Behavioral/Systems/Cognitive

Effector Immediate-Early Gene Arc in the Amygdala Plays a Critical Role in Alcoholism

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The immediate early gene, activity-regulated cytoskeleton-associated protein (Arc), has been implicated in synaptic plasticity. However, the role of Arc in alcoholism is unknown. Here, we report that the anxiolytic effects of acute ethanol were associated with increased brain-derived neurotrophic factor (BDNF) and tyrosine kinase B (trkB) expression, increased phosphorylation of extracellular signal-regulated kinases 1/2 (Erk1/2), Elk-1, and cAMP responsive element-binding protein (CREB), increased Arc expression, and increased dendritic spine density (DSD) in both the central amygdala (CeA) and medial amygdala (MeA) but not in the basolateral amygdala (BLA) of rats. Conversely, the anxiogenic effects of withdrawal after long-term ethanol exposure were associated with decreased BDNF and trkB expression, decreased phosphorylation of Erk1/2, Elk-1, and CREB, decreased Arc expression, and decreased DSD in both the CeA and MeA but not in the BLA of rats. We also showed that BDNF infusion into the CeA normalized phosphorylation of Erk1/2, Elk-1, and CREB, and normalized Arc expression, thereby protecting against the onset of ethanol withdrawal-related anxiety. We further demonstrated that arresting Arc expression in the CeA decreased DSD, thereby increasing anxiety-like and alcohol-drinking behaviors in control rats. These results revealed that BDNF–Arc signaling and the associated DSD in the CeA, and possibly in the MeA, may be involved in the molecular processes of alcohol dependence and comorbidity of anxiety and alcohol-drinking behaviors.

Key words: **Arc; BDNF; amygdala; anxiety; alcoholism; dendritic spines**

Introduction

Alcohol dependence is characterized by the development of withdrawal symptoms, such as anxiety-like behaviors, after cessation of long-term ethanol consumption in both humans and animals (National Institute on Alcohol Abuse and Alcoholism, 1993; Koob, 2003; Pandey, 2003; Cardoso et al., 2006). Innate anxiety or anxiety developing during ethanol withdrawal is crucial for the initiation and maintenance of alcohol abuse (Bibb and Chambless, 1986; Wilson, 1988; Koob, 2003; Cardoso et al., 2006). It has been shown that amygdaloid brain regions, particularly the central nucleus of amygdala (CeA), may be involved in anxiety related to ethanol withdrawal, or anxiety in general, thus reinforcing the negative effects of alcohol-drinking behaviors (Davis and Whalen, 2001; McBride, 2002; Koob, 2003). The molecular mechanisms involved in the modification of synaptic strength in the amygdala during ethanol exposure that mediate the development of alcohol withdrawal symptoms and promote alcohol intake remain to be identified. The effector immediate-early gene, activity-regulated cytoskeleton-associated protein (Arc), also known as Arg 3.1, may be relevant in synaptic plasticity related to alcoholism because it is rapidly induced by synaptic stimulation and localized to dendrites and spines in the brain (Link et al., 1995; Lyford et al., 1995; Steward and Worley, 2001; Rodriguez et al., 2005).

One of the neurotrophins, brain-derived neurotrophic factor (BDNF), has been implicated in the regulation of neuronal structure, function, and synaptic strengthening (Thoenen, 1995; Bibel and Barde, 2000; Poo, 2001). BDNF activates the tyrosine kinase B (trkB) receptor, which in turn phosphorylates and activates the extracellular signal-regulated kinases (Erk1/2) that ultimately phosphorylate cAMP responsive element-binding protein (CREB) at serine 133 (Impey et al., 1999; Sweatt, 2001). Furthermore, Erk1/2 directly phosphorylates Elk-1 to enhance Elk-1 complex formation with the serum response element (Gille et al., 1995). Thus, BDNF may regulate Arc gene expression in neurons via Erk1/2-dependent activation of both CREB and Elk-1 transcription factors (Waltereit et al., 2001; Ying et al., 2002; Ramanan et al., 2005). In addition, treatment with BDNF increases dendritic spines and synapse number in hippocampal and cerebellar neurons (Shimada et al., 1998; Tyler and Pozzo-Miller, 2001; Ji et al., 2005), suggesting a role for BDNF–Arc signaling in the regulation of neuronal architecture.

Previously, we have shown that ethanol withdrawal after chronic exposure decreased CREB phosphorylation in the CeA and medial nucleus of amygdala (MeA) but not in the basolateral amygdala (BLA) and that decreased CREB phosphorylation in the CeA may be involved in both ethanol withdrawal-related anxiety and a genetic predisposition to anxiety and alcoholism (Pan-

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dey et al., 2003, 2005). The purpose of this study was to explore the role of Arc and its relationship with BDNF–trkB signaling (Erk1/2, Elk-1, and CREB) and dendritic spine density (DSD) in the amygdala during the anxiolytic and anxiogenic effects of ethanol exposure and withdrawal, respectively. To directly link Arc-induced changes in synaptic plasticity in the CeA to anxiety and alcohol intake, we arrested the expression of Arc by infusion of Arc antisense oligodeoxynucleotides (ODNs) into the CeA and then measured DSD and alcohol-drinking and anxiety-like behaviors in rats.

Materials and Methods

Studies with ethanol-exposed rats

Acute ethanol exposure. All experiments were conducted in accordance with the National Institute of Health *Guidelines for the Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee. Adult male Sprague Dawley rats (280–300 g at the beginning of the experiment) were used in this study. For acute ethanol exposure studies, rats were injected with ethanol (1 g/kg) or *n*-saline intraperitoneally, and, after 1 h of injection, anxiety-like behaviors were measured by the elevated plus-maze (EPM) test described below. Immediately after anxiety measurements, rats were perfused to collect the brains for immunohistochemistry, *in situ* PCR, or Golgi–Cox staining as described below.

Long-term ethanol exposure. Rats were fed with ethanol and control diets as described previously by us (Pandey et al., 2001, 2003). Adult male Sprague Dawley rats were housed individually and offered 80 ml of the nutritionally complete Lieber-DeCarli control diet (Lieber-DeCarli Diet 82; Bio-Serve, Frenchtown, NJ) as their source of food and fluid. One group of rats was gradually introduced to ethanol and maintained on the ethanol-containing (9% V/V) Lieber-DeCarli liquid diet for 15 d (ethanol-fed group). Another group of rats continued to feed with the control liquid diet for 15 d (pair-fed control group). Ethanol diet-fed rats were withdrawn for 0 and 24 h, and anxiety-like behaviors were measured in ethanol diet-fed, ethanol-withdrawn (24 h), and control diet-fed rats as described below. Immediately after anxiety measurements, rats were perfused, and their brains were collected and used for immunohistochemistry, *in situ* PCR, or Golgi–Cox staining as described below. We chose 24 h as a time point of withdrawal because we had previously shown that peak anxiety occurred at this time after 15 d of ethanol treatment (Pandey et al., 1999). We generated another batch of chronic ethanol-fed rats to investigate the effect of CeA infusion of BDNF on the development of anxiety-like behaviors during ethanol withdrawal.

For this purpose, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and were implanted bilaterally with CMA/11 guide cannulas (CMA Microdialysis, North Chelmsford, MA) targeted 3 mm above the CeA. Cannulas were secured to the skull using dental cement and screws. The coordinates were as follows: 2.5 mm posterior and ±4.2 mm lateral to the bregma, and 5.1 mm ventral from the point of entry at the skull surface. Cannulas were covered with guided caps (CMA Microdialysis). After a recovery period of 1 week, rats were fed with ethanol or control diet as described above. After 15 d of ethanol liquid diet (containing 9% v/v ethanol) feeding and subsequent withdrawal (24 h), rats were infused twice (within 2 min at 24 h and 1 h before anxiety measurements) with 0.5 μl of artificial CSF (aCSF) or 50 ng of BDNF (in 0.5 μl of aCSF) using a microdialysis probe attached to an automatic pump. The microdialysis probe extended 3 mm beyond the guide cannulas into the CeA. The BDNF dose was based on our previous studies showing that this amount of BDNF infused into either the CeA or MeA was able to prevent the BDNF antisense ODN-induced anxiety-like and alcohol-drinking behaviors in rats (Pandey et al., 2006). All rats were used for measurement of anxiety-like behaviors, as described below, and their brains were processed for histochemistry to measure the levels of BDNF and Arc signaling proteins. We also measured the blood ethanol levels in ethanol-fed rats at the time of brain collection using an Analox Alcohol Analyzer (Analox Instruments, Lunenburg, MA).

Measurement of anxiety-like behaviors by the EPM test. The test procedure was the same as that described by us previously (Pandey et al., 2003, 2005, 2006). The EPM apparatus consisted of two open arms and two closed arms arranged directly opposite to each other and connected to a central platform. After a 5 min habituation period in the test room, each rat was placed on the central platform facing an open arm. The rat was observed for its exploration abilities to both open and closed arms during a 5 min test period. The number of entries made to each type of arm (open or closed) were recorded. The results were expressed as the mean ± SEM of the percentage of open arm entries and the mean percentage of time spent on the open arms (open arm activity). The general activity of rats was represented by the total number of closed arm entries as reported by other investigators (File, 1993; Rodgers and Johnson, 1995).

