Published in final edited form as:

Addict Biol. 2011 April ; 16(2): 238–250. doi:10.1111/j.1369-1600.2010.00275.x.

The role of amygdaloid brain-derived neurotrophic factor, activity-regulated cytoskeleton-associated protein and dendritic spines in anxiety and alcoholism

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Abstract

Innate anxiety appears to be a robust factor in the promotion of alcohol intake, possibly due to the anxiolytic effects of self-medication with alcohol. Brain-derived neurotrophic factor (BDNF) and its downstream target, activity-regulated cytoskeleton-associated (Arc) protein, play a role in the regulation of synaptic function and structure. In order to examine the role of the BDNF-Arc system and associated dendritic spines in the anxiolytic effects of ethanol, we investigated the effects of acute ethanol exposure on anxiety-like behaviors of alcohol-preferring (P) and -nonpreferring (NP) rats. We also examined changes in the expression of BDNF and Arc, and dendritic spine density (DSD), in amygdaloid brain regions of P and NP rats with or without ethanol exposure. It was found that in comparison to NP rats, P rats displayed innate anxiety-like behaviors, and had lower mRNA and protein levels of both BDNF and Arc, and also had lower DSD in the central amygdala (CeA) and medial amygdala (MeA), but not in the basolateral amygdala (BLA). Acute ethanol treatment had an anxiolytic effect in P, but not in NP rats, and was associated with an increase in mRNA and protein levels of BDNF and Arc, and in DSD in the CeA and MeA, but not BLA. These results suggest that innate deficits in BDNF-Arc levels, and DSD, in the CeA and MeA may be involved in the anxiety-like and excessive alcohol-drinking behaviors of P rats, as ethanol increased these amygdaloid synaptic markers and produced anxiolytic effects in P rats, but not NP rats.

Keywords

Alcoholism; Anxiety; Amygdala; BDNF; Arc; Dendritic spines

INTRODUCTION

Alcoholism is a complex multifactorial disease influenced by environmental and genetic factors (Cloninger, 1987; Crabbe, 2002; Enoch, 2003; Prescott and Kendler, 1999), as well

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Authors Contribution:

SCP was responsible for the study concept and design, and participated in writing the manuscript and interpreting the findings. SM, HZ, AJS were responsible for conducting behavioral and neurochemical studies. SM also performed calculations, summarized and statistically analyzed the data. He also participated in writing the manuscript and prepared photographs and bar diagrams. All authors read and approved the final version of the article.

as the presence of comorbid psychiatric disorders (Grant et al., 2004, 2005; Schuckit and Hesselbrock, 1994). Dysphoric psychiatric symptoms, such as negative emotionality, may be alleviated through the consumption of alcohol, thus promoting drinking behaviors and eventually resulting in the development of alcohol-use disorders (Koob, 2003; Novak et al., 2003; Pandey, 2003, 2004; Schuckit and Hesselbrock, 1994). Various animal models have been developed to study the neurobiological mechanisms underlying the genetic predisposition for alcoholism, including the alcohol-preferring (P) and -nonpreferring (NP) rat lines (Bell et al., 2006; Bennett et al., 2006; Crabbe, 2008; Li, Lumeng and Doolittle, 1993; McBride and Li, 1998; Tabakoff and Hoffman, 2000). P rats, which displayed greater voluntary ethanol intake than NP rats, exhibited innate anxiety-like behaviors and also, ethanol produced anxiolytic effects in P rats, but not in NP rats (Hwang et al., 2004; Jones et al., 2000; Pandey et al., 2005; Stewart et al., 1993). This suggests that P rats may serve as an appropriate model to investigate the neurobiological basis for the comorbidity of anxiety and alcoholism.

