (10, 11), and glycine has been reported to prime the NMDAR subunits for internalization (12).

NMDAR function can be regulated by changes in the phosphorylation state of the subunits via the activation of kinases or phosphatases (13). Protein phosphorylation and dephosphorylation also control the movement of NMDAR subunits to and from the plasma membrane. For example, activation of protein kinase C and cAMP-dependent protein kinase A, and the consequent phosphorylation of the NR1 subunit, increases forward trafficking of the receptor (14). Fyn kinase-mediated tyrosine phosphorylation of the NMDAR plays a role in the dopamine D1 receptor-mediated redistribution of NMDAR subunits from intracellular to postsynaptic compartments (15). Conversely, tyrosine dephosphorylation of the NR2A subunits leads to the internalization of the subunits, resulting in the rundown of channel activity (16). In addition, we recently found that H-Ras-mediated inhibition of Src kinase activity prevents the phosphorylation and membrane retention of NR2A subunits (17).

We, and others, reported that the activity of the NMDAR in the presence of ethanol is modulated via changes in the phosphorylation state of the subunits (18–20). NMDARs have been implicated in mediating ethanol-associated phenotypes such as tolerance, dependence, withdrawal, craving and relapse, and intoxication (5, 6); however, the mechanisms by which the actions of ethanol on the NMDAR underlie these phenotypes are not well understood. Because changes in the phosphorylation state of NMDAR subunits are important for both the trafficking of NMDARs and the actions of ethanol, we hypothesized that the modulation of NMDAR activity by ethanol may be due to changes in the trafficking of the subunits of the receptor. We found that, in the hippocampus, acute exposure to ethanol specifically increases the internalization of the NR2A subunit via ethanol-mediated activation of H-Ras. Importantly, as a consequence of the NR2A internalization, the remaining NR2B-containing NMDARs are predominant in mediating the excitatory postsynaptic currents (EPSCs) in the presence of ethanol.

EXPERIMENTAL PROCEDURES

Materials—The anti-NR2A, anti-NR2B, anti-actin antibodies, and all secondary antibodies, were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Protease inhibitor tablets were purchased from Roche Applied Science. Anti-GluR1 antibodies were purchased from Chemicon (Temecula, CA). Raf-1-Ras binding domain glutathione S-transferase agarose beads, H-Ras dominant-negative cDNA, Anti-Src, anti-TrkB, and pan anti-Ras antibodies were purchased from Upstate Biotechnologies (Lake Placid, NY). Anti-[pY418] Src was purchased from BIOSOURCE (Camarillo, CA). The cross-linking reagent bis(sulfosuccinimidyl)suberate (BS3) and protein determination kit (bicinchoninic acid assay (BCA)) were purchased from Pierce. Chymotrypsin was purchased from Worthington Biochemicals (Lakewood, NJ), and 4-(2-aminoethyl)benzenesulfonylfluoride (E64f) was purchased from Cal-

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† The abbreviations used are: NMDAR, N-methyl-d-aspartate receptor; EPSC, excitatory postsynaptic current; BS3, bis(sulfosuccinimidyl)suberate; BCA, bicinchoninic acid; E64f, 4-(2-aminoethyl)benzenesulfonylfluoride; AP-2, adaptor protein complex 2; DN, dominant negative; IP, immunoprecipitation.
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biochem. Lipofetamine Plus and Dulbecco's modified Eagle's medium were purchased from Invitrogen. Fetal bovine serum was purchased from HyClone (Logan, UT). Dexamethasone and phosphatase inhibitor mixtures were purchased from Sigma (St. Louis, MO). Ketamine was purchased from Abbott Laboratories (North Chicago, IL). L-(1-25)-cells stably transfected with NR1 plus NR2A or NR1 plus NR2B were a generous gift from Merck Sharp and Dehme, and PSD-95-GFP cDNA was synthesized by Syn Pep (Dublin, CA). The purity of the peptide was >90%, and the integrity was determined by mass spectroscopy. Mice—C57BL/6J; (B6.129S7-Fyntm1Sor) mice were purchased from Jackson Laboratories. The mean age of animals used for biochemical studies was 10–12 weeks. Experimental protocols involving the use of vertebrate animals were approved by the Gallo Research Center subcommittee on Research Animal Care and met National Institutes of Health guidelines.

Preparation of Tat-H-Ras Dominant Negative (DN) Fusion Protein—PTX sensitized DN was expressed in, and purified from, Escherichia coli as previously described (17).

Cell Culture—L-(1-25)-were cultured on 100-mm plates. When cells reached 75% confluency, they were transfected with 5 μg of PSD95-GFP cDNA using Lipofetamine Plus in accordance with the manufacturer's instructions. 24 h after transfection, the expression of the NMDAR cDNA using Lipofectamine Plus in accordance with the manufacturer's instructions. Homogenates were obtained as described in a previous study (22). The expression of NR1 and PSD-95 was routinely verified (data not shown).