Gold immunolabeling of BDNF, trkB, pCREB, total Erk1/2, pErk1/2, pElk-1, and Arc proteins in the rat brain. Protein levels were determined using the gold-immunolabeling histochemical procedure as described by us previously (Pandey et al., 2001, 2003, 2004). Rats were anesthetized with pentobarbital (50 mg/kg) and then perfused intracardially with *n*-saline (100 ml), followed by 400 ml of 4% ice-cold paraformaldehyde fixative. Brains were removed and placed in fixative for 20 h at 4°C. After fixation, brains were soaked in 10%, followed by 20% and then 30% sucrose (prepared in 0.1 M phosphate buffer, pH 7.4). Brains were then frozen, and 20 μm coronal sections were prepared using a cryostat. The brain sections were washed (two times for 10 min) with 0.01 M PBS and then blocked with RPMI 1640 (with L-glutamine) medium (Invitrogen, Auckland, New Zealand) for 30 min, followed by 10% normal goat serum (diluted in PBS containing 0.25% Triton X-100) for 30 min at room temperature. Sections were then incubated with 1% BSA (prepared in PBS containing 0.25% Triton X-100) for 30 min at room temperature. Sections were further incubated with various antibodies as follows: anti-pCREB (Ser133), total Erk1/2 (tErk1/2), pErk1/2, pElk-1 (Ser383), Arc, trkB or BDNF [1:500 dilution for pCREB (Millipore, Billerica, MA); 1:200 dilution for BDNF (N-20; H-117), trkB (SC-12), and Arc (H-300) (Santa Cruz Biotechnology, Santa Cruz, CA); 1:200 dilution for pElk-1, tErk1/2, and pErk1/2 (Cell Signaling Technology, Beverly. MA)] in 1% BSA (prepared in PBS containing 0.25% Triton X-100) for 18 h at room temperature. After two washes for 10 min each with PBS and two washes for 10 min each with 1% BSA in PBS, sections were incubated with gold particle (1.4 nm) conjugated anti-rabbit or anti-mouse secondary antibody (Nanoprobes, Yaphank, NY; 1:200 dilution in 1% BSA in PBS) for 1 h at room temperature. Sections were further rinsed several times in 1% BSA in PBS followed by rinsing in double-distilled water. The gold immunolabeling was developed using Silver Enhancement Solution (Ted Pella, Redding, CA) for 15–20 min, and then sections were washed several times using tap water. Sections were then mounted on slides. The quantification of gold-immunolabeled proteins was performed using the Image Analysis System (Loats Associates, Westminster, MD) connected to a light microscope. The threshold of each image was set up in such a way that an area without staining should give zero counts. Under this condition, immunogold particles in the defined areas of three adjacent brain sections of each rat were counted at high magnification (100×), and then values were averaged for each rat. The results were represented as the number of immunogold particles/100 μm^2 area of a defined amygdaloid structure.

In situ *reverse transcription-PCR for BDNF and Arc mRNA measurement in the rat brain.* Rat brain sections were used to determine the mRNA levels of BDNF and Arc using *in situ* reverse transcription-PCR as reported previously by us (Pandey et al., 2003, 2004, 2006). The free-floating brain sections (40 μm thickness) were treated with proteinase K (1 μg/ml in PBS containing 0.05% Triton X-100) for 15 min at 37°C. After washing with PBS, sections were subjected to DNase digestion. The sections were again washed with PBS and then transferred to PCR tubes containing 100 μl of reverse transcription reaction mixture (Applied Biosystems, Foster City, CA) and reverse transcribed with reverse transcriptase enzyme in the presence of oligo-dt_{16}. Reverse transcriptase enzyme was not added to the negative sections. PCR was performed using *Taq* DNA polymerase enzyme, 100 pmol of each set of primers (BDNF primers, 5′ TAACGGCGGCAGACAAAAAGACT 3′ and 5′ GTGTCTATCCTTATGAATCGCCAGCCAA 3′; Arc primers, 5′ ACAGAGGATGAGACTGAGGCAC 3′ and 5′ TATTCAGGCTGGGTCCTGT-

CAC 3′), and 1 mM each of NTP in which dTTP was replaced by digoxigenin (DIG)-11-dUTP (PCR conditions for BDNF: 94°C for 2 min; 94°C for 30 s; 57°C for 30 s; 72°C for 90 s; total of 30 cycles and then 72°C for 10 min; for Arc: 94°C for 5 min; 95°C for 15 s; 55°C for 30 s; 72°C for 30 s; total of 30 cycles and then 72°C for 3 min). The primer sequences we used were based on previous studies (Tokuyama et al., 2000; Huff et al., 2006). After PCR, sections were mounted on slides, and BDNF- or Arc-positive cell bodies were detected by using an alkaline phosphatase-conjugated anti-DIG antibody and subsequent staining of the complex with the specific substrate, nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate (Roche Diagnostics, Indianapolis, IN). The optical density (OD) of positive cell bodies was calculated with an Image Analyzer (Loats Associates). The OD from negative brain sections was subtracted from the OD from positive brain sections. The mean OD of Arc- or BDNF-positive cell bodies in the amygdaloid structures of three adjacent brain sections of each rat were calculated, and then values were averaged for each rat. The results were represented as mean OD/100 pixels of area for BDNF or Arc mRNA levels.

Golgi–Cox method for measurement of DSD in amygdaloid structures. DSD in the amygdaloid brain structures of rats exposed to ethanol was determined by the Golgi–Cox staining procedure as described in the FD Rapid Golgi Stain Kit manual (FD Neuro Technologies, Baltimore, MD). This procedure has been used extensively in the field and has proven to be sensitive and reproducible for demonstrating the morphological details of neurons, specifically dendritic spines (Robinson et al., 2001, 2002). For this purpose, brains were rapidly immersed in impregnation solution for at least 1 week. Afterward, brain sections (200 μm) were cut using a cryostat and then mounted and silver stained according to the instruction manual of the FD Rapid Golgi Stain Kit. After staining, sections were dehydrated and cleared in xylene solution and then coverslipped using mounting medium. Sections were observed under a microscope at high magnification (100×), and the DSD was counted by Sholl analysis using the Neurolucida Neuroexplorer program (MicroBrightField, Williston, VT). We counted DSD in only those neurons that had complete impregnation as observed by the labeling of dendrites connected to the soma. We counted the number of spines from 7 to14 dendrites (up to 100 μm length with 10 μm increment) for each rat (three brain sections per rat) in the CeA, MeA, and BLA, and values were averaged for each rat.

Studies with normal rats

Arc sense and antisense ODN infusion and anxiety-like behaviors. Rats were implanted bilaterally with CMA/11 guide cannulas (CMA Microdialysis) targeted 3 mm above the CeA as described above and used by us previously (Pandey et al., 2006). One week after surgery, rats were infused once with phosphorothioate-modified Arc sense or antisense ODNs. The ODN sequence was derived from bases 209–228 of the Arc gene sequence (Lyford et al., 1995) because Arc antisense ODNs derived from these bases have been shown to decrease Arc levels in the hippocampus and to impair the consolidation of long-term memory (Guzowski et al., 2000). The phosphorothioate-modified ODNs were synthesized by Integrated DNA Technologies (Coralville, IA). The following groups were generated for infusion into the CeA: (1) aCSF-infused (0.5 μl); (2) Arc antisense (50 pmol in 0.5 μl)-infused; and (3) Arc sense (50 pmol in 0.5 μl)-infused rats. Arc sense or antisense ODNs were infused for a period of 2 min. The anxiety-like behaviors were measured at 12 h and 3 d after infusion. Immediately after anxiety measurements, rats were perfused and brains were collected for measurement of mRNA levels of Arc using *in situ* PCR, and protein levels of BDNF, Arc, and neuron-specific nuclear protein (NeuN) (anti-NeuN primary antibody, 1:200 dilution; Millipore) in amygdaloid structures using the gold-immunolabeling technique, as described above. The number of NeuN-positive neurons in the CeA was counted at lower magnification (20×) using the Neurolucida program (MicroBrightField). Another batch of rats was generated to perform Golgi–Cox staining, as described above. DSD was counted from three to six dendrites in CeA for each rat in this batch, and values were averaged for each rat.

Arc sense and antisense ODN infusion and alcohol intake. Ethanol preference was measured using the two-bottle free-choice paradigm used by us previously (Pandey et al., 2003, 2006; Misra and Pandey, 2006). Rats were bilaterally implanted with cannulas targeted 3 mm above the CeA, as described above. After a recovery period of 1 week, rats were habituated to drink water from two bottles. Once they started drinking water equally from either bottle, rats were infused with 0.5 μl of aCSF or 50 pmol of Arc antisense or sense ODNs in 0.5 μl of aCSF using a microdialysis probe that was attached to an automatic pump. Arc sense/antisense ODNs were infused once for a period of 2 min, and rats were given 9% (v/v) ethanol in one bottle and water in the other bottle every day for 4 d at 6:00 P.M. The consumption of ethanol and water (milliliters) was also measured daily, and ethanol intake was calculated in terms of grams per kilogram per day. After measurement of alcohol-drinking behaviors, rats were perfused and their brains were processed for histochemistry to check the position of cannulas or tissue damage using cresyl violet staining.