Brain-derived neurotrophic factor (BDNF) is one of the neurotrophic factors involved in the regulation of synaptic function and morphology (Horch, 2004; Messaoudi et al., 2002; Poo, 2001). Human and animal studies have identified that aberrant BDNF signaling may contribute to both anxiety and alcoholism (Chen et al., 2006; Hensler, Ladenheim and Lyons, 2003; Jeanblanc et al., 2009; Lang et al., 2005; Matsushita et al., 2004; Moonat et al., 2010; Pandey et al., 1999). BDNF signaling via the tyrosine kinase B (TrkB) receptor ultimately results in phosphorylation of the cAMP-responsive element binding protein (CREB) gene transcription factor resulting in the upregulation of CREB-target genes, including the immediate-early gene activity-regulated cytoskeleton-associated (Arc) protein, also known as activity-regulated gene 3.1 (*Arg3.1*) (Bramham et al., 2008; Messaoudi et al., 2002; Pandey et al., 2008b; Pizzorusso et al., 2000; Soulé, Messaoudi and Bramham, 2006; Ying et al., 2002). Arc protein is translated locally within synaptically active dendritic spines, and participates in the regulation of dendritic spine density (DSD) and function (Bramham et al., 2010; Huang, Chotiner and Steward, 2007; Lyford et al., 1995; Messaoudi et al., 2007). Amygdaloid brain structures have been shown to serve as a neuroanatomical substrate for fear and anxiety (Davis et al., 2010; LeDoux, 2003), as well as negative emotionality related to alcohol use and abuse (Koob, 2003; Pandey, 2004). BDNF and Arc in amygdaloid structures have been implicated in anxiety-like and alcohol drinking behaviors (Pandey et al., 2006, 2008b).

Recently, we reported that P rats had lower levels of BDNF in the central amygdala (CeA) and medial amygdala (MeA), but not basolateral amygdala (BLA), compared to NP rats (Prakash, Zhang and Pandey, 2008), however innate differences in the downstream effectors of BDNF, such as Arc and DSD, have not yet been investigated in the amygdaloid structures of P and NP rats. Furthermore, the effects of acute ethanol exposure on amygdaloid BDNF-Arc signaling and DSD have not been studied in P and NP rats. In the present investigation, we examined the anxiety-like behaviors of P and NP rats at baseline and also following acute ethanol administration. We then examined changes in the mRNA and protein levels of BDNF and Arc in the CeA, MeA, and BLA of P and NP rats during ethanol exposure. Since BDNF and Arc may play a role in the regulation of dendritic spines, we also determined the DSD in the amygdaloid brain regions of P and NP rats during ethanol exposure.

METHODS

Animals and acute ethanol exposure

All experiments were conducted in accordance with the National Institute of Health's Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee. Adult male P and NP rats (62nd-67th generations) were

received from Indiana University, Indianapolis, IN. Animals were housed in a temperature-controlled facility under a 12 h light/dark cycle, with free access to food and water. Age-matched P and NP rats were used and there were no differences in body weight among the groups [mean $\pm$ SEM (n=18-19): NP + n-Saline = 377 ± 4.9 g, NP + Ethanol = 373 ± 4.6 g, P + n-Saline = 384 ± 3.9 g, P + Ethanol = 383 ± 4.4 g]. The P and NP rats were injected intraperitoneally with ethanol (diluted in n-saline; 1 g/kg) or n-saline alone. One hour after treatment, anxiety-like behaviors were measured using the elevated plus-maze (EPM) test or light-dark box (LDB) exploration test, as described below. Immediately following behavioral measurements, P and NP rats were anesthetized using pentobarbital (50 mg/kg) and brains were collected for immunohistochemistry, *in situ* RT-PCR, or Golgi-Cox staining, as described below. Blood was also collected to measure blood ethanol levels using an Analox Alcohol Analyzer (Analox Instruments, Lunenburg, MA).

Measurement of anxiety-like behaviors by the EPM test

The EPM test was performed as previously described (File, 1993; Pandey et al., 2006, 2008 a,b). In brief, each rat was placed on the central platform of the EPM apparatus facing an open arm. During the 5 min test period, exploration of the open and closed arms of the EPM was monitored and recorded. Results were represented as the mean $\pm$ SEM (n=10) of the percentage of open arm entries and the percentage of time spent on the open arms.

Measurement of anxiety-like behaviors by the LDB exploration test

The LDB exploration test procedure was performed as described previously (Pandey et al., 2008a; Zhang et al., 2010). Following a 5 min habituation period in the testing room, each rat was placed in the dark compartment of the LDB apparatus with its head facing away from the opening to the light compartment. During the 5 min test period, the movement of the rat was monitored via infrared sensors and results were recorded directly to a computer system. The percentage of time spent in either the dark compartment or light compartment was calculated for each animal. Results were represented as mean $\pm$ SEM (n=8-9) of the percentage of time spent in each compartment.