Preparation of Hippocampal Slice Homogenates—Transverse hippocampal slices (300 μm) were prepared from 10- to 12-week-old C57BL/6J mice. Slices were allowed to recover for 90 min in artificial cerebrospinal fluid (aCSF) saturated with 95% O2/5% CO2 containing (in mM): 126 NaCl, 1.2 KCl, 1.2 NaH2PO4, 1.2 MgCl2, 2.4 CaCl2, 18 NaHCO3, and 11 glucose. Following recovery, slices were treated with aCSF (control) or ethanol at room temperature, after which the slices were rinsed in cold aCSF, and used either for cross-linking experiments, for Ras activity assay, or for immunoprecipitation. In experiments using the cross-linking reagent BSA for 45 min at 4 °C while shaking. Slices were then rinsed three times with cold aCSF containing 20 mM Tris (pH 7.6), followed by sonication in cold homogenization buffer (10% SDS, 10 mM EDTA, 100 mM Tris (pH 8.0), and protease and phosphatase inhibitor mixtures). The protein concentration was determined using the BCA assay, followed by SDS-PAGE and Western blot analysis. Data are expressed as the ratio of normalized surface exposed or internalized receptors to the total receptor levels.

Chymotrypsin experiments were performed as described previously (22), with minor modifications. Following recovery, slices were treated with aCSF or 0.5 mg/ml chymotrypsin at 32 °C for 5 min. The chymotrypsin reaction was stopped by three washing steps in aCSF containing 20 mM Tris (pH 8.0), and protease and phosphatase inhibitor mixtures). The protein concentration was determined using the BCA assay, followed by SDS-PAGE and Western blot analysis.

Ras Activity Assay—The treated hippocampal homogenates (250 μg) were incubated with 15 μl of Raf-1-Ras binding domain glutathione S-transferase (GST) for 45 min at 4 °C while rotating, and then the manufacturer's instructions. The beads were then washed three times with lysis/wash buffer (provided by the manufacturer), resuspended in 2× sample buffer, resolved by SDS-PAGE, and analyzed by Western blot with pan anti-Ras antibodies.

Immunoprecipitation—The treated slices were homogenized in ice-cold buffer containing 1% deoxycholate, protease, and phosphatase inhibitors and (in mM): 250 sucrose, 20 Tris-HCl (pH 7.5), 2 EDTA and 10 EGTA. 500 μg of the protein was diluted with 1× immunoprecipitation buffer (1% Triton X-100 and (in mM): 150 NaCl, 10 Tris HCl (pH 7.4), 1 EDTA, 1 EGTA, and 0.2 sodium orthovanadate) and incubated with the appropriate antibody, followed by an overnight incubation at 4 °C with protein G-agarose beads. The beads were extensively washed, re-suspended in 2× sample buffer, and boiled for 5–10 min. The samples were then subjected to SDS-PAGE followed by Western blotting with the appropriate antibodies.

Western Blot Analysis—Proteins were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. After blocking, the membranes were incubated with the specific primary antibody, followed by horseradish peroxidase-conjugated secondary antibody. Detection was obtained following enhanced chemiluminescent reaction (Amersham Biosciences), and processed using the Storm PhosphorImager (Amersham Biosciences). Results were quantified by Image 1.61 (National Institutes of Health).

Electrophysiology—Electrophysiological recordings were performed in C57BL/6J mice ranging from P21 to P26. Briefly, mice were anesthetized with halothane and decapitated as per the University of California, San Francisco animal care guidelines. Horizontal sections of the hippocampus (230–300 μm) were prepared with a vibratome (Leica, Nussloch, Germany). Slices were placed in a holding chamber and allowed to recover for at least 1 h before being placed in the recording chamber and superfused with aCSF saturated with 95% O2/5% CO2 and 5% CO2 (in mM): 119 NaCl, 1.6 NaHCO3, 2.6 KCl, 10 Glucose, 2.5 CaCl2 (for EPSCs), or 0.01 MgCl2 (for field excitatory postsynaptic potentials (fEPSPs)), 2.5 CaCl2, 26.2 NaHCO3 and 11 glucose. Picrotoxin (100 μM) was added to block α-amino-3-hydroxy-5-methyl-4-isoxalepropionate (AMPA)kainate receptors. Cells were visualized using infrared differential interference contrast video microscopy.

Whole-cell voltage-clamp recordings were made using an Axopatch 1D or a MultiClamp 700A amplifier (Axon Instruments, Union City, CA). Electrodes (3.5–5.0 MΩ) contained (in mM): 120 cesium methanesulfonate, 20 Hepes, 0.4 EGTA, 8 NaCl, 5 TEA-Cl, 2.5 MgATP, and 0.25 NaGTP (pH 7.2–7.3), buffer at 270–285 mosM. Series resistance (10–30 MΩ) and input resistance were monitored on-line with a -4-mV depolarizing step (50 ms) given just after every afferent stimulus. Recordings from the CA1 region of the hippocampus were obtained with a bipolar stimulating electrode that was placed 100–300 μm rostral to the recording electrode and was used to stimulate excitatory afferents at 0.1 Hz. Neurons were voltage-clamped at +40 mV to evoke NMDAR-mediated EPSCs. At this potential, NMDARs have a maximal open probability, and the Mg2+ block is removed. EPSCs were filtered at 2 kHz, digitized at 5–10 kHz, and collected on-line using Igor Pro software (Wavemetrics, Lake Oswego, OR) or pClamp 9 (Axon Instruments). NMDAR example traces were constructed by averaging 20 EPSCs (200 s) elicited at +40 mV during baseline, ethanol application, washout, or drug application in the presence or absence of ethanol. To calculate the decay time (τ), Igor Pro software was used to fit exponential decay curves to averaged NMDAR-mediated EPSCs using the formula: y = A.exp(−invTau,x)+A.exp−invTau,x.

fEPSPs were recorded using a MultiClamp 700A amplifier (Axon Instruments) with whole-cell patch pipettes filled with 1 M NaCl and 25 mM HEPES. To evoke fEPSPs, Schaffer collateral/commissural afferents in hippocampal CA1 region were stimulated with 0.1-Hz pulses using steel bipolar microelectrodes at intensities adjusted to produce an evoked response that was 50% of the maximal recorded fEPSP for each recording. fEPSPs were filtered at 1 kHz, digitized at 10 kHz, and collected on-line using with pClamp 9 (Axon Instruments). NMDAR traces were constructed by averaging 6 fEPSPs (1 min) during the whole recording.

