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Chronic Intermittent Ethanol Treatment Selectively Alters *N*-Methyl-D-aspartate Receptor Subunit Surface Expression in Cultured Cortical Neurons

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ABSTRACT

A chronic intermittent ethanol (CIE) exposure regimen consists of repeated episodes of ethanol intoxication and withdrawal. CIE treatment has been reported to result in a significant enhancement of *N*-methyl-D-aspartate (NMDA) receptor-mediated synaptic responses in vivo, and trafficking of NMDA receptors is emerging a key regulatory mechanism that underlies the channel function. Therefore, in the present study, we examined the effects of CIE on NMDA receptor subunit surface expression. Cultured cortical neurons were exposed to 75 mM ethanol for 14 h followed by 10 h of withdrawal, repeated this cycle five times, and followed by 2 or 5 days of withdrawal. Surface-expressed NMDA receptor subunits and their endocytosis were measured by biotinylation and Western blots. CIE significantly increased NMDA receptor (NR) 1 and NR2B but not NR2A subunit surface expression after 5 days of treatment. However, CIE treatment did not reduce the NMDA receptor endocytosis. Quantification of immunocytochemistry confirmed CIE-induced increase in both the total number of NR1 and NR2B subunit clusters and their targeting to synaptic sites. It is noteworthy that this effect persisted even after ethanol withdrawal with a peak expression occurring between 0 and 2 days after withdrawal, and the expression on the plasma membrane was still at high levels after 5 days of withdrawal. In addition, this was accompanied by significant increases in postsynaptic density protein 95 clusters. Protein kinase A inhibitor completely reversed CIE-induced increase in NR1 and partially in NR2B surface level and a long-lasting effect. These changes may contribute to the development of ethanol-induced neurotoxicity and ethanol dependence.

NMDA receptor mediates excitatory neurotransmission in the central nervous system and plays a central role in the function of excitatory synapses, which are important in neuronal development, memory formation, and many forms of synaptic plasticity. The level of NMDA receptor function at the synapse critically regulates brain function and cell survival. At synapses, it is believed that the appropriate clustering of this receptor at the postsynaptic membrane is critical for efficient synaptic transmission, and a dynamic balanced process of NMDA receptors is key to remaining constant at the surface level of receptors (Wenthold et al., 2003). Trafficking of NMDA receptor has emerged as a key regulatory mechanism that underlies channel function. Recent experimental evidence supports that NMDA receptors are quite mobile within neurons via endocytosis and lateral diffusion in the membrane (Rao and Craig, 1997; Tovar and Westbrook, 1999; Crump et al., 2001; Snyder et al., 2005). For example, long-term treatment with NMDA receptor antagonists leads to an increase in surface clusters of NMDA receptors and a shift to a more synaptic localization (Rao and Craig, 1997).

The NMDA receptor system has emerged as an important site for the action of ethanol. Short-term ethanol exposure depresses NMDA receptor-activated ionic currents and Ca^{2+} influx in various brain regions (Hoffman et al., 1989; Lovinger et al., 1989). In contrast, long-term exposure to ethanol results in a compensatory increase in NMDA receptor binding density (Grant et al., 1990; Gulya et al., 1991) and elevated mRNA and protein expression of NMDA receptor subunits in the central nervous system (Follesa and Ticku, 1995, 1996; Kalluri et al., 1998; Chandler et al., 1999; Bao et al., 2001). As a result of increased NMDA receptor expression, altered NMDA receptor-mediated responses contribute to the

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; CIE, chronic intermittent ethanol; DIV, days in vitro; NR, *N*-methyl-D-aspartate receptor; PSD 95, postsynaptic density protein 95; PKA, protein kinase A; PBS, phosphate-buffered saline; Ctl, control; MK-801, 5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine (dizocilpine maleate); KT-5720, (9α,10β,12α)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo(1,2,3-*fg*:3′,2′,1′-*kl*)pyrrolo (3,4-*i*)(1,6)benzodiazocine-10-carboxylic acid, hexyl ester.

hyperexcitability and excitotoxicity associated with withdrawal from long-term ethanol exposure (Thomas and Morrisett, 2000; Carpenter-Hyland et al., 2004). More recent studies of short-term ethanol effect on NMDA receptor trafficking demonstrated an increased internalization of NR2A subunit through activation of H-Ras and inhibition of the tyrosine kinase Src (Suvarna et al., 2005), whereas prolonged ethanol exposure showed an increase in the NR1 and NR2B subunit targeting to synaptic but not to extrasynaptic localization in cultured hippocampal neurons (Carpenter-Hyland et al., 2004). These results indicate that the effect of ethanol treatment is involved in NMDA receptor trafficking.

Chronic intermittent ethanol (CIE) exposure regimen, a different model of long-term ethanol treatment, consists of repeated episodes of ethanol intoxication and withdrawal. In humans, long-term alcohol consumption typically follows an intermittent or repeat pattern characterized by regular bouts of intoxication interspersed with multiple periods of withdrawal from ethanol. Alcohol administration with a multiple intermittent paradigm in animals has been found to produce more persistent signs of withdrawal, dependence, and remarkable plastic changes in brain than one or a few periods of high alcohol administration (Becker and Hale, 1993; Kokka et al., 1993; McCown and Breese, 1993; Cagetti et al., 2003; Olsen et al., 2005). CIE-induced neuroadaptive changes in $GABA_A$ and NMDA receptors expression in cortical neurons also showed similar persistent pattern after ethanol withdrawal (Hu and Ticku, 1997). This differs from the long-term continuous ethanol treatment, in which such changes did not persist for more than a day after cessation of ethanol treatment (Sheela Rani and Ticku, 2006). Furthermore, CIE treatment is known to increase the severity of the subsequent withdrawal syndrome and duration (Becker, 1997). In the CIE animal model, alcohol withdrawal and dependence in rat leads to a kindling-like state of behavioral excitability that includes decreased seizure threshold and increased anxiety (Kokka et al., 1993; McCown and Breese, 1993), and these were accompanied by presumably causal changes in $GABA_A$ receptor function and expression of persistent decreases in hippocampus (Olsen et al., 2005). Synaptic responses mediated by NMDA receptor are known to be sensitive to ethanol and are involved in the development of alcohol dependence and withdrawal changes. However, the corresponding adaptive changes in NMDA receptor remain unclear, especially in the cerebral cortex. NMDA receptor-mediated field excitatory postsynaptic potentials investigated in the CIE rat model were reported recently to result in a significant enhancement of NMDA receptor-mediated synaptic response to NMDA (Nelson et al., 2005). Mechanisms underlying these changes have not yet been reported. In the present study, we investigated the alterations of NMDA receptor subunit surface expression after CIE treatment and withdrawal. Using a combination of biochemical and immunocytochemical approaches, we demonstrate that expression of NR1 and NR2B subunits is selectively increased at the membrane surface after CIE. Moreover, we observed that this effect was long-lasting after ethanol withdrawal in cultured cortical neurons.