Gold immunolabeling of BDNF and Arc proteins

Protein levels were determined using the gold-immunolabeling histochemical procedure as previously described (Pandey et al., 2008a,b; Prakash et al., 2008). Following perfusion and fixation with paraformaldehyde, rat brains were frozen at -80°C . Coronal sections ($20\text{ }\mu\text{m}$) were incubated in RPMI 1640 (with L-glutamine) medium (Invitrogen, Grand Island, NY) for 30 min, 10% normal goat serum (Vector Labs, Burlingame, CA) in 0.01 M phosphate buffered saline (PBS) containing 0.25% Triton X-100 (PBST) for 30 min and 1% bovine serum albumin (BSA) in PBST (BSA-PBST) for 30 min. Sections were then incubated for 18 h at room temperature in anti-BDNF (H-117, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Arc antibody (H-300, Santa Cruz Biotechnology) [1:200 in BSA-PBST]. Sections were washed and incubated for 1 h in gold particle-conjugated anti-rabbit secondary antibody (Nanoprobes, Yaphank, NY) [1:200 dilution in BSA-PBS] and developed using silver enhancement solution (Ted Pella, Redding, CA). Gold-immunolabeled BDNF and Arc protein levels were quantified using the Loats Image Analysis System (Loats Associates Inc., Westminster, MD) at high magnification (100x). For each brain region, immunogold particles from three fields in each of three adjacent brain sections (9 total object fields) were counted and values were averaged for each animal. Results were represented as mean $\pm$ SEM (BDNF protein: n=6; Arc protein: n=7-8) of the number of immunogold particles/ $100\text{ }\mu\text{m}^2$ area for each amygdaloid brain region.

In situ RT-PCR for BDNF and Arc mRNA measurement

BDNF and Arc mRNA levels were determined using *in situ* RT-PCR as previously described (Pandey et al., 2008a,b; Prakash et al., 2008). Following fixation and freezing of brains, coronal sections (40 μ m) were cut and treated with proteinase K (1 μ g/ml in PBST) for 15 min at 37°C. Sections were subjected to DNase digestion (Promega, Madison, WI) for 18 h and then transferred to PCR tubes containing 100 μ l of reverse transcription mixture (Applied Biosystems, Foster City, CA) for 1 h at 42°C. Negative sections were subjected to the same conditions, although in the absence of reverse transcriptase enzyme. Sections were then transferred to another tube containing PCR mixture (Applied Biosystems) consisting of 100 pmol of each set of primers (BDNF: 5' TAACGGCGGCAGACAAAAGACT 3' and 5' GTGTCTATCCTTATGAATCGCCAGCCAA 3'; Arc: 5' ACAGAGGATGAGACTGAGGCAC 3' and 5' TATTCAGGCTGGGTCCTGTCAC 3') and digoxigenin (DIG)-11-dUTP (Roche Diagnostics, Indianapolis, IN) instead of dTTP. Following PCR cycling, sections were mounted on slides, incubated with alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics, Indianapolis, IN), and stained with nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate (Roche Diagnostics). BDNF and Arc mRNA were quantified by calculation of optical density (OD) in the CeA, MeA and BLA using the Loats Image Analyzer (Loats Associates). The OD from negative sections were subtracted from the positive sections. For each brain region, the OD from three image fields in each of three adjacent brain sections (9 total fields) was quantified and the values were averaged for each animal. Results were represented as mean $\pm$ SEM (n=6) of the OD/100 pixels of area for each amygdaloid region.

Golgi-Cox method for measurement of DSD

DSD was determined by use of the Golgi-Cox staining procedure described in the FD Rapid Golgi Stain Kit manual (FD Neuro Technologies, Baltimore, MD) and described by us previously (Pandey et al., 2008b). Following removal, brains were immediately immersed in impregnation solution for 1 week. Frozen coronal sections (200 μ m) containing amygdaloid brain regions were mounted on slides and dried in the dark at room temperature. Sections were silver stained according the instruction manual, followed by dehydration and clearing in xylene solution. Sections were observed by a light microscope at high magnification (100x). Dendritic spines were counted using the Neurolucida Neuroexplorer program (MicroBrightField Bioscience, Williston, VT). DSD was calculated by Sholl analysis using a 10 μ m increment. Only dendrites which showed complete golgi impregnation from the soma were used for counting. For each brain region, dendrites were counted from three adjacent sections for a total of 9 dendrites which were averaged for each rat. DSD was represented as mean $\pm$ SEM (n=5) of the number of dendritic spines/10 μ m of dendritic length.