Statistical Analysis—For biochemistry experiments, results were obtained from at least three different animals as indicated in the figure legends. For electrophysiology experiments, n values represent the number of individual slices from at least three individual animals as indicated in the figure legends. Data are expressed as the mean ± S.D. (biochem) or S.E. (electrophysiology) and were analyzed using a Student’s t-test or one-way ANOVA followed by a Newman-Keuls post-hoc test.

RESULTS

Acute Ethanol Exposure Results in NR2A Subunit Internalization—To investigate whether acute ethanol treatment alters...
the surface expression of the NMDAR subunits, we utilized the membrane-impermeable cross-linking agent BS3. The cross-linker produces high molecular weight aggregates of the surface receptors that cannot enter the SDS-PAGE gel, and therefore the intracellular pool of receptors can be quantified (22). Incubation of hippocampal slices with ethanol (100 mM) did not alter the membranal localization of the NR2B subunit (Fig. 1a, left, lanes 2 versus 4) but led to a significant increase in the intracellular level of NR2A, which was accompanied by a corresponding decrease in surface expression of the subunit (Fig. 1a, right, lanes 2 versus 4). These changes were not due to alteration in the expression level of NR2A (Fig. 1b, lanes 1

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FIG. 1. Ethanol alters membrane surface expression of NR2A subunits. a, hippocampal slices were treated with aCSF (Ctl) or with 100 mM ethanol for 15 min, at room temperature followed by treatment with aCSF (−) = total, lanes 1 and 3) or cross-linker (BS3 = internalized pool, lanes 2 and 4) as described under "Experimental Procedures." Samples were analyzed by Western blot with anti-NR2B (left), anti-NR2A (right), and anti-actin antibodies. *, p < 0.05, n = 4 (one-way ANOVA). b, Li/tk− cells expressing (left) NR1 plus NR2B or (right) NR1 plus NR2A were treated with aCSF (Ctl) or with 100 mM ethanol for 15 min, at room temperature followed by treatment with aCSF (−) = total, lanes 1 and 3) or cross-linker (BS3 = internalized pool, lanes 2 and 4). Samples were analyzed by Western blot with anti-NR2B (left), anti-NR2A (right), and anti-actin antibodies. *, p < 0.05, n = 6 (Student’s t test). c and d, hippocampal slices were treated with aCSF (Ctl) or with 100 mM ethanol for 15 min, at room temperature followed by treatment with aCSF (−) = total, lanes 1 and 3) or cross-linker (BS3 = internalized pool, lanes 2 and 4) as described under "Experimental Procedures." Samples were analyzed by Western blot with anti-NR1 (c), anti-GluR1 (d), and anti-actin antibodies (c and d), n = 4. e, hippocampal slices were treated with aCSF (Ctl) or with 25 or 50 mM ethanol for 15 min at room temperature followed by treatment with aCSF (−) = total, lanes 1, 3, and 5) or cross-linker (BS3 = internalized pool, lanes 2, 4, and 6). Samples were analyzed by Western blot with anti-NR2A and anti-actin antibodies. **, p < 0.01, n = 3–5 (one-way ANOVA). f, hippocampal slices were treated with aCSF (Ctl) or with 100 mM ethanol for 15 min at room temperature. After ethanol treatment, slices were washed twice with aCSF, and then recovered for an additional 15 min, and treated with aCSF (−) = total, lanes 1 and 3) or cross-linker (BS3 = internalized pool, lanes 2 and 4). Samples were analyzed by Western blot with anti-NR2A and anti-actin antibodies. Control versus ethanol, **, p < 0.01; Eth versus washout, #, p < 0.01, n = 7 (one-way ANOVA). g, hippocampal slices treated with aCSF (Ctl) or with 100 mM ethanol for 15 min at room temperature followed by treatment with aCSF (−) = total, lanes 1 and 3) or chymotrypsin (Chy = internalized pool, lanes 2 and 4). Samples were analyzed by Western blot with anti-NR2A and anti-actin antibodies. **, p < 0.01, n = 3 (Student’s t test). For all experiments, the band intensity of NR2 subunits was normalized to actin, and histogram shows ratios of the internalized (BS3 or Chy) to total NR2A or NR2B plotted as means ± S.D. The n values are the number of individual experiments from individual animals. There were no changes in the total amount of protein in the samples, and equal amounts of proteins were loaded in each lane.
versus 3). To confirm that the ethanol-mediated effect on the subcellular localization of the NMDARs is specific for the NR2A subunit, we repeated the cross-linking experiments in mouse L(tk−) cell lines that stably express either the NR2A plus NR1 or NR2B plus NR1 under the control of a dexamethasone-inducible promoter (21). To ensure that the NMDARs were properly compartmentalized, the cells were also transiently transfected with the scaffolding protein postsynaptic density protein 95 (PSD-95). As shown in Fig. 1b, ethanol exposure resulted in an increase in the internalized pool of the NR2A subunit (Fig. 1b, right, lanes 2 versus 4), but not NR2B (Fig. 1b, left, lanes 2 versus 4) subunits. In addition, no change was observed in the surface expression of the NR1 subunit of the NMDAR, or the GluR1 subunit of the AMPAR (Fig. 1, c and d, lanes 2 versus 4). Next, we determined whether the ethanol-mediated NR2A internalization could still be detected at lower concentrations of ethanol and whether the effect could be reversed upon ethanol washout. We found that ethanol concentrations as low as 25 mM induced the reduction in the surface expression of the subunit and a corresponding increase in the immunoreactivity of the subunit in the intracellular compartment (Fig. 1e, lanes 2 versus 4 and 6). This change in the subcellular localization of NR2A was not observed after ethanol was treated for 15 min, and then washed out from the slice preparation, and the slices were allowed to recover for an additional 15 min (Fig. 1f, lanes 4 versus 6). Finally, we confirmed our results using another independent method. Following aCSF or ethanol treatment, the slices were treated with the serine protease, chymotrypsin, which is excluded from the interior of the cell due to its large size. Therefore, chymotrypsin cleaves the surface receptors, and only the intracellular pool of receptors is then detected by Western blot analysis (22). This method has previously been used to show long-term potentiation-mediated changes in surface expression of the NMDAR subunits (10). In Fig. 1g (lanes 2 versus 4), we demonstrate that ethanol exposure significantly increased the internalization of the NR2A subunits, however, ethanol had no effect on the surface or intracellular levels of NR2B (data not shown). Taken together, our results show that in hippocampal slice preparation, ethanol treatment results in the specific internalization of the NR2A subunit of the NMDARs.