Materials and Methods

Cell Culture and CIE Model. Primary cortical neurons were prepared from C57BL/6 mouse (Harlan, Indianapolis, IN) in high density ($\sim 2.8 \times 10^5/cm^2$) as described previously (Qiang et al., 2005). In brief, cells were dissociated by trituration from cortices of E15 mice and in low density ($\sim 1.2 \times 10^5/cm^2$) by trypsin treatment and trituration from newborn pup cortices. Cells were resuspended in minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 5% heat-inactivated horse serum (Gibco, Auckland, New Zealand), 100 μM L-glutamine, 28 mM D-glucose, and 1× antibiotic-antimycotic solution (Sigma, St. Louis, MO). Cells were plated onto poly(L-lysine)-coated tissue culture dishes and incubated under 5% CO_2 at 37°C. On the second day, a mixture of 5-fluoro-2′-deoxyuridine and uridine at concentrations of 20 and 40 μg/ml, respectively, was added into the medium to inhibit non-neuronal cell proliferation. From days 3 onward in vitro (DIV), cells were switched to a serum-free medium system consisting of Neurobasal medium, supplemented with B-27, 100 μM glutamine (Invitrogen), and 1× antibiotic-antimycotic solution. The cultured cells were assigned to one of four groups: 1) control (Ctl), in which neurons were kept in normal medium and subjected to the same media changes; 2) CIE, in which neurons were exposed to 75 mM ethanol (in medium) for 5 cycles, each cycle of 14-h ethanol exposure followed by 10 h of withdrawal, and in the last cycle, neurons were harvested after 14-h ethanol treatment; 3) CIEW2, CIE plus 2 days of withdrawal; and 4) CIEW5, CIE plus 5 days of withdrawal. The ethanol-treated neuronal cultures were incubated in an incubator saturated with ethanol, which maintained the ethanol concentration at the level added to the medium as determined using an alcohol assay kit (Sigma). During withdrawal cycles, cultures were kept in a separate ethanol-free incubator. Hippocampal neurons were also prepared in high density, treated in an identical manner, and used only for the comparison in total expression of NMDA receptors. In view of the developmental changes during culture, we started treatments in different groups at a different time to harvest cells at the same age of cultured neurons (e.g., Ctl, no ethanol treatment; CIE, starting ethanol treatment on DIV 8; CIEW2, starting ethanol treatment on DIV 6; and CIEW5, starting ethanol treatment on DIV 3). All cells were harvested at the end of DIV 13.

Surface Biotinylation Assay. Biotinylation was performed by using a Pinpoint Cell Surface Protein Isolation kit (Pierce, Rockford, IL). In brief, after treatment with or without CIE, high-density cortical neuronal cultures were placed on ice and rinsed in ice-cold PBS and then incubated in PBS containing 1.5 mg/ml Sulfo-NHS-SS-Biotin for 25 min at 4°C. The reaction was stopped with quench buffer, and the cells were collected. Neurons were lysed in 500 μl of lysis buffer containing complete protease inhibitor cocktail (Sigma). To determine the protein concentration and total expression of NMDA receptors by immunoblotting, 10% of the cell lysate was removed and kept at −80°C. To isolate biotinylated proteins, the cell lysates (from the remaining 90%) with equal amounts of protein in different treatments were incubated with immobilized streptavidin beads for 60 min at room temperature with rotation. Beads were washed, and proteins bound to beads were eluted in 1.5× sample buffer (62.5 mM Tris, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.01% bromphenol blue) by incubation in room temperature 60 min (Snyder et al., 2005). Samples were then separated by 4 to 12% SDS-polyacrylamide gel electrophoresis. Quantification was done by comparing the densitometric value of the specific antibody-probed biotinylated protein in the presence and absence of ethanol. The values in both CIE treatment and control were normalized to their total protein.

Biotinylation Assay of Receptor Endocytosis with Cleavable Biotin Reagent. Neuronal culture and treatment were carried out in the same way as described above. After incubation with cold 1.5 mg/ml cleavable biotin reagent in PBS (EZ-Link Sulfo-NHS-SS biotin) at 4°C for 25 min, the cultures were then returned to 37°C for 120 min to allow endocytosis to occur. Immediately after this, the remaining surface biotin was then cleaved by reducing its disulfide linkage with glutathione cleavage buffer (stripping buffer: 50 mM

glutathione, 75 mM NaCl, 75 mM NaOH, and 10% fetal bovine serum, pH 8.5–9.0). Neurons were then lysed, and the remainder of the assay was the same as that in surface expression (Snyder et al., 2005). In CIE treatment groups, ethanol was present throughout all steps before cell lysis except for the 4°C biotinylation reaction.