Statistical analyses

The differences between the groups were evaluated by a one-way ANOVA test. *Post hoc* comparisons were performed using Tukey's test. $p < 0.05$ was considered to be significant.

RESULTS

Effects of acute ethanol exposure on anxiety-like behaviors in P and NP rats

We examined the anxiety-like behaviors of P and NP rats one hour following intraperitoneal injection of either ethanol (1 g/kg) or *n*-saline. We first measured anxiety-like behaviors using the EPM test in one batch of P and NP rats (Fig. 1A). The percentage of open arm entries and percentage of time spent on the open arms was significantly different among the groups (percent of open arm entries: $F_{3,36}=21.3$, $p < 0.001$; percent of time spent in open

arms: $F_{3,36}=23.0$, $p<0.001$). At baseline, P rats displayed anxiety-like behaviors, in comparison to NP rats, as evidenced by the significantly ($p<0.001$) lower percentage of open arm entries and lower percentage of time spent on open arms by P rats. Acute ethanol exposure attenuated the anxiety-like behaviors of P rats, as shown by a significant ($p<0.001$) increase in the percent of open arm entries and percent of time spent on open arms by P rats, but not NP rats (Fig. 1A). The number of total arm entries (open plus closed arms) was significantly different among the groups ($F_{3,36}=5.5$, $p<0.01$). *Post hoc* analysis revealed that acute ethanol treatment significantly ($p<0.05$) increased total arm entries in P rats, but not NP rats (Fig. 1A), and that this effect was due to a significant ($p<0.001$) increase in open arm entries. In a second batch of P and NP rats, anxiety-like behaviors were measured by the LDB exploration test (Fig. 1B). The percentage of time spent in the light and dark compartments was significantly different among the groups ($F_{3,31}=107.9$, $p<0.001$). At baseline, we found that P rats spent significantly ($p<0.001$) less time in the light compartment and more time in the dark compartment, in comparison to NP rats, indicating that P rats also display anxiety-like behaviors using this testing paradigm. Acute ethanol exposure resulted in reduced anxiety-like behaviors in P rats as demonstrated by a significant ($p<0.001$) increase in the time spent in the light compartment and decreased time spent in the dark compartment, whereas acute ethanol exposure did not significantly change LDB exploration in NP rats (Fig. 1B). General activity was measured by the total number of ambulations in the LDB apparatus, and was similar among all groups (data not shown). Blood ethanol levels were not significantly different between P and NP rats treated with acute ethanol [mean $\pm$ SEM ($n=16$): NP = 93.5 ± 4.7 mg/dl, P = 95.1 ± 5.3 mg/dl]. These results indicate that, in comparison to NP rats, P rats exhibit anxiety-like behaviors which were attenuated by acute ethanol exposure. Interestingly, acute ethanol exposure had no effects on EPM or LDB test performance in NP rats.

Baseline levels and the effects of acute ethanol exposure on amygdaloid BDNF and Arc expression in P and NP rats

We previously reported that innate BDNF expression levels in the CeA and MeA, but not BLA, of P rats were lower than those of NP rats (Prakash et al., 2008). Here, we have confirmed those results and extended the study to examine the effects of acute ethanol exposure on amygdaloid BDNF expression. BDNF mRNA and protein levels in the CeA and MeA were significantly different among the groups (BDNF mRNA in CeA: $F_{3,20}=50.0$, $p<0.001$; BDNF mRNA in MeA: $F_{3,20}=29.0$, $p<0.001$; BDNF protein in CeA: $F_{3,20}=13.6$, $p<0.001$; BDNF protein in MeA: $F_{3,20}=8.5$, $p<0.001$). We found that, in comparison to NP rats, P rats had significantly ($p<0.01-0.001$) lower baseline levels of BDNF mRNA and protein in the CeA and MeA, but not BLA (Fig. 2A&B). Acute ethanol treatment significantly ($p<0.01-0.001$) increased BDNF mRNA and protein levels in the CeA and MeA, but not BLA, of P rats, however, no significant changes were observed in the amygdaloid BDNF expression of NP rats (Fig. 2A&B). These results suggest the possibility that innate anxiety-like behaviors of P rats may be related to reduced levels of BDNF expression in the CeA and MeA, and that the anxiolytic effects of acute ethanol exposure may be associated with increased BDNF expression.