Statistics

The differences between two groups were evaluated by Student's *t* test. The differences between more than two groups were evaluated by a one-way ANOVA test. The alcohol intake and DSD data were evaluated by repeated measures of ANOVA. *Post hoc* multiple comparisons were performed using Tukey's test.

Results

Arc induction and increased DSD in the amygdala by acute ethanol exposure

First, we examined the effect of acute ethanol exposure on anxiety-like behaviors, Arc expression, and DSD in various structures of the amygdala in rats. We injected ethanol (1 g/kg, i.p.) and measured anxiety-like behaviors 1 h after injection (mean ± SEM; blood ethanol levels, 93 ± 2.7 mg% at the time of brain collection). We found that acute ethanol significantly ($p < 0.001$) increased the percentage open arm entries and percentage time spent on open arms (Fig. 1*A*) as measured by the EPM test. We also found that acute ethanol significantly ($p < 0.001$) increased the total number of arm entries (closed plus open arm entries), but general activity, as measured by total closed arm entries, was not modulated by acute ethanol exposure (Fig. 1*A*).

We also measured mRNA and protein levels of Arc and DSD in the amygdaloid structures after 1 h of acute ethanol injection. We found that acute ethanol significantly increased ($p < 0.001$) the mRNA and protein levels of Arc (Fig. 1*B*,C) and DSD ($p < 0.05$–0.001) (Fig. 1*D*,F) in the CeA and MeA but not in the BLA of rats. Thus, acute ethanol exposure significantly increased Arc levels and DSD in the selected neurocircuitry of the amygdala. These results suggest the possibility that the anxiolytic effects of ethanol may be related to an increase in Arc-induced synaptic strengthening in the amygdaloid structures of rats.

Arc induction in the amygdala is related to increased BDNF signaling during acute ethanol exposure

Arc expression is regulated by phosphorylation of Elk-1 and CREB because of the phosphorylation and activation of Erk1/2 via BDNF-mediated stimulation of trkB (Gille et al., 1995; Davis et al., 2000; Waltereit et al., 2001; Yin et al., 2002). In addition, BDNF and trkB are CREB-regulated target genes (Tao et al., 1998; Duman, 2004). Therefore, we measured both the mRNA and protein levels of BDNF and the protein levels of trkB, tErk1/2, pErk1/2, pElk-1, and pCREB in the amygdaloid structures of rats treated with acute ethanol. The trkB receptor can exist either as a full-length catalytic receptor or in a truncated form (Klein et al., 1990; Middlemas et al., 1991). Here, we measured the protein levels of the full-length trkB receptor in the amygdaloid structures of rats during ethanol exposure. We observed that the mRNA and protein levels of BDNF were significantly increased ($p < 0.001$) in the CeA and MeA but not in the BLA of rats (Fig.

2*A*,*B*). We also found that increased BDNF expression was associated with increased protein levels of trkB ($p < 0.001$) and increased phosphorylation of Erk1/2, Elk-1, and CREB as indicated by significantly increased ($p < 0.001$) protein levels of pErk1/2, pElk-1, and pCREB in the CeA and MeA, but not in the BLA, of rats exposed to acute ethanol (Fig. 2*A*,*B*). Acute ethanol had no significant effect on tErk1/2 protein levels in the CeA, MeA, or BLA of rats (Fig. 2*A*,*B*). Also, the pattern of labeling of tErk1/2 in amygdaloid structures was different from pErk1/2 labeling; tErk1/2 was primarily localized in the cytosol, whereas pErk1/2 was localized in the nuclei of neurons (Fig. 2*A*). These results were similar to other studies related to the distribution of tErk1/2 and pErk1/2 in the cytosol and nucleus of neurons (Jiang et al., 2001). Furthermore, these results indicate that an increase in Arc levels might be attributable to increased BDNF and trkB levels in addition to an associated increase in the phosphorylation of Elk-1 and CREB attributable to activation of Erk1/2 in both the CeA and MeA of rats during acute ethanol exposure.

Withdrawal from chronic ethanol exposure reduced BDNF signaling, Arc expression, and DSD in amygdaloid structures

We reported previously that ethanol withdrawal, but not treatment, significantly decreased CREB phosphorylation in both CeA and MeA structures (Pandey et al., 2003). Here, we extended these studies and examined the effect of chronic ethanol treatment and its withdrawal (24 h) on the levels of BDNF, trkB, and Arc, in addition to DSD in CeA, MeA, and BLA. We found that ethanol-withdrawn rats displayed anxiety-like behaviors as demonstrated by significant decreases in both percentage open arm entries ($F_{(2,33)} = 49.39$; $p < 0.001$) and percentage time spent on open arms ($F_{(2,33)} = 36.38$; $p < 0.001$) (Fig. 3*A*). Long-term ethanol treatment had no effect on the mRNA and protein levels of BDNF, protein levels of trkB or Arc (Fig. 3*B*,*C*), or on DSD (Fig. 4*A*,*B*). However, ethanol withdrawal produced significant decreases ($p < 0.05$–0.001) in the levels of BDNF, trkB, and Arc (Fig. 3*B*,*C*), in addition to DSD ($p < 0.05$–0.001) in CeA and MeA of rats (Fig. 4*A*,*B*). Chronic ethanol treatment and withdrawal had no significant effect on BDNF, trkB, or Arc levels (Fig. 3*B*,*C*) and also had no effect on DSD in the BLA structures (Fig. 4*A*,*B*). These results suggest that ethanol withdrawal, after chronic ethanol exposure, decreased the