Western Blotting. Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis on 4 to 12% NuPAGE Bis-Tris gels and transferred to polyvinylidene difluoride membranes. For immunodetection of the NMDA receptor subunits, membranes were probed using subunit-specific antibodies against mouse NR1, NR2B subunits (BD PharMingen, San Diego, CA), and against rabbit NR2A subunit (Sigma). The chemiluminescence signal developed using Western Lighting Plus reagent (PerkinElmer Life and Analytical Sciences, Waltham, MA) was captured on X-ray film (Kodak BioMax-Light; Eastman Kodak, Rochester, NY), and the band intensity was quantitated using the UN-SCAN-IT software. Data were normalized to the intensity of β-actin bands and were represented as a percentage of the control basal group. The effects of ethanol exposure on the amount of each NMDA receptor subunit were analyzed by one-way analysis of variance. When the results were significant, Bonferroni post hoc tests were used.

Immunocytochemistry and Quantitation. Neurons isolated from the newborn pup cortices were plated on poly(L-lysine)-coated glass coverslips in 24-well plates at a lower density ($\sim 1.2 \times 10^5/cm^2$). The cells (DIV 13) were fixed with 4% paraformaldehyde in PBS for 15 min and permeabilized with 0.25% Triton X-100 for 30 min; nonspecific staining was blocked for 30 min in 0.1% Triton X-100, 5% goat serum, and 0.2% bovine serum albumin in PBS. Double-label immunostaining was done with combinations of polyclonal subunit-specific antibodies rabbit anti-NR1 (Abcan, Cambridge, MA), anti-NR2A (Sigma), and anti-NR2B (Upstate Biotechnology, Lake Placid, NY) and mouse antibodies against presynaptic marker Synaptophysin (Abcan), and postsynaptic marker PSD95 (BD PharMingen), overnight incubation at 4°C. After washing, the cells were incubated with a mixture of secondary antibodies conjugated to Alexa 488 or 568 (Invitrogen) at 1:200 for 30 min. Neurons were imaged on an Olympus FV500 confocal laser scanning microscopy at 60× magnification and a 1.5-fold zoom setting (Olympus, Tokyo, Japan). Laser and detector settings were retained for all images collected within experimental groups. Images (512 × 512 pixels) spaced by 300 to 500 nm were recorded at a planar resolution of 200 nm/pixel. For evaluation of synaptic colocalization, antibody-positive receptor clusters were defined as clusters of fluorescence that were at least twice the background. For the quantitative comparison of the number in synaptic localization, colocalization of NMDA receptor subunits and synaptophysin positive puncta was defined as synaptic localization (Rao and Craig, 1997). Generally 20 to 30 randomly chosen dendrites from 10 to 15 neurons of two coverslips per culture, three cultures each condition, were randomly selected on the basis of healthy morphology. Puncta density was expressed as puncta number per 20 μm of dendrite length. All immunocytochemical analysis was done under blinded conditions.

Results

Comparison of CIE-Induced Expression Levels of NMDA Receptor Subunits in Cultured Cortical and Hippocampal Neurons. We compared the expression patterns of NMDA receptor subunits using Western blot analysis in cultured cortical and hippocampal neurons in the preliminary experiments. The results indicated that 5 days CIE regimen induced an increased expression of total NR1 and NR2B, but not NR2A, subunits in a very similar pattern in cultured cortical and hippocampal neurons (Fig. 1). Furthermore, to test whether this effect was long-lasting in cultured cortical neurons like that described in the changes of NMDA-mediated synaptic response in hippocampal neurons (Nelson et al., 2005), we examined expression levels after CIE withdrawal. We found that this effect persisted during the period