Induction of the Arc immediate-early gene is a downstream consequence of BDNF signaling that may mediate the effects of BDNF on synaptic structure and function (Bramham et al., 2010; Messaoudi et al., 2002; Pandey et al., 2008b; Ying et al., 2002). We measured mRNA and protein levels of Arc in P and NP rats following injection with either ethanol or *n*-saline. Arc mRNA and protein levels in the CeA and MeA were significantly different among the groups (Arc mRNA in CeA: $F_{3,20}=31.0$, $p<0.001$; Arc mRNA in MeA: $F_{3,20}=8.4$, $p<0.001$; Arc protein in CeA: $F_{3,26}=31.4$, $p<0.001$; Arc protein in MeA: $F_{3,26}=15.5$, $p<0.001$). We found that P rats innately expressed significantly ($p<0.01-0.001$) lower levels of Arc mRNA

and protein in the CeA and MeA, but not BLA, in comparison to NP rats (Fig. 3A&B). Acute ethanol injection significantly ($p<0.01-0.001$) increased Arc mRNA and protein levels in the CeA and MeA, but not BLA, of P rats, without having any significant effects on Arc expression in NP rats (Fig. 3A&B). These results indicate that BDNF signaling via Arc induction in the amygdala may play a role in the anxiety-like behaviors and anxiolytic effects of ethanol observed in P rats, but not NP rats.

Amygdaloid DSD corresponds to BDNF and Arc expression in P and NP rats at baseline and following acute ethanol exposure

BDNF signaling via the induction of the Arc immediate-early gene has been shown to result in structural and functional synaptic changes, including the proliferation and lengthening of dendritic spines (Bramham et al., 2008; Horch, 2004; Messaoudi et al., 2007; Pandey et al., 2008b). To study the potential structural effects of amygdaloid BDNF signaling and Arc expression in P and NP rats, we measured the DSD within amygdaloid brain regions of a group of P and NP rats at baseline and following acute ethanol administration. DSD in the CeA and MeA was found to be significantly different among the groups (DSD in CeA: $F_{3, 16}= 21.3, p<0.001$; DSD in MeA: $F_{3, 16}= 24.7, p<0.001$). P rats were found to have significantly ($p<0.001$) lower DSD in the CeA and MeA, but not BLA, in comparison to NP rats. Following injection of acute ethanol, DSD was significantly ($p<0.001$) increased in the CeA and MeA, but not BLA, of P rats. Acute ethanol did not significantly change DSD in amygdaloid regions of NP rats (Fig. 4A&B). These findings correspond with amygdaloid BDNF and Arc levels suggesting the possible involvement of BDNF-Arc signaling in the modulation of dendritic spine morphology. Lower amygdaloid DSD may play a role in innate anxiety-like behaviors seen in P rats, as compared to NP rats, and increased DSD may be related to the anxiolytic effects of acute ethanol in P rats (Fig. 5).

DISCUSSION

The present investigation provides evidence that lower BDNF and Arc levels, and DSD, in the CeA and MeA may be involved in the anxiety-like behaviors of P rats, in comparison to NP rats, and that increased amygdaloid BDNF-Arc signaling and DSD by ethanol exposure may be related to the anxiolytic effects of ethanol in P rats (Fig. 5). Several previous studies have used the EPM test to determine anxiety-like behaviors of P and NP rats at baseline and following acute ethanol exposure (Hwang et al., 2004; Pandey et al., 2005; Stewart et al., 1993). In the present investigation, we have confirmed previous results that have shown anxiety-like behaviors using the EPM test (Pandey et al., 2005; Stewart et al., 1993) and have extended these findings using another behavioral test of anxiety, the LDB exploration test. Alcohol exposure has previously been shown to have anxiolytic effects in humans (Lipscomb et al., 1980). Epidemiological studies have also shown a significant relationship between anxiety and alcoholism (Grant et al., 2004; Novak et al., 2003; Schuckit and Hesselbrock, 1994). It has been suggested that individuals experiencing anxiety may consume alcohol as an attempt to reduce dysphoria associated with anxiety (Bolton, Robinson and Sareen, 2009; Robinson et al., 2009; Valentin, Mounts and Deacon, 2004; Wilson, 1988). Since P and NP rats are selectively bred for higher and lower alcohol preference, respectively, these findings may suggest that the high voluntary ethanol consumption reported in P rats (Bell et al., 2006; Li et al., 1993; Pandey et al., 2005), in comparison to NP rats, may be related to the anxiolytic effects produced by ethanol exposure. Further mechanistic study into the role of BDNF signaling in the anxiolytic effects of acute ethanol in P rats is needed to provide insight into the common neurobiological mechanisms underlying anxiety and alcoholism.