Ethanol Exposure Increases the Association between the NR2A Subunit and the AP-2 Adaptor Protein Complex—The intracellular tails of the NR2 subunits contain internalization motifs that mediate clathrin-dependent receptor endocytosis (23). To determine if ethanol exposure potentiates the endocytic pathway to internalize NR2A subunits, we tested whether in the presence of ethanol, the subunit interacts with adaptin β2 (a component of the adaptor protein complex 2 (AP-2) involved in clathrin-dependent receptor endocytosis). Similar to glycine, which was recently shown to prime the receptor for internalization (12) (Fig. 2a, lane 3), exposure to ethanol (100 mM, 15 min) significantly increased the association of adaptin β2 with NR2A and vice versa (Fig. 2a, lanes 1 versus 2). To address the question of specificity of adaptin interaction with the NR2A, we conducted similar studies in the mouse L(tk−) cells that express either the NR1 plus NR2A (Fig. 2b, left) or NR1 plus NR2B (Fig. 2b, right) subunits together with PSD-95. Ethanol treatment resulted in an increase in the association of NR2A with adaptin β2 (Fig. 2b, left, bottom panel), but no change in NR2B-adaptin association was observed (Fig. 2b, right, bottom panel). Finally, to determine if the association with adaptin β2 is specific for an NMDAR subunit, we tested whether ethanol alters the association of adaptin β2 with the AMPAR subunit GluR1, and the brain derived neurotrophic factor receptor subunit TrkB, and found no change in the interaction between these proteins and adaptin β2 (Fig. 2c, middle and low panels). These results suggest that ethanol treatment caused the recruitment of the endocytic machinery to the NMDAR, leading to NR2A endocytosis.

**Fig. 2.** Ethanol exposure increases the association of the NR2A subunits with adaptin β2. a, hippocampal slices were treated with aCSF (control, lane 1), 100 mM ethanol for 15 min at room temperature (lane 2), or 100 μM glycine for 5 min (lane 3). Slices were homogenized as described under “Experimental Procedures,” and NR2A and adaptin β2 were immunoprecipitated (IP) with 5 μg of anti-NR2A (left), or anti-adaptin β2 (right) antibodies (top panels, IP). Co-immunoprecipitations of adaptin β2 (left) or NR2A (right) were determined by immunoblotting with anti-adaptin β2 or anti-NR2A antibodies (bottom panels, IB). Data are expressed as the ratio of NR2A-adaptin β2 association to the IP of either NR2A or adaptin β2 and calculated as the percent increase in NR2A-adaptin β2 association over control and plotted as means ± S.D. *, p < 0.05, n = 4 (Student’s t test). b, L(tk−) cells expressing NR1 plus NR2A (left) or NR1 plus NR2B and PSD95 (right) as described under “Experimental Procedures” were treated with aCSF (control, lane 1) or 100 mM ethanol for 15 min at room temperature (lane 2). The cells were homogenized as described under “Experimental Procedures,” and NR2A, or NR2B were immunoprecipitated with 5 μg of anti-NR2A (left) or NR2B (right) antibodies (top panels, IP). Co-immunoprecipitation of adaptin β2 was determined by immunoblotting with anti-adaptin β2 antibodies (bottom panels, IB), n = 3. c. hippocampal slices were treated with aCSF (lane 1) or 100 mM ethanol (lane 2) for 15 min at room temperature. Slices were homogenized, and adaptin β2 was immunoprecipitated with anti-adaptin β2 antibodies (top panel, lanes 1 and 2). Co-immunoprecipitations of TrkB (middle panel), or GluR1 (bottom panel) were determined by immunoblotting with anti-TrkB and antiGluR1 antibodies n = 3. Also shown are the input (lane 3), and a control for the immunoprecipitation experiments (lane 4). The n values for all the experiments are the number of individual experiments from individual animals. There were no changes in the total amount of protein in the samples.
ethanol-mediated NR2A internalization. Tyrosine dephosphorylation of NR2A subunits can lead to the internalization and rundown of NMDAR activity (16), and we recently observed that the surface expression of the NR2A but not the NR2B subunit is negatively regulated via the association of the small GTP-binding protein H-Ras with Src kinase, and consequent inhibition of Src activity (17). Both Src and H-Ras are important regulators of NMDAR function and synaptic plasticity (24–26). We therefore hypothesized that, in the presence of ethanol, NR2A is internalized as a result of H-Ras activation and the subsequent inhibition of Src activity that prevents the tyrosine phosphorylation of the subunit.