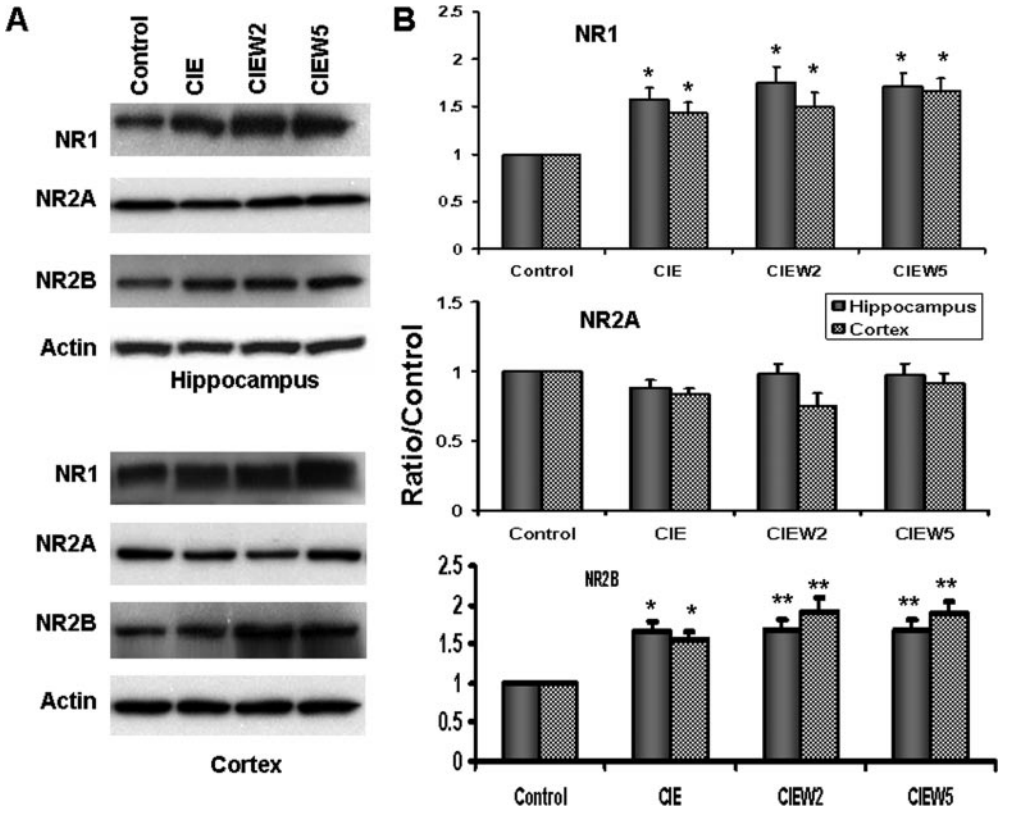

Fig. 1. Comparison of CIE-induced NMDA receptor subunit expression in cultured cortical neurons and hippocampal neurons. Primary cultured neurons isolated from fetal cortices and hippocampi, respectively, were subjected to 5 days of CIE treatment followed by 2 to 5 days of ethanol withdrawal. A, the representative immunoblots for NMDA receptor subunits NR1, NR2A, and NR2B expression are shown. Lysates with equal amount of protein from cultured neurons with indicated treatments were separated by SDS-polyacrylamide gel electrophoresis. B, densitometric assessments of the blots are presented as the ratio of each treatment to the matched control. Data (mean ± S.E.) shown are from three to five independent experiments. One-way analysis of variance revealed significant ethanol response effects ($p < 0.01$); *, $p < 0.05$, and **, $p < 0.01$, compared with their nonethanol control in each brain region (post hoc comparisons).

of 1 to 5 days after CIE withdrawal, which is consistent with CIE-induced long-lasting changes in synaptic current.

CIE Treatment Selectively Increased Surface Expression of NR1 and NR2B, but Not NR2A, Subunits in Cultured Cortical Neurons. To understand the role of NMDA receptor subunits as the molecular basis underlying CIE-induced synaptic efficacy changes, we examined the CIE-induced alterations of the NMDA receptor subunit surface expression in cultured cortical neurons. Surface-expressed proteins were labeled using biotinylation with 1.5 mg/ml sulfo-NHS-SS-biotin, and the biotinylated receptors were detected by immunoblot analysis (Fig. 2A). Quantification of the band intensities demonstrated that CIE significantly increased the levels of surface expression of NR1 and NR2B to 127 ± 11 and 136 ± 16%, respectively, immediate after 5 days of CIE treatment and a robust increase to 203 ± 22 and 316 ± 29% of control amounts 2 days after withdrawal, respectively. In contrast, no significant change was observed in the level of NR2A expression (96 ± 10% immediately after 5 days CIE treatment and 102 ± 12% of control amounts 2 days after withdrawal, respectively). It is interesting that the increased expression of NR1 and NR2B subunits persisted (196 ± 18 and 288 ± 21% of control amount) 5 days after replacement of the ethanol-containing media with the normal media (Fig. 2B). A similar effect of CIE (i.e., an increase in NR1 and NR2B surface expression) was also observed in rat cultured cortical neurons (data not shown). Further analysis of the ratio of surface expression versus its total expression in control and CIE-treated neuronal cells indicated an increase in distributions in the NR1 and NR2B membrane pools to 1.19 ± 0.07 and 1.28 ± 0.09 of that in control, respectively, immediate after 5 days of CIE treatment and to 1.36 ± 0.1 and 1.64 ± 0.12 of that in control 2 days after withdrawal, but not in the case of NR2A (0.95 ± 0.15 after CIE treatment, 1.11 ± 0.11 after 2 days of withdrawal, and 0.9 ± 0.09 after 5 days of withdrawal), suggesting that CIE does not only induce an increase in total expression but also significantly promotes NR1 and NR2B subunit delivery to the surface membrane (Fig. 2B).

To investigate whether PKA activity is involved in regulating CIE-induced increase of NR1 and NR2B surface expression, neuronal cultures were exposed to the PKA activator 8-Br-cAMP (10 μM) for 24 h, which led to a large increase in surface NR1 and NR2B subunit expression. Effect of the PKA inhibitor KT-5720 was examined on NR1 and NR2B surface expression. KT 5720 was added into control cells for 48 h and CIE culture only during the last 2 days of the 5-day exposure period. We found that KT-5720 reduced NR1 and NR2B surface expression in control cultures and completely reversed the ethanol-induced increase in NR1 and partially reversed the ethanol-induced increase in NR2B expression in CIE and CIEW5 groups (Fig. 3), suggesting that PKA activity is involved in CIE-induced targeting of NMDA receptors.

CIE-Induced Increase of NMDA Receptor Level on Membrane Does Not Involve a Reduction in Endocytosis. Ethanol could increase NMDA receptor surface expression by either promoting surface delivery or preventing endocytosis of receptor proteins. Therefore, to determine whether one or both mechanisms are involved in the CIE effect, we examined whether CIE treatment alters the receptor subunit endocytosis. Likewise, cultured cortical neurons were treated with the CIE paradigm, and endocytosis of NMDA receptor subunits was examined using a cleavable biotin assay. Normalized to the levels of surface receptors, the results showed no decrease in the endocytosis rate of biotin-bound NMDA receptor subunits after CIE treatment or ethanol withdrawal (Fig. 4). Our results suggest that endocytosis of NMDA receptors is not involved in the mechanism of CIE-induced increase of NMDA receptor surface expression.

CIE Treatment Promoted NMDA Receptor Subunits Clustering and Synaptic Targeting in Cultured Cortical Neurons. We determined the levels of neuronal surface expression of NMDA receptor subunits. To confirm the above