Studies in humans and animal models have implicated a role for BDNF and related signaling in both anxiety and alcoholism (Chen et al., 2006; Davis, 2008; Hashimoto, 2007; Uhl et al.,

2001). A single nucleotide polymorphism in the BDNF gene has been found to result in a reduced BDNF secretion phenotype (Chen et al., 2008; Egan et al., 2003) that has been linked with the development of anxiety (Chen et al., 2006; Jiang et al., 2005; Lang et al., 2005) and alcoholism (Matsushita et al., 2004). BDNF haplodeficient mice have been found to voluntarily consume greater amounts of ethanol compared to wild-type mice (Hensler et al., 2003; McGough et al., 2004). BDNF signaling in the hippocampus and striatum has also been implicated as a possible homeostatic pathway that may be operative in preventing the development of alcohol dependence and may also contribute to the regulation of ethanol consumption (Jeanblanc et al., 2006; Logrip, Janak and Ron, 2008; McGough et al., 2004). Specifically, a more recent study suggested that endogenous BDNF signaling in the dorsolateral striatum may regulate alcohol-drinking behaviors (Jeanblanc et al., 2009). Conditional deletion of BDNF has been shown to result in increased anxiety-like behaviors (Rios et al., 2001). Also, deletion of TrkB, the BDNF receptor, increased anxiety-like behaviors, which was associated with a reduction in DSD (Bergami, Berninger and Canossa, 2009; Bergami et al., 2008). In contrast, overexpression of brain TrkB resulted in decreased anxiety-like behaviors, suggesting a role for BDNF-TrkB signaling in the regulation of anxiety-like behaviors (Koponen et al., 2004).

Previously, we identified a role for amygdaloid BDNF signaling in anxiety-like and alcohol-drinking behaviors via exogenous manipulation of amygdaloid BDNF levels in SD rats (Pandey et al., 2006). We found that infusion of BDNF antisense oligodeoxynucleotides (ODNs) into the CeA and MeA, but not BLA, provoked anxiety-like behaviors and increased ethanol consumption, and that these effects could be rescued by co-infusion of BDNF (Pandey et al., 2006). These studies demonstrated a mechanistic role for amygdaloid BDNF signaling in the regulation of anxiety-like behaviors and ethanol consumption. Although the findings of the present investigation are correlative in nature, in light of evidence from previous studies, it could be possible that reduced BDNF expression and signaling in the CeA and MeA may be involved in anxiety-like behaviors of P rats. Also, an increase in amygdaloid BDNF levels may be responsible for the observed reduction in anxiety-like behaviors following acute ethanol exposure.

BDNF signaling has been implicated in the regulation of synaptic function and structure, which may be mediated by the induction of the downstream effector immediate-early gene *Arc* (Bramham et al., 2008; Messaoudi et al., 2002; Pandey et al., 2008b; Ying et al., 2002). Binding of BDNF to the high-affinity TrkB receptor results in the activation of extracellular-signal regulated kinase (Erk1/2), which subsequently phosphorylates and activates transcription factors CREB and Elk-1, resulting in the downstream upregulation of *Arc* (Pandey et al., 2008b; Pizzorusso et al., 2000; Poo, 2001; Reichardt, 2006; Tao et al., 1998). The *Arc* gene contains response elements with unique binding sites for CREB and Elk-1, such that regulation of *Arc* gene transcription occurs through BDNF signaling and synaptic activity (Bramham et al., 2010; Kawashima et al., 2009; Pintchovski et al., 2009; Waltereit et al., 2001). Following transcription, *Arc* mRNA is transported to distal dendrites where local translation occurs within synaptically active dendritic spines, resulting in spine proliferation (Huang et al., 2007; Messaoudi et al., 2007; Steward and Worley, 2001; Steward et al., 1998). Recently, we found that SD rats withdrawn after chronic ethanol exposure, which exhibit anxiety-like behaviors in comparison to controls, displayed reduced BDNF expression and signaling in the CeA and MeA, but not BLA, that was associated with a decrease in the downstream BDNF effectors, *Arc* and DSD (Pandey et al., 2008b). We further demonstrated that exogenous BDNF infusion into the CeA of these rats had an anxiolytic effect that was associated with an increase in BDNF signaling and *Arc* expression (Pandey et al., 2008b). We also found that acute ethanol exposure in SD rats resulted in increased BDNF signaling components, and *Arc* and DSD (Pandey et al., 2008b). In the present investigation, we found that lower amygdaloid BDNF expression levels of P rats, in