To measure H-Ras activity, we determined the level of GTP-bound active Ras association with the Raf-1-Ras binding domain, in the presence or absence of ethanol. Similar to KCl treatment (Fig. 3a, lanes 1 versus 3), which increases Ras activity, ethanol exposure (100 mM, 15 min) increased Ras-GTP levels by at least 2-fold over the control (Fig. 3a, lanes 1 versus 2), suggesting that ethanol treatment elevates total Ras activity. The Ras activity assay does not differentiate between the Ras family members (H-Ras, K-Ras, and N-Ras). However, because in the adult mouse brain, H-Ras expression is two to five times higher than the other isoforms (27), we assume that at least part of the Ras activity detected in the presence of ethanol is that of H-Ras. We reasoned that if H-Ras activity is important for NR2A internalization, then inhibiting H-Ras should prevent the actions of ethanol on NR2A endocytosis. To inhibit H-Ras activity, we transduced the dominant negative form of H-Ras (H-Ras DN) into hippocampal slices using the Tat fusion protein transduction system (28). The H-Ras DN sequesters the guanine nucleotide exchange factors necessary for Ras activation and thereby inhibits the activation of endogenous Ras (29). First, we determined whether Tat-H-Ras DN inhibits the ethanol-mediated increase in endogenous Ras activity. As shown in Fig. 3b (lanes 2 versus 3), preincubation of hippocampal slices with 2 μM Tat-H-Ras DN decreased endogenous Ras activity, but the Tat-peptide (2 μM), which was used as a control, did not (Fig. 3b, lanes 2 versus 4). Next, measured the association of the NR2A subunits to adaptin β2 in the absence or presence of Tat-H-Ras DN. Tat-H-Ras DN alone had no effect on the interaction between NR2A and adaptin β2 or adaptin β2 or vice versa (Fig. 3c, lane 3); however, Tat-H-Ras DN blocked the ethanol-mediated association of adaptin β2 with NR2A or adaptin β2 (Fig. 3c, lanes 2 versus 4), and ethanol-mediated internalization of the NR2A subunits (Fig. 3d, lanes 4 versus 8). Taken together, these results suggest that the ethanol-mediated activation of H-Ras is required for NR2A internalization.

Src Inhibition Is Necessary for the Internalization of NR2A—We previously found that active H-Ras interacts with Src to inhibit the activity of the kinase, resulting in the reduction of phosphorylation and surface expression of NR2A (17). To test whether Src is inhibited in the presence of ethanol, we measured the activity of the kinase in the absence or presence of ethanol by immunoprecipitating Src and subsequently detected the level of the enzyme with anti-pY418Src antibody that recognizes only the kinase in its active state. Following ethanol exposure, there was an ~40% decrease in the levels of active phosphorylated Src (Fig. 4a, lanes 1 versus 2), suggesting that, in the presence of ethanol, Src kinase activity is indeed inhibited. Next, to determine whether the ethanol-mediated NR2A internalization was dependent upon the inhibition of Src, we tested the surface expression of NR2A in the presence or absence of ethanol in hippocampal slices from Src heterozygote mice (Src+/−), which express 50% of the Src gene, and their corresponding littermate controls (Src+/+). Similar to the results obtained from C57BL/6J mice (Fig. 1b), ethanol significantly increased the internalization of the NR2A subunits in Src+/+ mice (Fig. 4b, left, lanes 2 versus 4). In contrast, NR2A was internalized under basal conditions in Src+/− mice, and no additional changes were observed upon exposure to ethanol (Fig. 4b, right, lanes 2 versus 4), suggesting that the reduction of Src expression occludes ethanol-mediated internalization of NR2A. To determine whether ethanol-induced internalization is mediated specifically via the inhibition of Src, the experiment was repeated in Fyn−/− and Fyn+/+ mice. Ethanol exposure resulted in a significantly enhanced NR2A internalization in both the Fyn+/+ and Fyn−/− mice (data not shown, and Fig. 4c, lanes 2 versus 4). These results suggest that the inhibition of Src, but not of Fyn, activity is necessary for the internalization of the NR2A subunit in the presence of ethanol.