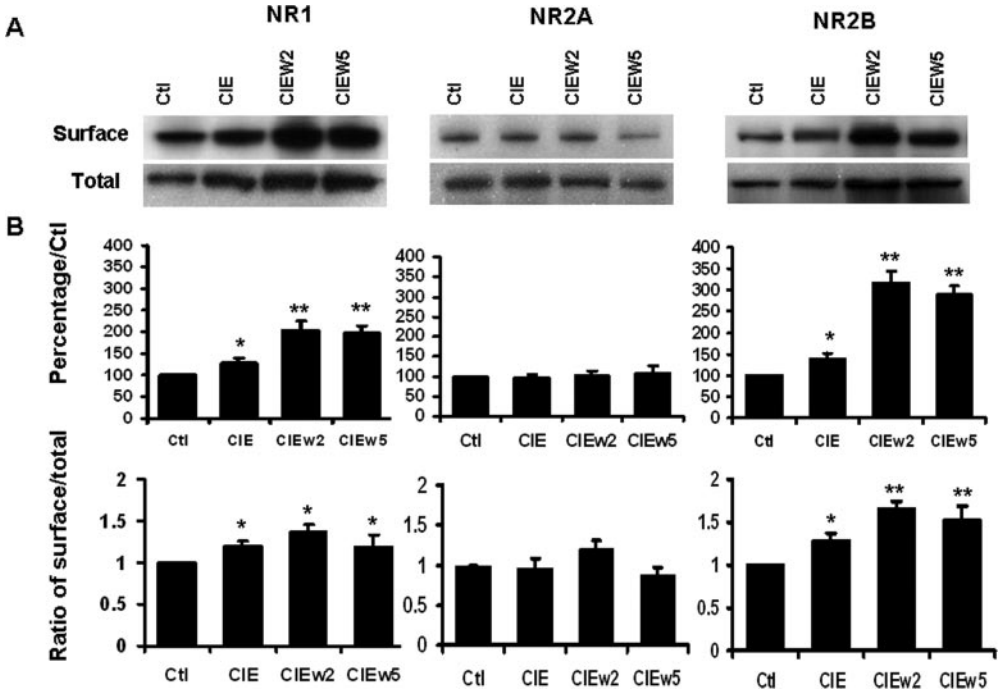

Fig. 2. CIE treatment selectively increased surface expression of NR1 and NR2B subunits. Cultured cortical neurons in high density were treated with CIE regimen. Surface protein levels were measured by biotinylation array. A, representative blots (top) showing the samples of biotinylated surface and total NR1 and NR2B subunits from control and CIE treatments. B, quantification of biotinylation immunoblots (n = 3–5 per group). The results are presented as the ratio of percentage to control (middle) and of surface to total (bottom), respectively. Data (mean ± S.E.) shown are from three to five independent experiments. One-way analysis of variance revealed significant CIE effects ($p < 0.01$); *, $p < 0.05$, and **, $p < 0.01$ compared with matched control (post hoc comparisons).

results and further examine whether CIE-induced increase of NMDA receptor subunits in plasma membrane expression may contribute to enhancement of their synaptic localization, low-density cortical neuronal cultures were subjected to CIE treatment and withdrawal regimen. Control and CIE-treated neurons were then subjected to immunocytochemical staining at the same age (DIV 13). Most of the neurons exhibited typical pyramidal cell morphology, and thus only pyramidal-like neurons were selected for subsequent image analysis. Numerous punctate clusters containing NR1, NR2A, and NR2B immunoreactivity were observed along the dendritic arbors (Fig. 5A). We compared the number and location of NMDA receptor clusters in sets of randomly selected control and CIE-treated neurons. As shown in Fig. 5B, the quantitation confirmed that exposure of cortical cultures to 75 mM CIE for 5 days greatly increased the density of NR1 and NR2B clustering ($p < 0.05$, $n = 30$) but not for NR2A, which is consistent with the results of biotinylation assay. Because the total clusters of NR1 and NR2B subunits contain non-surface clusters, therefore, we used double immunocytochemical staining of the NMDA receptor subunits NR1, NR2A, and NR2B combined with presynaptic marker synaptophysin to assay the density of NMDA receptor subunits clusters at the synapse. CIE treatment significantly increased the density of colocalizing clusters of NR1/synaptophysin and NR2B/synaptophysin. There was no change in the pattern of synaptophysin immunoreactivity or in the number of synaptophysin-labeled presynaptic terminals after CIE treatment (Fig. 5). The results suggest that the CIE treatment increased NR1 and NR2B receptor subunits expressed in plasma membrane and targeted to synapse. More importantly, this effect was long-lasting (persisting during ethanol withdrawal for 5 days). In addition, we also observed the parallel increased clustering of PSD 95 (Fig. 6) after CIE treatment, suggesting a possible association of PSD95 in NMDA receptor targeting to the membrane.

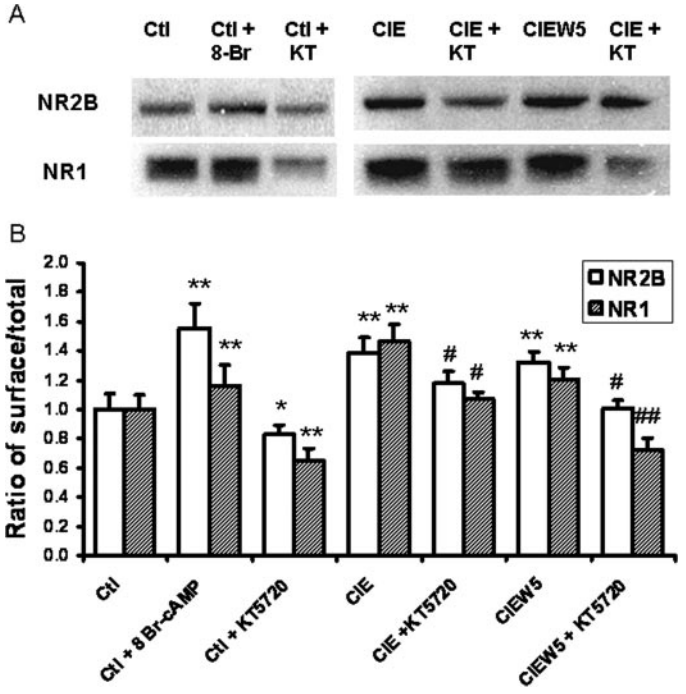

Fig. 3. PKA is involved in the CIE-induced increase in NR1 and NR2B subunits surface expression. PKA activator 8-Br-cAMP (10 μM) and inhibitor KT-5720 (1 μM) were used in cultured neuronal cells, and surface protein levels were measured by biotinylation array. A, the representative immunoblots for NMDA receptor subunits NR2B and NR1 expression are shown. Left, PKA activator 8-Br-cAMP (8-Br) was used for 24 h, and inhibitor KT-5720 (KT) was used for 48 h in control cultures. Right, the addition of KT-5720 on the last 2 days during CIE exposure completely prevented the CIE-induced increase of NR1 and partially reversed the CIE-induced increase of NR2B surface expression. B, quantification of biotinylation immunoblots ($n = 3$). The results are presented as ratio of surface to total (mean ± S.E.). One-way analysis of variance revealed significant difference ($p < 0.01$); *, $p < 0.05$, and **, $p < 0.01$ compared with Ctl; #, $p < 0.05$, and ##, $p < 0.01$compared with matched CIE-treated culture (post hoc comparisons).