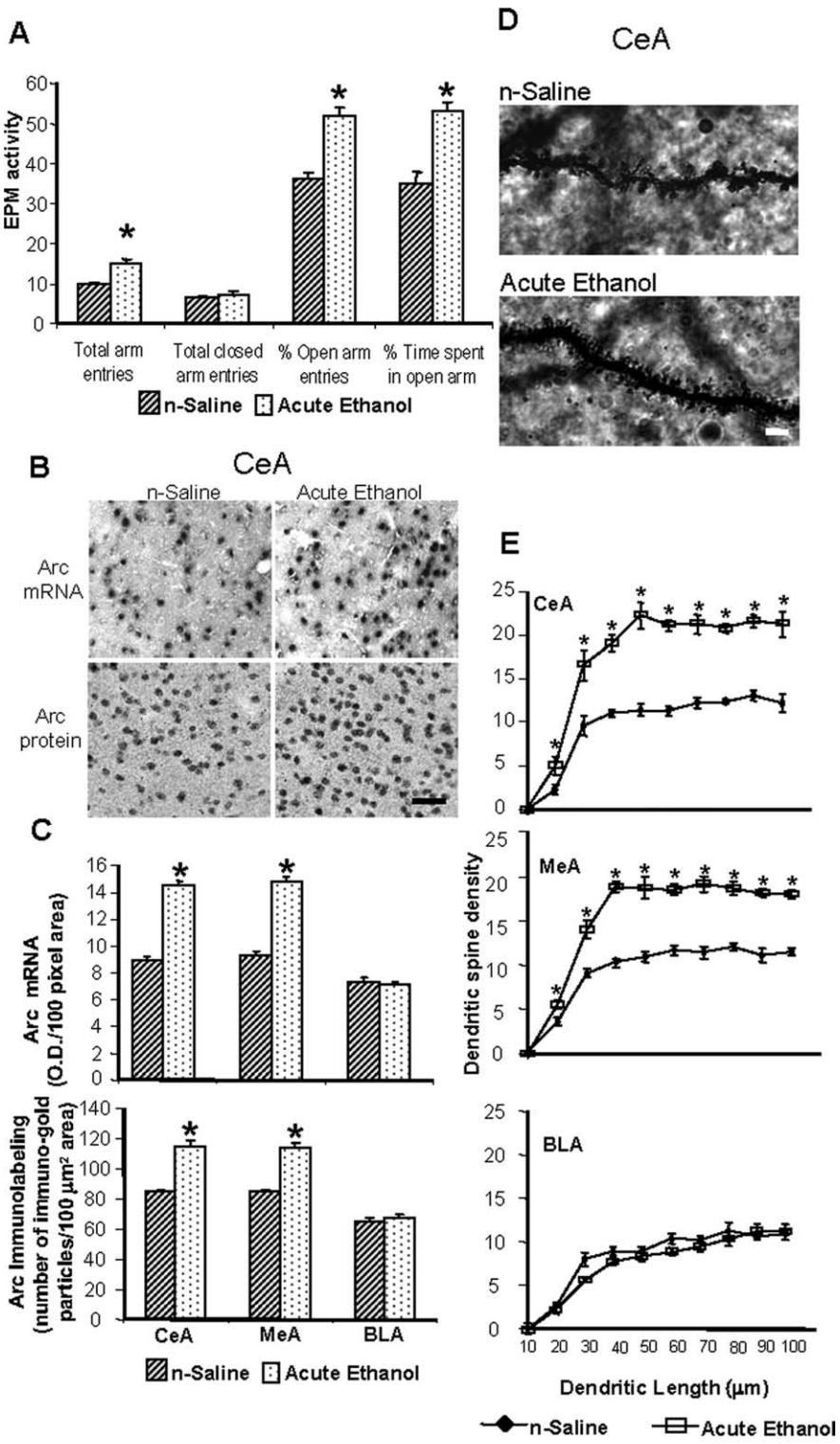

Figure 1. Acute ethanol produced an anxiolytic response, induced Arc expression, and increased DSD in both the CeA and MeA of rats. ***A***, The effect of acute ethanol exposure (1 g/kg, i.p.) on open arm and closed arm activity in the EPM test. Values are the mean ± SEM of 11 rats in each group. *$p < 0.001$, significantly different from *n*-saline-treated rats (Student's *t* test). ***B***, Low-magnification views of Arc mRNA (*in situ* PCR) and Arc gold immunolabeling (protein) in the CeA of *n*-saline- and ethanol-treated rats (scale bar, 40 μm). ***C***, Effect of acute ethanol treatment on mRNA and protein levels of Arc in various amygdaloid (CeA, MeA, and BLA) structures of rats. Values are mean ± SEM of five to six rats in each group. *$p < 0.001$, significantly different from the *n*-saline-treated rats (Student's *t* test). As can be seen, acute ethanol exposure increased Arc expression in both the CeA and MeA of rats. ***D***, Photomicrographs with Golgi-impregnated neurons showing dendritic spines in the CeA of *n*-saline- and acute ethanol-treated rats (scale bar, 10 μm). ***E***, Effect of acute ethanol treatment on DSD in various amygdaloid (CeA, MeA, and BLA) structures of rats. Values are mean ± SEM of five rats in each group. *$p < 0.05$–0.001, significantly different from the *n*-saline-treated rats (repeated measures of ANOVA followed by Tukey's test). Acute ethanol exposure increased DSD in both the CeA and MeA.

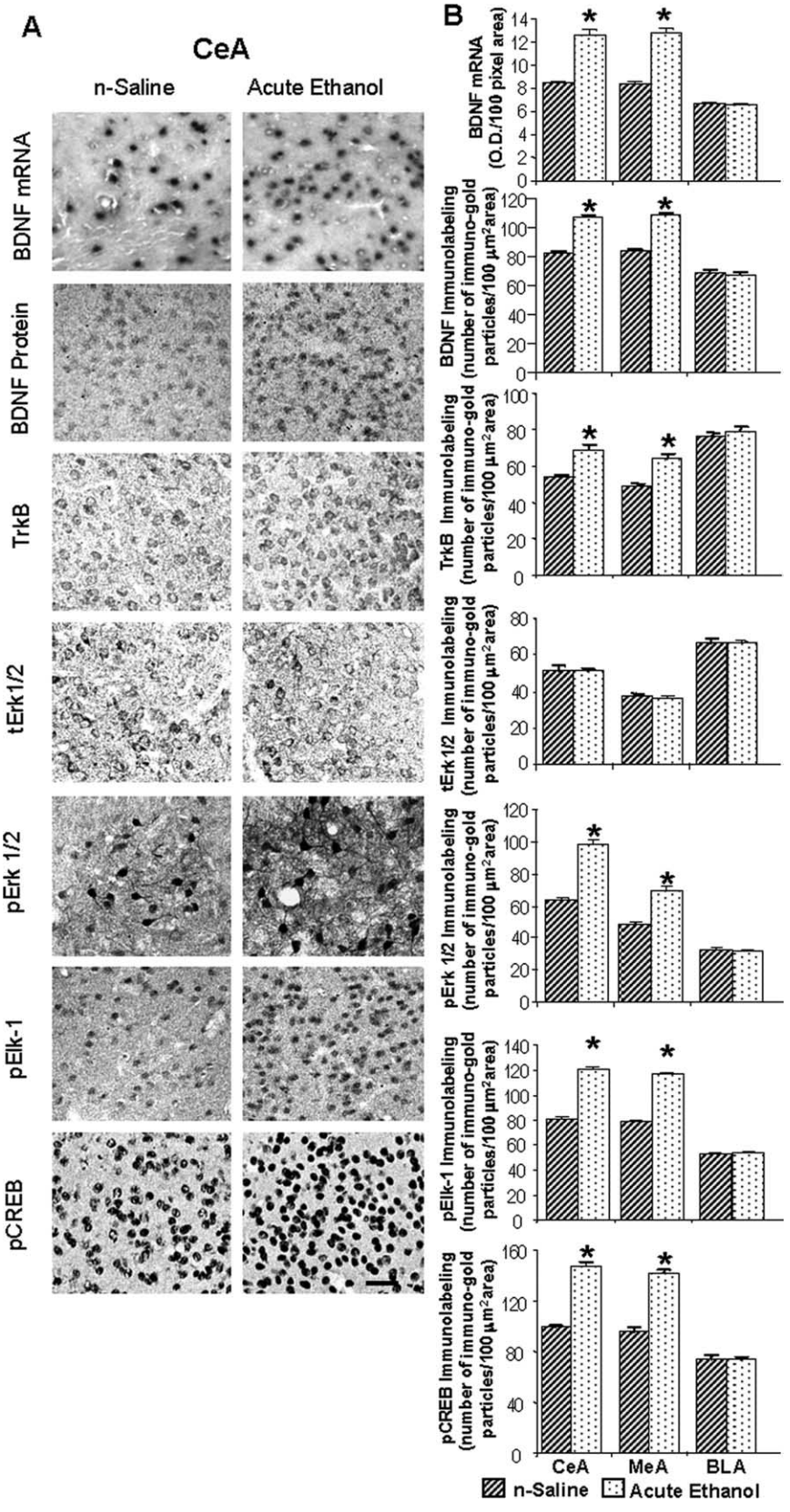

Figure 2. ***A***, Low-magnification views of BDNF mRNA (*in situ* PCR) and BDNF, trkB, tErk1/2, pErk1/2, pElk-1, and pCREB gold immunolabeling (protein) in the CeA of *n*-saline- and ethanol-treated rats (scale bar, 40 μm). ***B***, Effect of acute ethanol treatment on mRNA and protein levels of BDNF and protein levels of trkB, tErk1/2, pErk1/2, pElk-1, and pCREB in various amygdaloid (CeA, MeA, and BLA) structures of rats. Values are mean ± SEM of five to six rats in each group. *$p < 0.001$, significantly different from *n*-saline-treated rats (Student's *t* test). Acute ethanol exposure increased BDNF and trkB levels and increased phosphorylation of Erk1/2, Elk-1, and CREB in both the CeA and MeA of rats.

levels of BDNF, trkB, and Arc and also decreased DSD in both the CeA and MeA, but not in the BLA, of rats.

To examine whether or not decreased Arc levels were related to the decreased phosphorylation of Erk1/2 and Elk-1, we determined the protein levels of these signaling molecules during ethanol treatment and its withdrawal. We found that long-term ethanol treatment had no effect on the protein levels of tErk1/2, pErk1/2, or pElk-1 in the CeA, MeA, or BLA. Conversely, the protein levels of pErk1/2 and pElk-1 significantly decreased ($p < 0.001$), without any changes observed in tErk1/2 protein levels, in both the CeA and MeA, but not the BLA, during ethanol withdrawal (Fig. 3*B*,*C*). These results suggest that the decreased phosphorylation of Erk1/2, attributable to the decreased expression of BDNF and trkB, may contribute to the decreased phosphorylation of both CREB and Elk-1, subsequently leading to decreased Arc expression in both the CeA and MeA during ethanol withdrawal.

BDNF infusion into the CeA prevented development of anxiety-like behaviors and normalized Arc expression during ethanol withdrawal

We implanted bilateral cannulas targeting the CeA, and 1 week after cannulation surgery, rats were fed with either a liquid control diet or an ethanol diet (9% ethanol v/v) for 15 d. The ethanol diet-fed groups (blood ethanol levels in ethanol diet-fed rats were 182 ± 11 mg% at the time of brain collection, mean ± SEM) were withdrawn for 0 and 24 h, and at the same time, these rats, along with control diet-fed rats, were infused with BDNF (50 ng in 0.5 μl of aCSF) or aCSF (0.5 μl) twice [at 24 h (initiation of withdrawal) and 1 h before anxiety measurements]. There were no significant differences in the body weights of rats among the various groups. As shown in Figure 5*A*, ethanol withdrawal (24 h) produced a significant reduction in open-arm activity [percentage entries ($F_{(4,39)} = 39.01$; $p < 0.001$) and percentage time ($F_{(4,39)} = 33.30$; $p < 0.001$) spent on open arms] during the EPM test, whereas BDNF infusion into the CeA protected against the development of anxiety-like behaviors in ethanol-withdrawn rats (Fig. 5*A*). Interestingly, BDNF infusion into the CeA of ethanol-withdrawn rats significantly increased ($p < 0.001$) the phosphorylation of Erk1/2, CREB, and Elk-1 and also increased ($p < 0.001$) the mRNA and protein levels of Arc in the CeA but not in the MeA or BLA (Fig. 5*B*,*C*). BDNF infusion into the CeA of control rats had no significant effect on anxiety levels, Arc expression, or the phosphorylation states of Erk1/2,