comparison to NP rats, were associated with lower Arc levels and DSD, and acute ethanol exposure increased BDNF, Arc and DSD in the CeA and MeA of P, but not NP rats. Taken together along with previous evidence (Pandey et al., 2006, 2008b), these data suggest that lower amygdaloid BDNF expression may be responsible for the lower Arc levels and DSD that may be involved in anxiety-like and excessive alcohol-drinking behaviors of P rats.

Arc protein has several functions at the level of the dendritic spine, which may underlie the effects of BDNF signaling on synaptic function and structure (Bramham et al., 2008, 2010; Soulé et al., 2006). Examination of learning tasks in Arc knockout mice and rats subjected to intra-hippocampal infusion of Arc antisense ODNs suggests that Arc protein is critical to the processes underlying learning and memory, including long-term potentiation (LTP) (Guzowski et al., 2000; McIntyre et al., 2005; Messaoudi et al., 2007; Plath et al., 2006). However, the mechanisms underlying the regulatory processes governing spine morphology via Arc have been less explored. It has been shown that blockade of LTP induction via the infusion of Arc antisense ODNs results in decreased F-actin that can be reversed by the actin stabilizing drug, jasplakinolide (Messaoudi et al., 2007). The stabilization of F-actin has been suggested as a key downstream mediator of dendritic spine enlargement and an increase in DSD (Bramham, 2008; Fischer et al., 1998). Recently, we have examined the role of Arc and associated changes in DSD in anxiety-like and alcohol-drinking behaviors (Pandey et al., 2008b). We found that the infusion of Arc antisense ODNs into the CeA of SD rats had anxiogenic effects and resulted in a reduction in DSD (Pandey et al., 2008b). We further demonstrated that this reduction in Arc expression and DSD was associated with increased alcohol intake (Pandey et al., 2008b). These data suggest that reduced Arc signaling and DSD in the CeA may be responsible for anxiety-like behaviors and increased alcohol consumption. Since P rats display innately lower amygdaloid Arc expression and lower DSD compared to NP rats, and in light of our previous studies in SD rats, reduced Arc signaling and DSD in the CeA and MeA of P rats may play a critical role in the anxiety-like and alcohol-drinking behaviors of P rats. Furthermore, we found that acute ethanol exposure resulted in increased BDNF, Arc and DSD in the CeA and MeA of P rats, but not NP rats, suggesting that amygdaloid BDNF-Arc signaling associated with spine proliferation may be related to the anxiolytic effects of acute ethanol. The effects of chronic ethanol exposure on dendritic spine number and morphology have been studied by various researchers (Chandler, 2003; Ferrer et al., 1986; Zhou et al., 2007). Chronic ethanol exposure has been found to result in the modification of dendritic spine morphology and DSD in various brain regions of both mice and rats (Carpenter-Hyland and Chandler, 2006; Lescaudron, Jaffard and Verna, 1989; Riley and Walker, 1978; Wenisch et al., 1998; Zhou et al., 2007). A reduction in DSD has also been shown in the cortical pyramidal neurons of human alcoholics (Ferrer et al., 1986). These studies suggest that neuroadaptations resulting in changes in DSD and spine morphology due to chronic ethanol exposure may play a role in altered synaptic plasticity during alcoholism.