Discussion

There is considerable evidence indicating the critical role of NMDA receptors in the development of ethanol dependence. Administration of MK-801, a noncompetitive antagonist of the NMDA receptor, attenuated ethanol withdrawal seizures (Grant et al., 1990; Morrisett et al., 1990). In contrast, administration of NMDA at a dose that did not produce seizures in control mice potentiated ethanol withdrawal seizures (Grant et al., 1990). Cerebral cortex is a high-order brain region involved in many plasticity activities, including learning and memory, and drug addiction. NMDA receptors are highly enriched in the cortex and contribute to cortical excitation. Although brain regional specificity of ethanol-induced

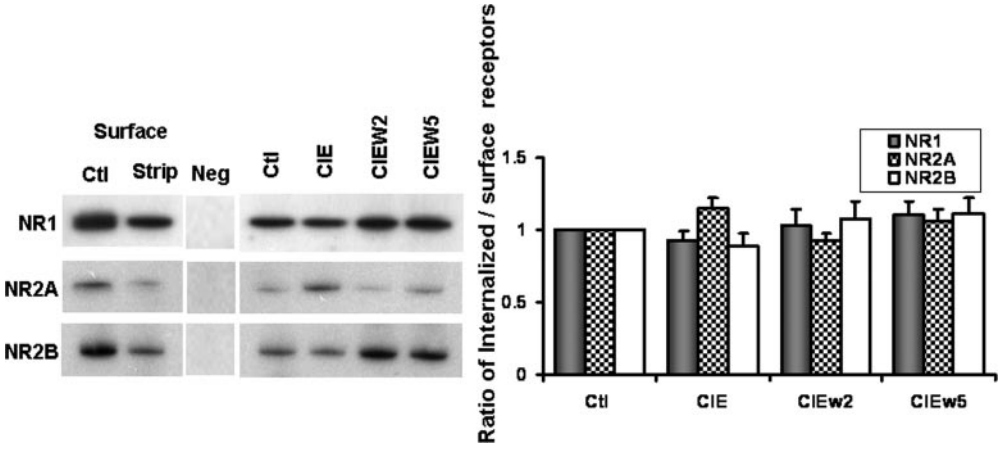

Fig. 4. CIE does not reduce NMDA receptor endocytosis. A, left, stripping removes most of the biotin-labeled surface receptors NR1, NR2A, and NR2B. Negative controls are cytoplasmic protein isolated from cultured neuronal cells after biotin labeling. Right, the amount of internalized surface receptors under indicated conditions, which is measured by biotinylating surface receptors, stripping after 120-min incubation at 37°C in normal medium or medium containing 75 mM ethanol, and probing by antibodies against NMDA receptor subunits. B, quantification of immunoblots for each subunit ($n = 3$–5 per group). The ratio of internalized receptors to matched surface receptors is shown. Error bars indicate S.E.M. One-way analysis of variance for each subunit was used.