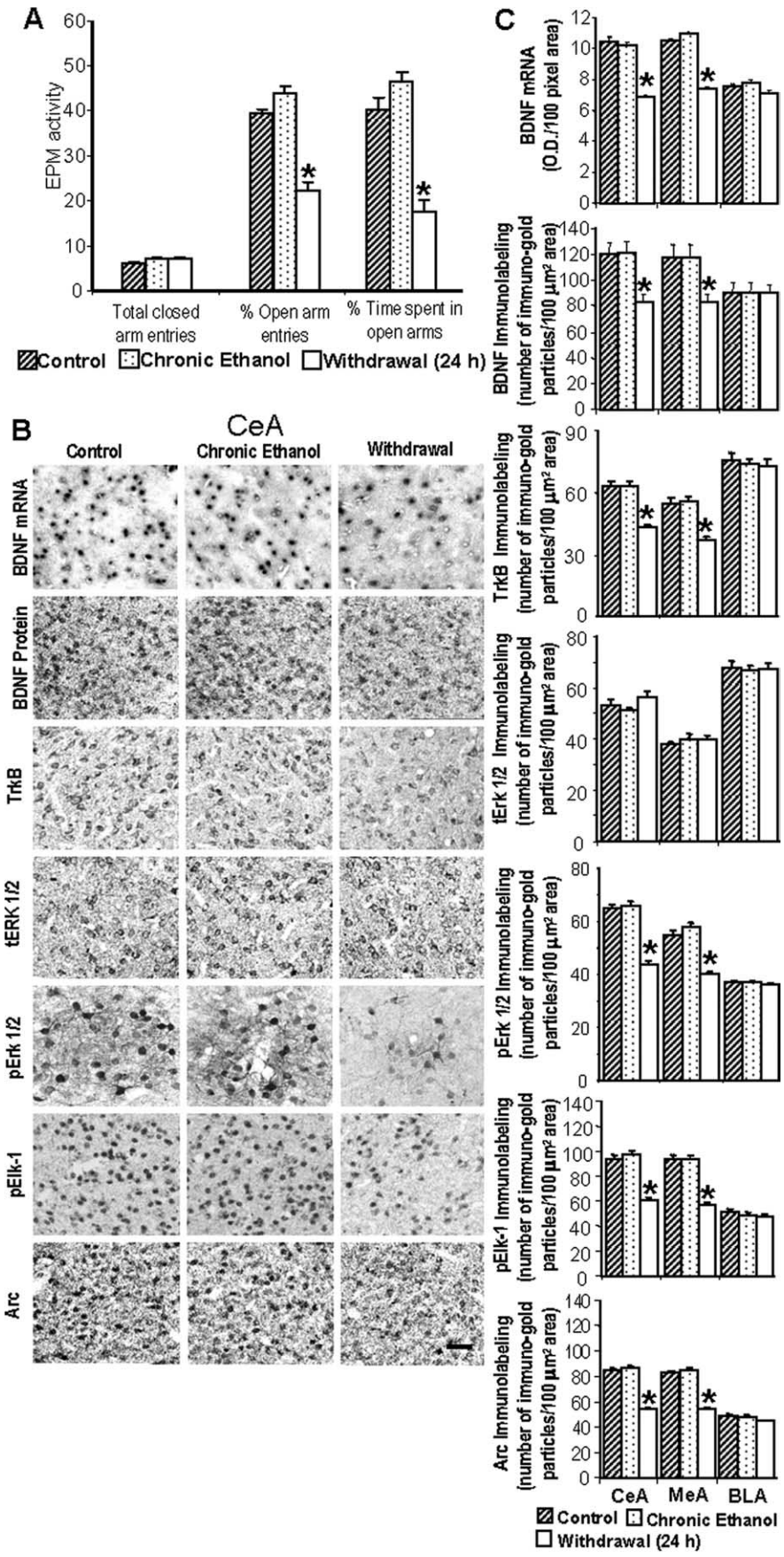

Figure 3. Withdrawal from long-term ethanol exposure produced anxiety-like behaviors and decreased BDNF–Arc signaling in both the CeA and MeA of rats. ***A***, The effect of long-term ethanol exposure and withdrawal on open arm and closed arm activity in the EPM test. Values are the mean ± SEM of 12 rats in each group. *$p < 0.001$, significantly different from control diet-fed rats (ANOVA followed by Tukey's test). ***B***, Low-magnification views of BDNF mRNA (*in situ* PCR) and BDNF, trkB, tErk1/2, pErk1/2, pElk-1, and Arc gold immunolabeling (protein) in the CeA of control diet-fed, ethanol diet-fed, and ethanol-withdrawn rats (scale bar, 40 μm). ***C***, Effect of long-term ethanol treatment and withdrawal on mRNA and protein levels of BDNF and protein levels of trkB, tErk1/2, pErk1/2, pElk-1, and Arc in various amygdaloid (CeA, MeA, and BLA) structures of rats. Values are mean ± SEM of five to seven rats in each group. *$p < 0.05$–0.001, significantly different from the control diet-fed rats (ANOVA followed by Tukey's test). As can be seen, ethanol withdrawal decreased BDNF–Arc signaling in both the CeA and MeA.

Elk-1, or CREB in CeA, MeA, or BLA structures (Fig. 5*B*,*C*). These results suggest that decreased BDNF-induced Arc expression, specifically in the CeA, may be involved in ethanol withdrawal-related anxiety-like behaviors.

Infusions of Arc antisense ODNs into the CeA provoked anxiety-like behaviors

To examine the direct association between decreased expression of Arc in the CeA and anxiety-like behaviors, we implanted bilateral cannulas targeting the CeA. After 1 week of recovery, rats were infused with 0.5 μl of aCSF, Arc sense, or Arc antisense ODNs (50 pmol). We found that intra-CeA infusion of Arc antisense ODNs produced a significant reduction of percentage open arm entries ($F_{(5,54)} = 24.53$; $p < 0.001$) and percentage time ($F_{(5,54)} = 15.62$; $p < 0.001$) spent on the open arm at 12 h but not at 3 d after infusion compared with aCSF-infused rats (Fig. 6*A*). However, rats infused with Arc sense ODNs did not show any significant changes in their open or closed arm activities at 12 h or 3 d after infusion compared with aCSF-infused rats (Fig. 6*A*). The overall general activity (total number of closed arm entries) was similar in all groups. These results indicate that Arc antisense ODN infusion into the CeA provoked anxiety-like behaviors at 12 h. Furthermore, these effects were reversible in that, 3 d after infusion, the anxiogenic effects of Arc antisense ODNs disappeared in rats.

Decreased dendritic spines in the CeA after infusion of Arc antisense ODNs

Immediately after measuring anxiety, brains were used to examine protein and mRNA levels of Arc as well as DSD. Figure 6*B* shows the expression of Arc protein and mRNA in the CeA after Arc manipulations. We found that, 12 h after Arc antisense ODN infusion into the CeA, both the protein and mRNA levels of Arc significantly decreased ($p < 0.001$) in only the CeA but

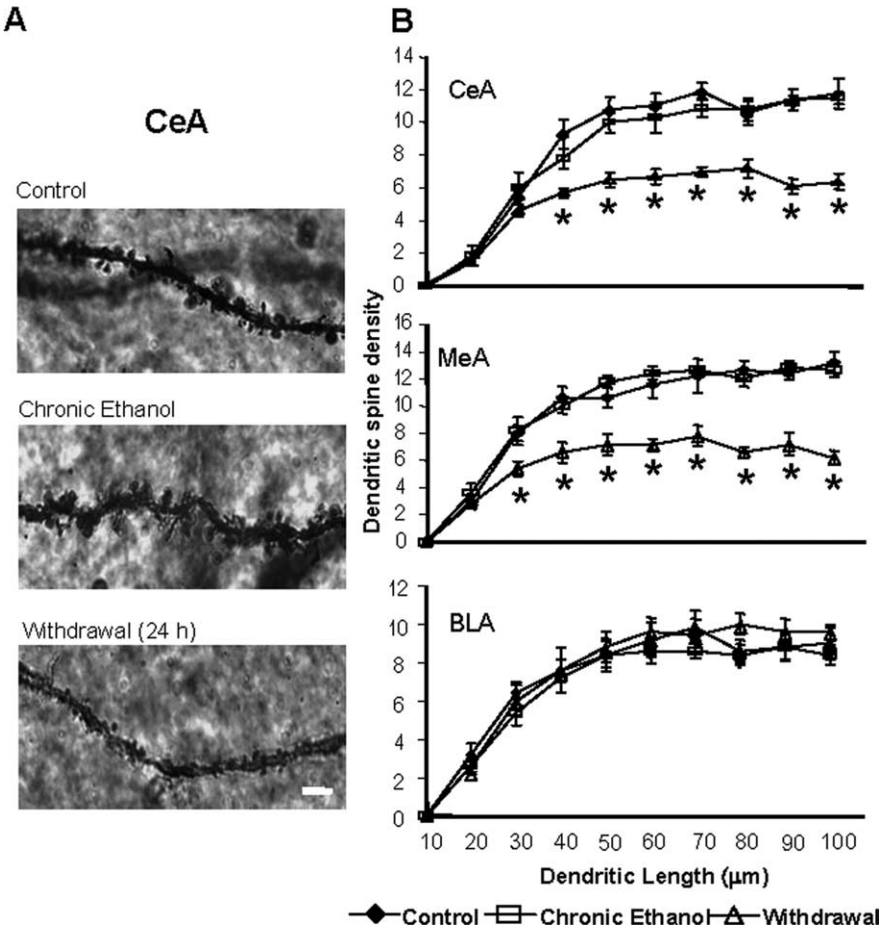

Figure 4. Withdrawal from long-term ethanol exposure decreased DSD in both the CeA and MeA of rats. ***A***, Photomicrographs showing dendritic spines of Golgi-impregnated neurons in the CeA of control diet-fed, ethanol diet-fed, and ethanol-withdrawn rats (scale bar, 10 μm). ***B***, Effect of long-term ethanol treatment and withdrawal on DSD in various amygdaloid (CeA, MeA, and BLA) structures of rats. Values are mean ± SEM of five rats in each group. *$p < 0.05–0.001$, significantly different from the control diet-fed rats (repeated measures of ANOVA followed by Tukey's test). Ethanol withdrawal decreased DSD in both the CeA and MeA.