Characterization of the Actions of Ethanol on NMDAR-mediated Activity in the CA1 Region of the Hippocampus—It is well established that ethanol exposure inhibits NMDAR-mediated activity in neuronal preparations (30–32). Similarly, we found that bath application of 80 mM ethanol inhibited NMDAR-mediated fEPSPs (47 ± 7%, n = 11, Fig. 5, a and b, white circles and white bar) and EPSCs (37 ± 7%, n = 9, data not shown) in the hippocampus. In line with these previous studies (30–32) and others, incubation with low ethanol concentration (25 mM) inhibited NMDAR-mediated-EPSCs by 24 ± 6% (n = 18) (data not shown), and as has previously been reported (18, 20, 33–35), we observed an acute desensitization to the inhibitory effects of ethanol during field-recording of channel activity (Fig. 5a).

Inhibition of H-Ras Reduced the Inhibitory Actions of Ethanol on NMDAR-mediated fEPSP—We hypothesized that ethanol-induced internalization of the NR2A subunit via the activation of H-Ras contributes to the inhibitory actions of ethanol on the NMDAR in the hippocampus. NMDAR-mediated fEPSPs were therefore measured in the presence and absence of ethanol and in the presence and absence of Tat-H-Ras DN. We found that ethanol-mediated inhibition of fEPSPs was reduced by preincubation of the slices with 1 μM Tat-H-Ras DN (22 ± 8%, n = 8, *p < 0.05, Fig. 5, a and b, black circles and black bars), suggesting that H-Ras-mediated internalization of the NR2A subunit contributes to the inhibitory effects of ethanol on NMDAR function.

Ethanol Exposure Alters the Decay Time of the NMDARs—Because ethanol exposure decreased the surface expression of the NR2A but not NR2B subunits, we hypothesized that the internalization of NR2A in the presence of ethanol would reduce the contribution of this subunit to the activity of the NMDARs. Alteration in NMDAR subunit composition is reflected by a specific change in the decay time of evoked NMDAR-mediated EPSCs; in particular, the NR2A-containing NMDAR exhibits a faster decay time relative to the NR2B-containing NMDAR (2, 36). Therefore, we examined whether ethanol would slow the decay of NMDAR EPSCs. As predicted, the average values of the fast and slow components of the decay-time constant (τf and τs, respectively) of the NMDAR EPSCs were slower after 15- or 30-min application of 80 mM ethanol (Table I and Fig. 5, a and d). Ethanol slowed the weighted decay time by 33 ± 14% (τf = 62 ± 10 ms, n = 6) after 15 min and 70 ± 29% (τs = 78 ± 14 ms; n = 6; *, p < 0.05) after 30 min compared with the control (τf = 47 ± 7 ms; n = 6; *, p < 0.05) (Fig. 5, c and d), suggesting that the contribution of the NR2A-containing NMDARs to the activity of the channel is reduced.

Ethanol Treatment Alters NMDAR Subunit Composition of Functional Channels—If the inhibition of channel activity is due to ethanol-induced internalization of the NR2A subunits, then the majority of the remaining NMDARs should consist of...
Fig. 3. Ethanol increases Ras activity and dominant negative Tat-H-Ras inhibits ethanol-mediated NR2A endocytosis and internalization. a, hippocampal slices were treated with aCSF (control, lane 1), 100 mM ethanol for 15 min (lane 2), or 50 mM KCl for 5 min (lane 3), at room temperature. Ras activity was determined with Raf-1-Ras binding domain glutathione S-transferase-agarose binding assay as described under “Experimental Procedures.” Data are expressed as the ratio of Ras-GTP over total Ras levels, normalized to the control. **, p < 0.01 (Student’s t test). b, hippocampal slices were pre-treated for 2 h with aCSF (lanes 1 and 2) or with 2 μM Tat-H-RasDN (lane 3), and 2 μM Tat-peptide (lane 4) at room temperature. Slices were then treated with aCSF (lane 1, control), 100 mM ethanol for 15 min (lanes 2–4), and Ras activity was determined as described above. n = 2. c, hippocampal slices were treated with aCSF (control, lane 1), 100 mM ethanol for 15 min (lane 2), 2 μM Tat-H-RasDN for 2 h (lane 3), or Tat-H-RasDN followed by 100 mM ethanol for 15 min (lane 4), as described in b. Immunoprecipitations were conducted as described in Fig. 2, and co-immunoprecipitations were determined by probing the membranes with anti-NR2A (left) and anti-adaptin β2 antibodies (right). Data are expressed as percent increase over control in the ratio of co-immunoprecipitated protein to immunoprecipitated protein. For IP of NR2A (left), Ctl versus Eth, *, p < 0.05; Eth versus Tat-H-RasDN plus Eth, #, p < 0.05, n = 4. For IP of adaptin (right), Ctl versus Eth, *, p < 0.05; Eth versus Tat-H-RasDN plus Eth, #, p < 0.05, n = 4 (Student’s t test). d, following treatment as described for c, the slices were incubated with either aCSF (lanes 1, 3, 5, and 7) or cross-linker (BS3) (lanes 2, 4, 6, and 8), the levels of NR2A subunit were determined and analysis was performed as described in Fig. 1. Data are expressed as means ± S.D. of at least three experiments.