In summary, the findings of the present investigation implicate a role for reduced amygdaloid BDNF expression associated with reduced Arc expression and DSD in the comorbidity of anxiety and alcoholism. Furthermore, our findings suggest that alcohol-induced increases in amygdaloid BDNF expression which may be related to Arc induction and spine proliferation may play a role in the anxiolytic effects of ethanol. Since the anxiolytic effects of acute ethanol were observed in P rats, but not NP rats, these rats may serve as an adequate model for the self-medicating effects of alcohol consumption in those with comorbid anxiety disorders. These findings along with our previous findings (Pandey et al., 2008b; Prakash et al., 2008) clearly indicate that the amygdaloid BDNF-Arc signaling pathway may be an effective pharmacotherapeutic target for the treatment of anxiety-spectrum disorders that may be associated with the development of alcohol abuse and dependence.

Acknowledgments

This study was supported by the grants from the National Institute on Alcohol Abuse and Alcoholism (AA-013341; AA-010005; AA-016690) and the Department of Veterans Affairs (Merit Review Grant; Research Career Scientist award) to SCP. The Alcohol Research Resource Award (R24AA015512) to Indiana University also supported this study by providing us with selectively bred P and NP rats.

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Fig.1.

The effects of acute ethanol exposure (1 g/kg; I.P.) on anxiety-like behaviors in alcohol preferring (P) and non-preferring (NP) rats, as measured by the elevated plus-maze (A) and light/dark box exploration test (B). Values are the mean $\pm$ SEM of 8-10 rats in each group.

*Significantly different from their respective control groups ($p < 0.05$ - 0.001 ; ANOVA followed by Tukey's test).

Fig. 2.

A) Representative low-magnification photomicrographs (Scale bar = 50 μm) of brain-derived neurotrophic factor (BDNF) protein gold-immunolabeling and *in-situ* RT-PCR (BDNF mRNA labeling) in central amygdala (CeA) and medial amygdala (MeA) of alcohol preferring (P) and non-preferring (NP) rats treated with either *n*-saline or ethanol (1 g/kg).

B) The effects of acute ethanol exposure on the protein and mRNA levels of BDNF in the amygdaloid structures of P and NP rats. Values are the mean $\pm$ SEM of 6 rats in each group.

*Significantly different from their respective control groups ($p < 0.01$ - 0.001 ; ANOVA followed by Tukey's test).

Fig.3.

A) Representative low-magnification photomicrographs (Scale bar = 50 μm) of activity-regulated cytoskeleton-associated (Arc) protein gold-immunolabeling and *in situ* RT-PCR (Arc mRNA labeling) in central amygdala (CeA) and medial amygdala (MeA) of alcohol preferring (P) and non-preferring (NP) rats treated with either *n*-saline or ethanol (1 g/kg).

B) The effects of acute ethanol exposure on the protein and mRNA levels of Arc in the amygdaloid structures of P and NP rats. Values are the mean $\pm$ SEM of 6-8 rats in each group. *Significantly different from their respective control groups ($p < 0.01$ - 0.001 ; ANOVA followed by Tukey's test).

Fig. 4.

A) Representative low-magnification photomicrographs (Scale bar = 50 μ m) showing Golgi-impregnated neurons in the central amygdala (CeA) of alcohol preferring (P) and non-preferring (NP) rats treated with either *n*-saline or ethanol (1 g/kg). The boxed areas of the low magnification photomicrographs are shown at high magnification (Scale bar = 10 μ m) in the adjacent photomicrograph showing dendritic spines. B. The effects of acute ethanol exposure on DSD (number of dendritic spines/10 μ m of dendritic length) in the CeA, medial amygdala (MeA), and basolateral amygdala (BLA) of P and NP rats. Values are mean $\pm$ SEM of 5 rats per group. *Significantly different from control groups ($p < 0.001$; ANOVA followed by Tukey's test).

Fig. 5.

A hypothetical model of the anxiolytic response of acute ethanol, and brain-derived neurotrophic factor (BDNF)-induced changes in synaptic plasticity in the amygdaloid circuitry. In comparison to NP rats, P rats have lower levels of BDNF, activity-regulated cytoskeleton-associated protein (Arc), and dendritic spine density (DSD) in the central amygdala (CeA) and medial amygdala (MeA), which is associated with innate anxiety-like behaviors in P rats, but not NP rats. Acute ethanol administration results in an anxiolytic response, and increases BDNF, Arc and DSD in the CeA and MeA in P rats, but not NP rats. This suggests the possibility that innately low levels of BDNF and Arc, and associated DSD, in the CeA and MeA, may play a role in the anxiety-like and excessive alcohol-drinking behaviors of P rats. (↓) Decrease; (↑) Increase; (—) Normal.