not in the MeA or BLA compared with aCSF-infused rats (Fig. 6*B*,*C*). The reductions in mRNA and protein levels of Arc in the CeA were normalized 3 d after infusion of Arc antisense ODNs in rats. Infusion of Arc sense ODNs into the CeA did not produce any significant changes in the protein or mRNA levels of Arc in the CeA, MeA, or BLA either 12 h or 3 d after infusion compared with the aCSF-infused group (Fig. 6*B*,*C*). Next, we measured DSD in the CeA using Golgi–Cox staining. We found that Arc antisense ODN infusion into the CeA significantly decreased ($p < 0.05–0.001$) the DSD in the CeA at 12 h after infusion. Infusion of Arc sense ODNs into the CeA did not produce any significant change in DSD compared with the aCSF-infused group (Fig. 6*E*,*F*). Arc sense and antisense ODN infusions also did not produce any change in the protein levels of NeuN or BDNF or in the number of NeuN-positive neurons in the CeA (data not shown) at 12 h after infusion. These results indicate that Arc antisense, but not sense ODN, infusion into the CeA reduced DSD but produced no change in the total number of NeuN-positive neurons in the CeA.

Relationship between decreased Arc expression in the CeA and alcohol intake

As described above, decreased Arc levels in the CeA were associated with the development of anxiety-like behaviors in rats. Because high anxiety levels promoted alcohol intake, we also measured alcohol preference using a two-bottle free-choice paradigm after Arc expression manipulations in the CeA to establish an association between anxiety-like and alcohol drinking behaviors. A 3 × 4 (group × day) repeated measure using a two-way ANOVA was performed on changes in alcohol consumption that yielded a significant group effect ($F_{(2,76)} = 14.56$; $p < 0.001$), a significant day effect ($F_{(3,76)} = 8.08$; $p < 0.001$), and a significant interaction ($F_{(6,76)} = 3.83$; $p < 0.01$) of these variables in rats. We found that Arc antisense ODN-infused, but not sense ODN-infused, rats consumed significantly more ethanol on days 1 ($p < 0.001$) and 2 ($p < 0.05$) of the postinfusion period compared with aCSF-infused rats (Fig. 6*D*). The alcohol intake on either the third or fourth day after infusion in Arc antisense ODN-infused rats was similar to the aCSF group of rats (Fig. 6*D*). The total fluid intake (milliliters per day) was similar in all groups (Fig. 6*D*). These results indicate that decreased Arc levels and DSD in the CeA increased both alcohol-drinking and anxiety-like behaviors. These results further suggest that, when the Arc levels were normalized (Fig. 6*B*,*C*), 3 d after infusion, both alcohol-drinking (Fig. 6*D*) and anxiety-like (Fig. 6*A*) behaviors were also normalized.

Discussion

Previous studies have demonstrated that BDNF signaling serves as a homeostatic pathway in both the hippocampus and striatum to regulate ethanol consumption (McGough et al., 2004; Jeanblanc et al., 2006). Chronic ethanol exposure either decreased or had no effect on BDNF levels in the rat hippocampus and forebrain regions (MacLennan et al., 1995; Tapia-Arancibia et al., 2001; Miller et al., 2002). It has also been found that a BDNF gene polymorphism may be linked with a vulnerability to alcohol abuse (Uhl et al., 2001; Matsushita et al., 2004) and that a deficiency in BDNF may promote alcohol intake in mice (Hensler et al., 2003; McGough et al., 2004). The present investigation extended these studies to reveal a novel molecular mechanism suggesting that increases in both Arc expression and DSD in the CeA may elicit the anxiolytic effects of acute ethanol, whereas decreases in both Arc expression and DSD in the CeA may be involved in the development of anxiety-like behaviors during withdrawal after chronic ethanol exposure. This investigation also suggests that Arc may be involved in the regulation of DSD and that decreases in both Arc expression and DSD in the CeA may be involved in the neuronal plasticity associated with alcohol-drinking and anxiety-like behaviors (Fig. 7).

Acute ethanol exposure significantly increased levels of BDNF and trkB and induced Arc expression in the CeA and MeA, which may be related to the BDNF-linked activation of Erk1/2 and subsequent phosphorylation of both Elk-1 and CREB during acute ethanol exposure. Acute ethanol also produced anxiolytic effects

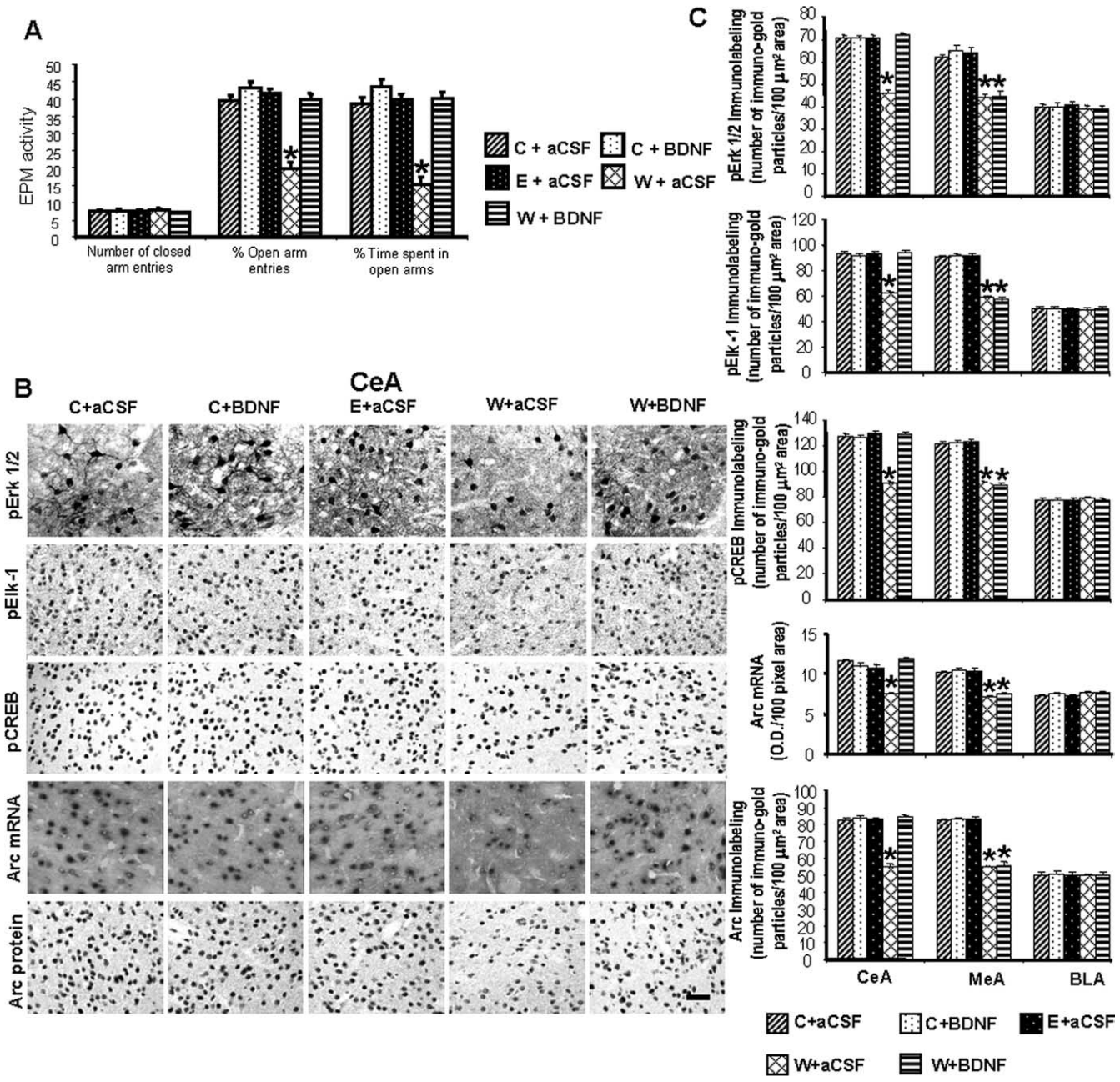

Figure 5. BDNF infusion into the CeA prevented development of anxiety-like behaviors and normalized the reduction in Arc expression and BDNF signaling during ethanol withdrawal. ***A***, The effect of central amygdaloid BDNF infusions on open arm and closed arm activity in the EPM test during ethanol withdrawal. Values are the mean ± SEM of eight to nine rats in each group (control diet-fed rats plus aCSF infusion, C+aCSF; control diet-fed rats plus BDNF infusion, C+BDNF; ethanol diet-fed rats plus aCSF infusion, E+aCSF; ethanol-withdrawn rats (24 h) plus aCSF infusion, W+aCSF; ethanol-withdrawn rats (24 h) plus BDNF infusion, W+BDNF). *$p < 0.001$, significantly different from their respective control group (ANOVA followed by Tukey's test). ***B***, Low-magnification views of Arc mRNA (*in situ* PCR) and Arc, pErk1/2, pElk-1, and pCREB gold immunolabeling (protein) in CeA structures of control diet-fed, ethanol diet-fed, and ethanol-withdrawn rats infused with BDNF or aCSF (scale bar, 40 μm). ***C***, Effect of BDNF infusions into the CeA on mRNA and protein levels of Arc and protein levels of pErk1/2, pElk-1, and pCREB in various amygdaloid (CeA, MeA, and BLA) structures of rats. Values are mean ± SEM of six rats in each group. *$p < 0.001$, significantly different from their respective control group (ANOVA followed by Tukey's test).