Fig. 4. Inhibition of Src kinase is necessary for ethanol-mediated NR2A internalization. a, hippocampal slices were treated with aCSF (control, lane 1) or with 100 mM ethanol for 15 min (lane 2). IP was conducted using 5 μg of anti-Src (lanes 1 and 2) or control anti-IgG antibodies (lane 4). Also shown is the input (lane 3). The membranes were probed with anti-[pY418] Src (top) and anti-Src (bottom) antibodies. The bar histogram shows ratios of anti-[pY418] Src to total Src, *, p < 0.05; Eth, **, p < 0.01, n = 3 (Student’s t test). b and c, hippocampal slices from b: Src+/+ (left) and Src−/− (right) and c: Fyn−/− mice were treated with aCSF (Ctl) (lanes 1 and 2) or with 100 mM ethanol (lanes 3 and 4) for 15 min at room temperature, followed by cross-linking as described in Fig. 1. Membranes were probed with anti-NR2A and anti-actin antibodies. Analysis was performed as described in Fig. 1, and the data are normalized to actin, and surface expressed or internalized to total NR2A subunits, plotted as means ± S.D. of at least three experiments. b, Src+/+, *, p < 0.05, n = 3 (Student’s t test). c, Fyn−/−, **, p < 0.01, n = 5 (Student’s t test).
FIG. 5. Ethanol exposure alters functional NMDAR properties. a, NMDAR-mediated fEPSPs measured in the absence (C, 11 slices from 8 mice) or presence (●, 8 slices from 4 mice) of 1 μM Tat-H-Ras DN (which was preincubated with slices for 2 h), followed by the application of 80 mM ethanol as indicated by the horizontal line. Inset shows example traces of NMDAR fEPSPs in control (left) and Tat-H-Ras DN treated slices.
The forward and inward trafficking of the NMDAR subunits has emerged as playing a central role in the regulation of NMDAR activity (7, 8). Here we present data to suggest that the trafficking of NMDARs is altered by ethanol. We found that acute exposure of hippocampal neurons to ethanol leads to the specific internalization of NR2A subunits via the endocytic pathway. Our data also suggest that ethanol-induced internalization of the NR2A subunits is mediated via the activation of H-Ras and the inhibition of Src kinase activity. Importantly, our results imply that, as a consequence of the internalization, the contribution of the NR2A subunits to the activity of the NMDARs is markedly reduced.

**Ras Is Activated in the Presence of Ethanol**—We observed that the activity of the small GTP-binding protein, Ras, is increased in the presence of ethanol. We previously reported that H-Ras acts as a molecular “switch off” mechanism for Src-mediated NR2A (but not NR2B) phosphorylation and membrane retention (17). In the current study, we observed that ethanol increases Ras activity, and treatment with the dominant negative H-Ras mutant (Tat-H-Ras DN) blocked the ethanol-mediated increase in Ras activity and NR2A internalization—endoctyosis. In addition, we also observed that inhibition of H-Ras activity reverses the ethanol-mediated inhibition of NMDAR-mediated iEPSPs, suggesting that increased Ras activity is necessary for ethanol-mediated NR2A internalization. For clarity, the stimulus artifacts on the EPSC traces have been removed. g, NMDAR-mediated EPSCs measured before and after stable application of ethanol in the presence of ifenprodil (3 μM; n = 7, patterned bar) and the respective baselines (white bars). *p < 0.05, paired t test comparing change in decay time induced by each drug to their respective baselines.
internalization. The mechanism by which ethanol exposure activates Ras is currently unknown; however, Ras activity can be stimulated by various signaling pathways, such as the activation of cAMP/protein kinase A cascade (43, 44). Because ethanol mediates its actions in part via the cAMP/protein kinase A pathway (45), it is possible that protein kinase A signaling is mediating Ras activation in the presence of ethanol. In addition, Ras activity can be increased via the activation of receptor tyrosine kinases (46), and we recently found that acute exposure of neurons to ethanol increases the expression level and function of the brain-derived neurotrophic factor (47), suggesting that activation of the brain-derived neurotrophic factor signaling pathway may lead to the activation of Ras.

Ethanol Exposure Prevents Src Activation—Previous studies have suggested that in order for the NR2 subunits to be internalized, the protein needs to be in a dephosphorylated state (16). Our current results imply that the specific inhibition of Src activity in the presence of ethanol contributes to the internalization of NR2A subunits, presumably by preventing Src from phosphorylating the subunits. Previously, we showed that the association between H-Ras and Src leads to the inhibition of Src activity (17); therefore, the inhibition of Src activity is likely to be mediated through the activation of H-Ras.

Both Src and Fyn kinases phosphorylate the NR2 subunits, thereby modulating the activity of the channel (26). Our previous study (20) and the present results suggest opposing actions of ethanol on the activity of these two closely related kinases, leading to differential regulation of the NR2A subunit via the inhibition of Src and the NR2B subunit via the activation of Fyn. The possible selective regulation of NR2B-containing NMDARs by Fyn, and NR2A-containing receptors by Src, is not entirely surprising, because Src and Fyn are activated by different signaling pathways and have different modes of compartmentalization within the NMDAR complex (26).

Other Possible Mechanisms for Internalization of the NR2A Subunit in the Presence of Ethanol—Previously, ethanol has been shown to mediate its effects by increasing protein phosphatase activity, thereby dephosphorylating NR2A and NR2B subunits and decreasing NMDAR activity (19). This study raises the possibility that the dephosphorylation of NR2A subunits in the presence of ethanol may also contribute to the endocytosis of the NMDAR subunits. In addition, NMDARs are localized at the synapse by protein-protein interactions with the scaffolding protein PSD-95 (48), and this interaction is thought to prevent subunit internalization via masking the AP-2 binding site (49). Ethanol has been shown to alter the compartmentalization of scaffolding proteins (50), and recently anesthetics that resemble ethanol in their pharmacological properties were found to disrupt the interaction of PSD-95 with NMDARs (51). Therefore, it is possible that ethanol exposure disrupts the specific interaction between the NR2A subunits and PSD-95, thereby allowing the interaction of NR2A with AP-2; we are currently investigating this possibility.