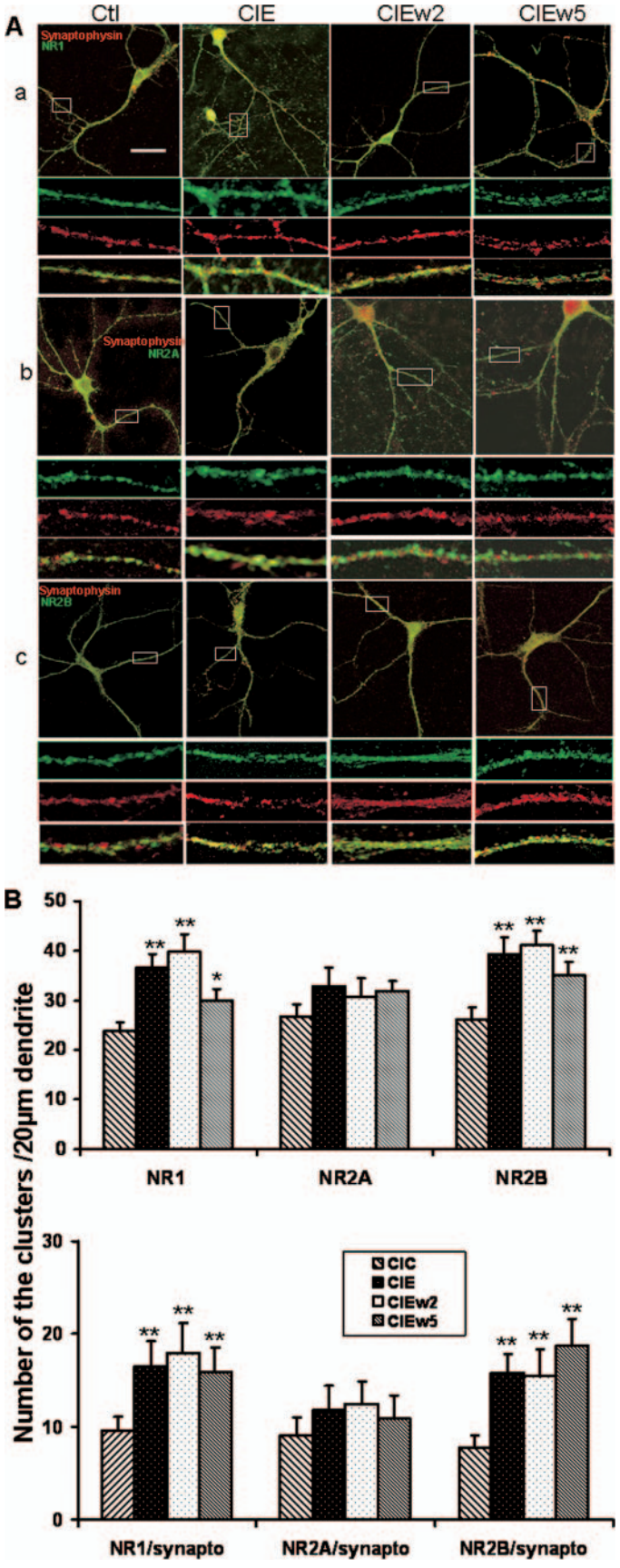

Fig. 5. CIE increased dendritic and synaptic targeting of NR1 and NR2B subunits. A, representative lower magnification confocal images of double-labeled neurons with positive clusters stained with antibodies against indicated NMDA receptor subunits (green) and synaptophysin (red) on the pyramidal-like neurons with dendrites. Under each neuron, higher-magnification images of the same cell representing the subunits NR1 (a), NR2A (b), and NR2B (c) and synaptophysin expression and their colocalization in a single dendrite, respectively. B, quantification of the clusters counting in indicated subunits NR1, NR2A, and NR2B. The graphs illustrate the number of clusters of NMDA receptor subunits (top) and colocalized clusters with synaptophysin (bottom)/20-μm dendrite length for control and different CIE-treated neurons. The numbers of total clusters of synaptophysin were not significantly different between different subunit samples. Data (mean ± S.E.M.) shown are from 20 to 30 dendrites per group. One-way analysis of variance revealed significant CIE-induced effects ($p < 0.01$); *, $p < 0.05$, and **, $p < 0.01$ compared with the matched control (post hoc comparisons). Scale bar, 20 μm.

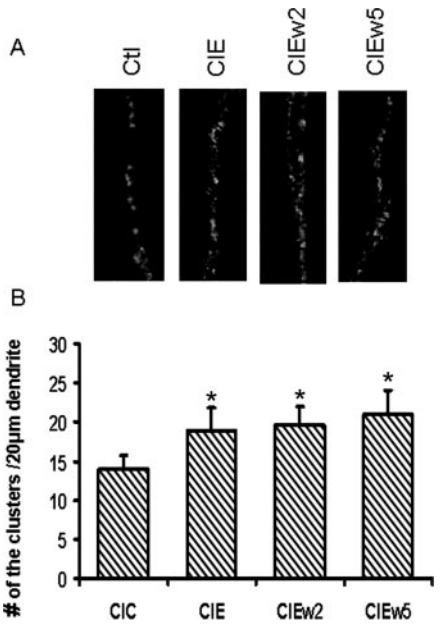

Fig. 6. CIE treatment increased PSD 95 clustering on plasma membrane. A, representative confocal images of PSD 95-positive clusters come from the neuronal dendrites in the indicated treatment groups. B, quantification of the clusters counted. Error bars indicate S.E.M. The graphs illustrate the number of clusters of PSD 95 per 20-μm dendrite length for control and different CIE-treated neurons; n = 20–30 dendrites per group. One-way analysis of variance revealed significant CIE-induced effects ($p < 0.01$); *, $p < 0.05$ compared with the control (post hoc comparisons).

adaptation and signaling mechanisms have been reported previously (Kalluri and Ticku, 1999; Yaka et al., 2003), one study pointed out that ethanol-dependent mice were accompanied by an up-regulation of NMDA receptor expression in many brain regions, including cerebral cortex, hippocampus, thalamus, and striatum, implicating that ethanol withdrawal seizures may be the result of multiple brain regions undergoing NMDA receptor hyperexcitation (Gulya et al., 1991). Alcohol alters synaptic transmission at NMDA synapses, which might disrupt cortical processing or the information the cortex relays to other brain regions, thereby playing an important role in ethanol-withdrawal behaviors. Previous studies of CIE-induced alterations were almost derived from the hippocampus of CIE rat (Kokka et al., 1993; Cagetti et al., 2003; Nelson et al., 2005), and it is important to elucidate the CIE-induced alterations of NMDA receptor in the cortical region in view of its role in ethanol-withdrawal seizures. First, we compared whether CIE-induced neuroadaptive increase of NMDA receptor subunit expression will occur in a similar pattern in hippocampus and cortex. The results, as measured by Western blotting, revealed that CIE-

caused changes in NMDA receptor expression were generally similar in these two brain regions. We therefore used cortical neuronal culture as a model to examine possible molecular and cellular mechanisms underlying CIE-induced up-regulation of NMDA receptor surface expression. We observed that CIE treatment significant increased NR1 and NR2B subunit surface levels, and this effect persisted after CIE withdrawal, suggesting a critical role of NMDA receptors in cerebral cortex on the development of ethanol dependence.