in rats, which were similar to other studies in rodents (Pandey et al., 2004, 2005; Wilson et al., 2004) and humans (Lipscomb et al., 1980). These results suggest that a BDNF-induced increase in Arc expression in the CeA and MeA may be involved in producing anxiolytic effects as a result of ethanol exposure. Interestingly, long-term ethanol exposure produced neuroadaptations at the level of BDNF–Arc signaling. The expression of BDNF, trkB, and Arc, as well as the phosphorylation levels of Erk1/2, Elk-1, and CREB, were decreased in both the CeA and MeA during ethanol withdrawal but were maintained at normal levels during chronic ethanol exposure. Behaviorally, ethanol-withdrawn but not ethanol-fed rats displayed anxiety-like behaviors. The protein levels of tErk1/2, as shown here, and CREB (Pandey et al., 2003) were not modulated by ethanol withdrawal, and only the phosphorylated forms of Erk1/2 and CREB were decreased during ethanol withdrawal after chronic ethanol exposure; this may be attributable to the decreased levels of BDNF and trkB in the CeA and MeA. These results indicate that BDNF → trkB → Arc sig-

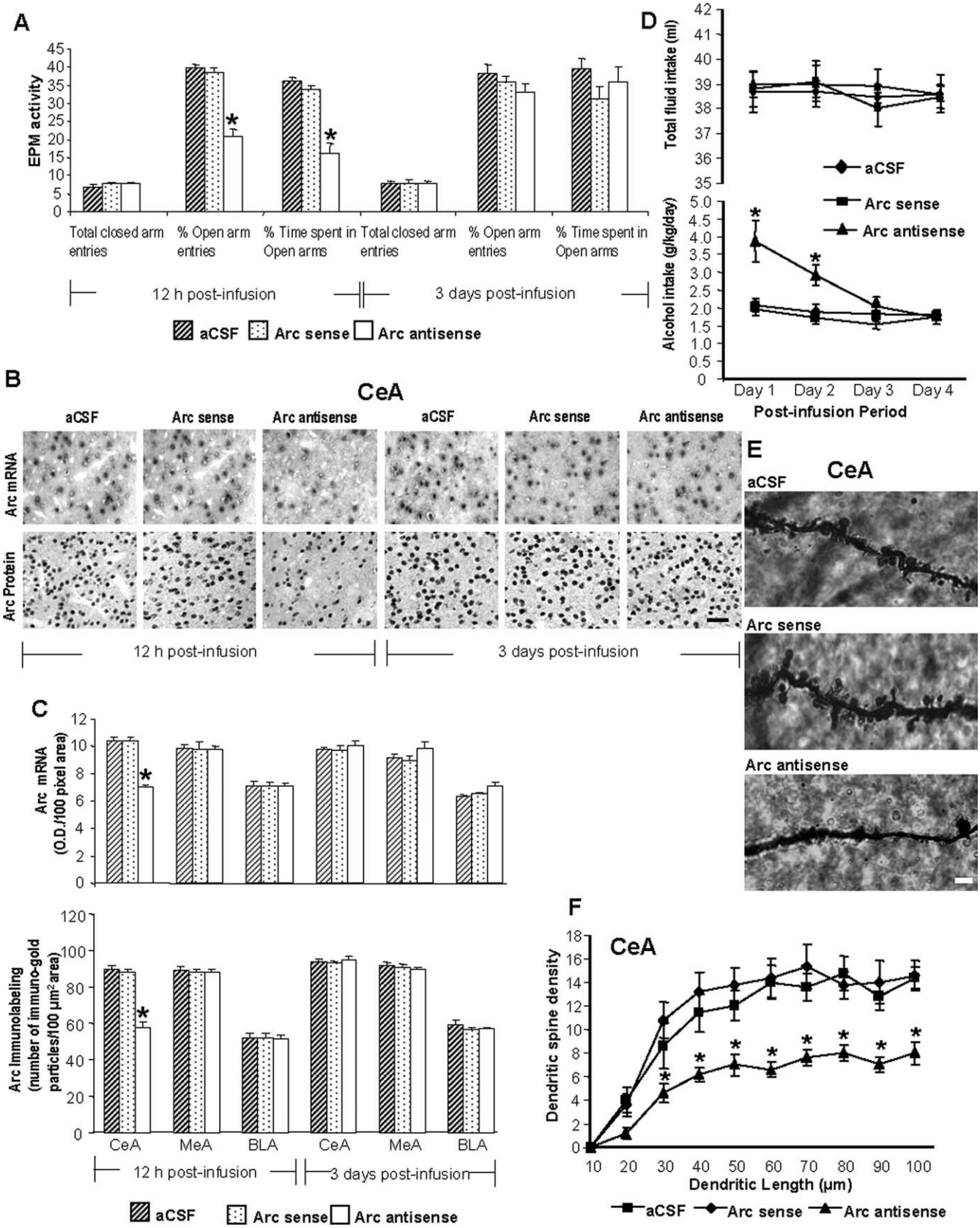

Figure 6. Reduction in Arc expression in the CeA decreased the DSD, provoked anxiety-like behaviors, and promoted alcohol intake in rats. ***A***, Effect of CeA infusion (once) of aCSF, infusion of Arc sense ODNs, or Arc antisense ODNs on open and closed arm activities of the EPM test as a measure of anxiety-like behaviors. Values are the mean ± SEM of 14 rats in each group at 12 h after infusion and six rats in each group at 3 d after infusion. *$p < 0.001$, significantly (ANOVA followed by Tukey's test) different from aCSF-infused rats (12 h after infusion). ***B***, Photomicrographs at low magnification showing Arc mRNA and protein positive cells in the CeA at 12 h and 3 d after a single infusion of aCSF and Arc sense ODNs and Arc antisense ODNs into the CeA (scale bar, 40 μm). ***C***, Quantitation of Arc gold immunolabeling (number of immunogold particles/100 μm^2 area) and mRNA levels of Arc in the amygdaloid structures at 12 h and 3 d after single infusion of aCSF and Arc sense ODNs and Arc antisense ODNs into the CeA. Infusion of Arc antisense ODNs led to decreased Arc expression in the CeA. Values are the mean ± SEM of (*Figure legend continues*.)

naling in the amygdala may act as a homeostatic pathway mediating anti-anxiety and anxiety-like responses attributable to ethanol exposure and withdrawal (Fig. 7).

Preexisting high anxiety or anxiety developing during ethanol withdrawal appears to play an important role in the development and maintenance of alcohol-drinking behaviors in some human alcoholics and in animal models (Weiss and Rosenberg, 1985; Wilson, 1988; Koob, 2003; Pandey et al., 2005). It has been shown that bilateral lesioning of CeA but not of BLA decreased experimental anxiety-like and alcohol-drinking behaviors in rats (Moller et al., 1997). The CeA functions as a major output station for information processing and receives afferent connections from the hypothalamus, thalamus, various cortical structures, and brainstem and also sends efferent connections to the lateral hypothalamus, midbrain, bed nucleus of the stria terminalis, and ventral tegmental area (Koob, 2003; Sah et al., 2003). Because of these crucial connections, the CeA serves as an important neuroanatomical substrate for anxiety and alcoholism (Davis and Whalen, 2001; McBride, 2002; Koob, 2003). Here, we demonstrated that decreased Arc expression in the CeA acts as a neuronal marker for the development of anxiety-like behaviors during ethanol withdrawal. The bilateral infusion of BDNF into the CeA restored Arc expression and also prevented the ethanol withdrawal-related anxiety. BDNF-induced normalization of Arc expression may be attributable to activation of Erk1/2 and the subsequent phosphorylation of both Elk-1 and CREB in the CeA of ethanol-withdrawn rats. These findings suggest that decreased BDNF–Arc signaling in the CeA may be involved in the development of anxiety-like behaviors during ethanol withdrawal.