The NR2A Subunit Contributes to the Inhibitory Actions of Ethanol on the NMDAR—Previous studies have clearly demonstrated that ethanol exposure inhibits the activity of the NMDARs (for reviews see Refs. 50, 52, and 53). We found that in hippocampal slices, a portion of the NR2A-containing subunits is internalized after ethanol treatment. We therefore hypothesized that the reduction of membranial expression of the NR2A subunit may contribute to the inhibitory actions of ethanol on the activity of the channel. The main regulatory NR2 subunits in the hippocampus are the NR2A and NR2B subunits (54), which have different pharmacological properties such that NR2A-containing NMDARs have a faster decay time compared with the NR2B-containing receptors (2). We found that the NMDAR EPSCs exhibit a slower decay time in the presence of ethanol, suggesting that the contribution of the NR2A subunit to the remaining activity of the channel is decreased. This possibility is further supported by the experiments in which application of the NR2A inhibitor, NVP-AAM077, occluded the ethanol-induced inhibition of NMDAR-mediated EPSCs, whereas the NR2B-specific inhibitor, ifenprodil, did not.

Our results, suggesting that the NR2A subunit contributes to the inhibitory actions of the NMDARs by ethanol, are different from previous studies that suggested greater contribution of the NR2B subunit, or equal contribution of both. However, most of these experiments were done in heterologous systems such as HEK293 cells (55–60), or in Xenopus oocytes (61–64). Although these studies provide important information of the actions of ethanol on channels containing individual subunits, it is difficult to directly relate these findings to ours, because these studies were conducted in non-neuronal systems that do not contain proteins such as PSD-95 that are required for the localization and proper function of the NMDARs. Woodward, and colleagues (58) used three expression systems, L(tk−) cells, HEK293 cells, and Xenopus oocytes, and two methods, electrophysiological recording and Ca2+ imaging, to evaluate the inhibition of ethanol on NR1/NR2B and NR1/NR2A. They found NR1/NR2A and NR1/NR2B have different sensitivity to ethanol in HEK293 and L(tk−) cells but not in oocytes, suggesting that the assay system used influenced the degree of ethanol inhibition of recombinant NMDA receptors. Although some experiments were also done in cultured cortical (59), striatal (60), or cerebellar (65) neurons, these experiments cannot be directly related to ours, because we conducted the experiments in hippocampal slices, and therefore the type of neurons and their developmental stage are different. Furthermore, experiments using slice electrophysiology to determine the effects of the specific NR2A and NR2B inhibitors on NMDAR sensitivity to ethanol have not been conducted before. Finally, although our experiments suggest an important contribution of the NR2A subunit, they do not negate the studies showing the contribution of the NMDAR subunits. For example, studies in non-neuronal systems identified the C0 region within the NR1 subunit as an important region for regulating NMDAR sensitivity to ethanol (40, 41). It is therefore possible that NMDARs that contain both NR2A or NR2B and the NR1-C0 region are highly sensitive to ethanol. It is also possible that the NR1 subunit splice variants in hippocampus may be different from those in cortical, striatal, or cerebellar neurons, and play a role in the ethanol sensitivity of NR2B. Finally, we previously reported that the sensitivity of the NMDAR to ethanol in different brain regions depends on intracellular protein compartmentalization (20). Therefore, further studies will determine whether the mechanism presented here is unique for the hippocampus or shared by other brain regions.

Implications—In summary, our studies suggest the NR2A-containing receptors, but not NR2B, are internalized upon exposure to ethanol. Thus, ethanol treatment leads to a switch of subunit composition of active channels from NR2B- and NR2A-containing NMDARs to mainly NR2B-containing receptors. The subunit compositional switch of functional NMDARs has been previously detected during development and spontaneous synaptic activity (66). Here we propose that exposure of neurons to drugs of abuse such as ethanol also alters the ratio of functional NR2-containing NMDA receptors.

The NMDAR subunits play a role in synaptic plasticity, and a single exposure to drugs of abuse produces NMDA-dependent synaptic plasticity (67). Therefore, NR2B-mediated...
NMDAR functions are likely to contribute to the long-lasting synaptic plasticity processes that ultimately result in phenotypes that underlie alcohol abuse. Recently, Liu and colleagues (37) found that NR2A-containing NMDARs mediate long-term potentiation, whereas the activation of NR2B-containing NMDAR triggers long-term depression in the CA1 region of the hippocampus. Interestingly, ethanol exposure was found to inhibit long-term potentiation (68), and to enhance long-term depression (69), raising the possibility that the observed inhibition of long-term potentiation is mediated via a decrease in active NR2A-containing channels, and the increase in long-term depression is mediated via the remaining NR2B-containing channels.

NMDARs are known to play an important role in various phenotypes that contribute to the development of alcohol addiction (5), and studies have suggested that NR2B subunit-selective antagonists may be effective against alcohol dependence (70). We therefore propose that the changes in subunit composition of active NMDARs in the presence of ethanol contribute to the changes in synaptic plasticity that lead to the phenotypes associated with alcohol addiction.

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Ethanol Alters Trafficking and Functional N-Methyl-D-aspartate Receptor NR2 Subunit Ratio via H-Ras
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