In recent years, there has been increasing evidence demonstrating that NMDA receptor surface expression is regulated by trafficking between intracellular and plasma membrane pools (Standley et al., 2000) and lateral movement of surface NMDA receptors through the membrane between synaptic and extrasynaptic sites (Rao and Craig, 1997; Rumbaugh and Vicini, 1999; Prybylowski et al., 2002). The trafficking of NMDA receptor was found to mediate various brain functions such as development, long-term potentiation formation, and activity-dependent changes (Rao and Craig, 1997; Crump et al., 2001; Grosshans et al., 2002) and play a role in pathological conditions, such as Alzheimer's disease and drug abuse (Carpenter-Hyland et al., 2004; Snyder et al., 2005). Therefore, we hypothesized that as the molecular basis of CIE-induced alterations in NMDA receptor function, long-term repeated ethanol treatments increase NMDA receptor expression in the plasma membrane through altering the receptor trafficking, which may serve as a mechanism of the development of alcohol dependence. In present study, by using biotinylation assay and immunochemistry analysis, the results demonstrate that CIE caused a significant increase in NR1 and NR2B but not NR2A subunits on the surface of plasma membrane in cortical neuronal culture. After being normalized to the total expression of NR1 and NR2B subunits, the ratio of surface to total expression of NR1 and NR2B subunits seemed significantly higher in CIE-treated cells versus the control, indicating that CIE promotes NMDA receptor targeting to the surface of membrane. These results from cortical neurons add to the mechanisms that the increased surface expression of NMDA receptors after long-term ethanol exposure would cause the increase in the density and synaptic function of the receptor, which is expected to significantly enhance neuronal excitability and consequently lead to ethanol-withdrawal seizures. At synapses, because of the trafficking of NMDA receptors, the insertion and removal of receptors are dynamic, so that the amount of surface receptors remains in a balance. Increased endocytosis has been reported to be involved in the mechanisms of decrease of membrane level of NMDA receptor in short-term ethanol treatment (Suvarna et al., 2005) and in Alzheimer's disease model (Snyder et al., 2005). To examine the relative alteration in the balances after CIE treatment, we used both BS^3 cross-linking approach (Suvarna et al., 2005) and biotinylation assay to examine internalized NMDA receptor subunits levels, respectively. We found that there was no decreased ratio of NR1 and NR2B endocytosis detected immediately after CIE or during 5 days of ethanol withdrawal compared with matched controls. Taken together, these results suggest that short- and long-term ethanol treatments may alter NMDA receptor trafficking differentially or through different mechanisms. It is likely that CIE increases NR1 and NR2B surface expression by promoting the synaptic targeting without reducing the rate of endocytosis.

It is important to note that the CIE-induced increase in NR1 and NR2B surface expression was persistent after ethanol withdrawal, which is considered critically important in maintaining ethanol dependence and relapse. We observed a significant increase of NR1 and NR2B subunit surface expression levels immediately after ethanol withdrawal, with the peak detected between 0 and 2 days after withdrawal. Moreover, it remained during 5 days of withdrawal. Quantification of immunocytochemistry confirmed that CIE induced a long-lasting increase both in the total number of NR1 and NR2B subunit clusters and in their targeting to the synaptic location, suggesting that CIE-induced persisting high level of NMDA receptors in surface during withdrawal may serve as a molecular mechanism of withdrawal hyperexcitation. It is also important to emphasize that the repeated withdrawals are a critical part of the CIE treatment, which results in longer lasting increased NMDA receptors on surface after the last ethanol dose. This differs from the long-term, continuous ethanol administration paradigms, in which similar changes in the increase of NMDA receptor expression and function are observed, but such changes did not persist for more than a day after cessation of ethanol treatment (Sheela Rani and Ticku, 2006). Repeated alcohol-withdrawal episodes are known to increase the severity and duration (Becker et al., 1997). There are similar reports with the CIE paradigm using the behavioral model. An early withdrawal period starts immediately after the cessation of alcohol exposure and can last for several days (Fadda and Rossetti, 1998). In Becker's observation, a single withdrawal did not cause persistent withdrawal-seizure response, whereas multiwithdrawal caused the longer withdrawal-seizure responses, peaking between 8 and 24 h and lasting more than 48 h (Becker et al., 1997). Therefore, our results indicated that CIE-induced NMDA receptor surface expression up-regulation in cortical neuronal culture is consistent with those behavioral data from CIE animals. Drug addiction, including alcoholism and molecular and cellular adaptations are believed to lead to persistent changes in transcription, translation, and synaptic morphology and function that are extremely long-lived and are analogous to the plastic processes that underlie learning and memory (Nestler, 2001; Ron and Jurd, 2005). Long-lasting adaptive changes after CIE exposure in NMDA receptor expression on the surface may account for a result of CIE-enhanced receptor response to NMDA and thus may contribute to the hyperexcitability that occurs within neural circuitry when long-term ethanol administration is withdrawn.

The signal transduction mechanisms underlying CIE-induced long-lasting effect of surface/synaptic targeting of NMDA receptors remain poorly defined. Recent studies showed that ethanol activates PKA signaling (Diamond and Gordon, 1997; Carpenter-Hyland et al., 2004). PKA inhibitor completely reversed the ethanol-induced increase in NMDA receptor clustering (Carpenter-Hyland et al., 2004). To investigate whether PKA is involved in the CIE-induced long-lasting effect, we used PKA inhibitor during CIE treatment and found that PKA inhibitor reversed CIE-induced increase of the NMDA receptor surface level and long-lasting effect, suggesting PKA signaling as a regulatory role in the CIE-induced long-lasting alteration of NMDA receptors. In addition, in the present study, we also observed that CIE treatment produced a parallel increase of PSD 95, which is

consistent with previous study results that the increase in NMDA receptors at the synapse was accompanied by an increased clustering of PSD95 after the long-term ethanol exposure (Chandler et al., 2006). Although our study did not address the direct association between NMDA receptor delivery and PSD 95 clustering, several findings suggest that the NMDA receptor interacts with PDZ proteins before it reaches the synapses (Setou et al., 2000; Standley et al., 2000), and PSD 95 itself contains targeting information, which is important for its synaptic localization (Roche et al., 2001). In another study, PSD 95 was found to be involved in NMDA receptor internalization. NR2B-mediated internalization was blocked by coexpression with the synaptic protein PSD 95 (Roche et al., 2001). However, we did not detect a reduced internalization of the receptors induced by CIE in this study. Together, the mechanisms of the CIE-induced increased surface expression are likely through multiple regulations, because ethanol is a small, polar, highly membrane-permeable agent that does not have a defined pharmacological site of action. Therefore, additional analyses of signaling events that control the long-lasting changes in NMDA receptor subunits are required to elucidate fully the mechanisms involved in CIE-induced neuroadaptive changes of the NMDA receptor expression.

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