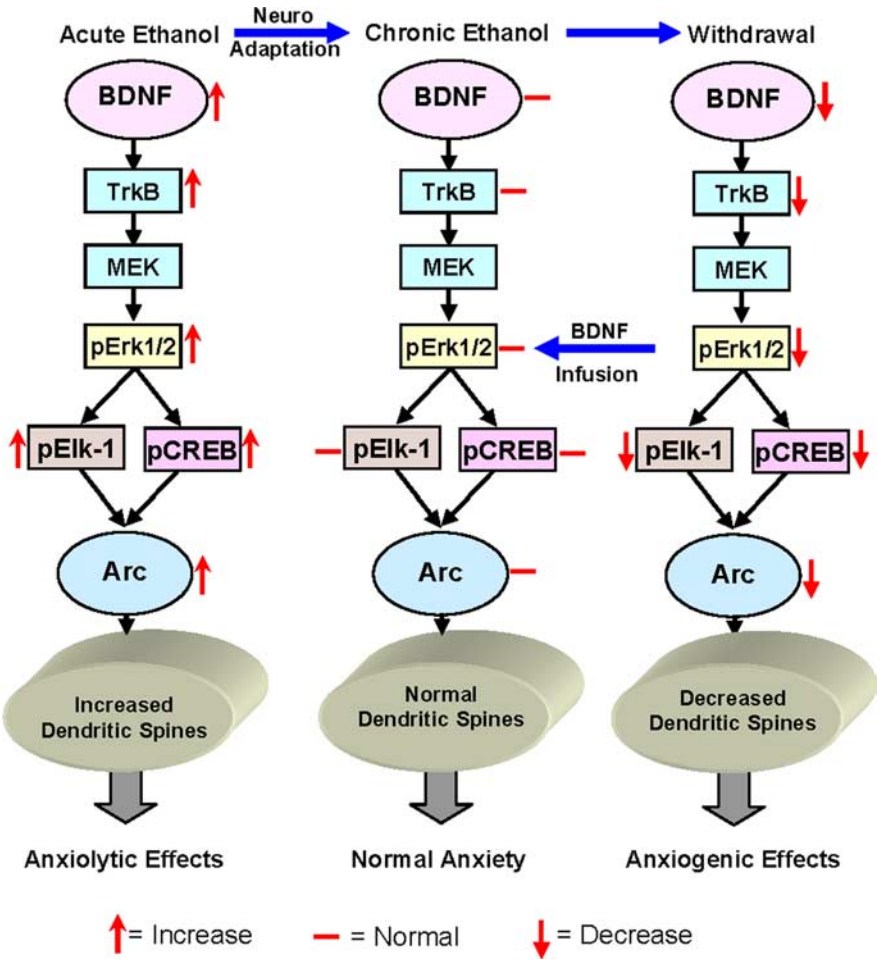

Figure 7. Proposed model for Arc modulation of synaptic strengthening in the CeA during alcohol dependence. The present investigation revealed that increased BDNF⟶trkB⟶Arc signaling in the CeA might be involved in increased synaptic strengthening (increased DSD) that may be operative in the anxiolytic effects of acute ethanol exposure in rats. Conversely, development of anxiety-like behaviors might be related to decreased BDNF⟶trkB⟶Arc signaling associated with decreased DSD in the CeA during ethanol withdrawal after chronic exposure. BDNF infusion into the CeA attenuated the anxiety-like behaviors and normalized the reduction in BDNF–Arc signaling during ethanol withdrawal. Arc antisense ODN infusion into the CeA decreased the DSD in the CeA, provoked anxiety-like behaviors, and promoted alcohol intake. Thus, BDNF–Arc signaling and its relationship to dendritic spines in the CeA is crucial in understanding the underpinning mechanisms of anxiety and alcoholism. MEK, Mitogen-activated protein kinase kinase.

Synaptic activation has been shown to cause Arc mRNA to selectively localize to activated postsynaptic sites on dendrites and to spines in the hippocampus (Steward et al., 1998; Steward and Worley, 2001; Rodriguez et al., 2005), and blocking Arc expression by Arc antisense ODN infusion into the hippocampus impaired long-term potentiation (LTP) and learning and memory (Guzowski et al., 2000; McIntyre et al., 2005). It has previously been shown that BDNF induced LTP in the hippocampus via activation of Erk1/2, phosphorylation of CREB, and through increased Arc expression (Ying et al., 2002). Furthermore, BDNF–trkB signaling has been shown to regulate dendritic spine formation in hippocampal neurons (Ji et al., 2005). These studies suggest that BDNF-induced synaptic consolidation may be mediated via the BDNF–trkB–Arc signaling pathway in the hippocampus. Here, we found that acute ethanol increased Arc levels and DSD in the CeA and MeA. Conversely, we also found that ethanol withdrawal after chronic ethanol exposure decreased Arc levels and DSD in both the CeA and MeA. We suggest the notion that decreased Arc levels may be responsible for the decreased DSD observed in both the CeA and MeA and that this decrease may be involved in producing anxiety-like behaviors and in promoting alcohol-drinking behaviors. A more causal approach in-

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(*Figure legend continued.*) six to nine rats in each group. *$p < 0.001$, significantly (ANOVA followed by Tukey's test) different from aCSF-infused rats (12 h after infusion). ***D***, Effects of central amygdaloid infusion (once) of Arc sense ODNs and antisense ODNs on alcohol intake as measured by the two-bottle free-choice paradigm. Results represent a 9% (ethanol in water solution) alcohol intake (grams per kilogram per day). Decreasing Arc expression via infusion of Arc antisense ODNs into the CeA led to increased alcohol intake, whereas mean total fluid intake (milliliters per day) remained the same. Values are the mean ± SEM of seven to eight rats in each group. *$p < 0.05–0.001$, significantly (repeated measures of ANOVA followed by Tukey's test) different from aCSF-infused rats. ***E***, Photomicrographs showing dendritic spines of Golgi-impregnated neurons in the CeA of aCSF, Arc sense ODNs, and Arc antisense ODNs (12 h after infusion) infused control rats (scale bar, 10 μm). ***F***, Effect of CeA infusion of Arc sense ODNs and antisense ODNs (12 h after infusion) on the DSD in CeA brain structures of rats. Values are mean ± SEM of five rats in each group. *$p < 0.05–0.001$, significantly different from the CeA aCSF-infused rats (repeated measures of ANOVA followed by Tukey's test). Arc antisense ODNs decreased DSD in the CeA.

dicated that Arc antisense ODN, but not sense ODN, infusion into the CeA significantly decreased Arc levels and DSD. Also, rats infused with Arc antisense ODNs into the CeA displayed anxiety-like behaviors and consumed higher amounts of ethanol. Interestingly, when the expression of Arc returned to normal in the CeA, 3 d after infusion of Arc antisense ODNs, anxiety levels and alcohol intake were also normalized. Although there may be several mechanisms involved in regulating DSD, the BDNF-induced expression of Arc may be involved in the regulation of DSD, because Arc interacts with cytoskeletal proteins such as microtubule-associated protein-2 and filamentous actin (Lyford et al., 1995; Fujimoto et al., 2004). It has also been shown that Arc mutant mice failed to form long-lasting memories but had normal anxiety-like behaviors and DSD in hippocampal CA1 neurons (Plath et al., 2006). It is possible that other homeostatic mechanisms may be compensating for the global deletion of Arc from birth, thereby maintaining both normal anxiety and DSD in Arc mutant mice. Previous studies have also shown that ethanol and exposure to other drugs of abuse, such as morphine and cocaine, caused alterations in DSD in the cortex, hippocampus, cerebellum, and nucleus accumbens of rats (Ferrer et al., 1986; Tarelo-Acuna et al., 2000; Robinson et al., 2001, 2002; Zhou et al., 2007).

Recently, we found that decreased BDNF expression attributable to infusion of BDNF antisense ODNs into the CeA and MeA, but not in BLA, was involved in anxiety-like and alcohol-drinking behaviors in rats, which was rescued by coinfusion with BDNF (Pandey et al., 2006). It may be possible that BDNF antisense ODN infusion into the CeA and MeA may have reduced Arc expression and DSD attributable to decreased levels of pErk1/2 and pCREB (Pandey et al., 2006). Future studies will explore the possibility that lower Arc levels and DSD may be operative in the regulation of anxiety and alcohol-drinking behaviors during BDNF antisense ODN-infused conditions. It is also important to mention that infusion of a physiological concentration of exogenous BDNF (50 ng) into the CeA, as we used in the present study and in a previous study (Pandey et al., 2006), produced no effect on anxiety-like behaviors or on BDNF signaling in control rats but reversed the anxiety-like behaviors and deficits in BDNF function in both ethanol-withdrawn rats and BDNF antisense ODN-infused rats. Similarly, treatment of hippocampal slices using a low concentration of BDNF, in the nanogram range, reversed the deficits in basal synaptic transmission in BDNF-deficient but not in wild-type mice (Patterson et al., 1996). Whereas, infusion with a high concentration of BDNF, in the microgram range, into rat hippocampus triggered long-term potentiation, in addition to increasing phosphorylation of Erk1/2 and CREB and inducing Arc expression (Ying et al., 2002). Together, our results suggest the possibility that a physiological concentration of exogenous BDNF may reverse the anxiety-like behaviors and deficits in BDNF signaling under BDNF-deficient conditions, whereas higher concentrations of exogenous BDNF may be required to produce effects on both anxiety-like behaviors and BDNF signaling in control rats.

Conclusions

The findings of the present investigation implicate decreased BDNF–Arc signaling in both the CeA and MeA, as the molecular mechanism that may be involved in the development of anxiety-like behaviors during ethanol withdrawal. Because decreased Arc in the CeA produced a reduction in DSD, provoked anxiety-like behaviors, and increased alcohol intake in normal rats, we suggest that BDNF–Arc signaling in the amygdala may be a common molecular target for the comorbidity of anxiety and alcoholism and, more importantly, may be used as a therapeutic target in developing better treatments for these brain disorders.

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