Neuropsychopharmacology (2006) 31, 1406–1419

www.neuropsychopharmacology.org

The Decreased Cyclic-AMP Dependent-Protein Kinase A Function in the Nucleus Accumbens: A Role in Alcohol Drinking but not in Anxiety-Like Behaviors in Rats

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The nucleus accumbens (NAc) brain structures have been implicated in the reward and reinforcing properties of ethanol. The present study investigated the role of nucleus accumbal cyclic AMP (cAMP)-dependent protein kinase A (PKA) signaling in alcohol drinking and anxiety-like behaviors of rats. It was found that infusion of PKA inhibitor (Rp-cAMP) into the NAc shell significantly increased the alcohol but not the sucrose intake, without modulating the anxiety-like behaviors, as measured by elevated plus maze test in rats. PKA inhibitor infusion into the NAc shell significantly decreased the protein levels of α-catalytic subunit of PKA (PKA-Cα) and phosphorylated cAMP response element-binding protein (p-CREB) as well as decreased the protein levels of neuropeptide Y (NPY) in the shell but not in the NAc core of rats. On the other hand, infusion of PKA activator (Sp-cAMP) or NPY alone into the NAc shell did not produce any changes in alcohol intake; however, when these agents were coinfused with PKA inhibitor, they significantly attenuated the increases in alcohol preference induced by pharmacological inhibition of PKA. Interestingly, PKA activator coinfusion with PKA inhibitor into the NAc shell significantly normalized the PKA inhibitor-induced decreases in the protein levels of PKA-Cα and p-CREB as well as of NPY in the NAc shell of rats. Taken together, these results provide the first evidence that decreased PKA function in the NAc shell is involved in alcohol drinking but not in anxiety-like behaviors of rats. Furthermore, decreased function of PKA may regulate alcohol drinking behaviors via CREB-mediated decreased expression of NPY in the NAc shell of rats.
Neuropsychopharmacology (2006) **31,** 1406–1419. doi:10.1038/sj.npp.1300900; published online 28 September 2005

Keywords: CREB; PKA; NPY; nucleus accumbens; alcohol preference; anxiety

INTRODUCTION

Alcohol is one of the most widely abused drugs in the world (NIAAA, 1993). Understanding the molecular mechanisms in the specific neurocircuitry associated with alcohol drinking behaviors is important to find a pharmacological means of halting the progression of alcohol addiction. The nucleus accumbens (NAc) is a brain region commonly associated with reward, motivation, and reinforcing effects of ethanol (Imperato and Di Chiara, 1986; McBride *et al*, 1995, 1999; Koob and LeMoal, 1997, 2001). The NAc represents a key area in the sense that it regulates limbic–motor interactions in the brain (Kalivas *et al*, 1999; Mogenson *et al*, 1980). This makes it a vital component for reward-seeking behaviors and thus represents a neurocircuitry involved in positive affective states of addiction (Koob 2003a, b; Pandey, 2004). Functionally and anatomically, the NAc has been categorized into two subdivisions, with the more dorsally located NAc core associated with motor areas such as the substantia nigra and the more ventrally located NAc shell associated with more limbic brain regions (Heimer *et al*, 1991; Zahm, 1999, 2000). The NAc shell receives dopaminergic efferents of the ventral tegmental area (VTA). This pathway is often referred to as the mesolimbic dopaminergic system and has been implicated in alcohol and drug addiction (Robbins and Everitt, 1996; Wise, 1996; Weiss *et al*, 1993; Nestler, 2001; Hodge *et al*, 1997; Zocchi *et al*, 2003).

The gene transcription factor, cyclic AMP (cAMP) response element-binding (CREB) protein, is a nuclear protein that is activated by phosphorylation (at serine-133) by several different protein kinase pathways such as protein kinase A (PKA), Ca^{2+}/calmodulin-dependent protein kinase IV (CaMK IV), and ribosomal S6 kinase (RSK) via the mitogen-activated protein (MAP) kinase pathway (Impey *et al*, 1999; Silva *et al*, 1998; Soderling, 1999). Phosphorylated CREB (pCREB) regulates the expression of several CREB target genes including neuropeptide Y (NPY) (Mayr

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Received 31 January 2005; revised 12 July 2005; accepted 15 August 2005
Online publication: 18 August 2005 at http://www.acnp.org/citations/Npp081805050070/default.pdf

and Montminy, 2001; Lonze and Ginty, 2002; Shieh *et al*, 1998; McClung and Nestler, 2003; Pandey *et al*, 2004). Most of the studies associated with CREB and the NAc have focused on cocaine and morphine dependence (Barrot *et al*, 2002; Carlezon *et al*, 1998; McClung and Nestler, 2003; Self and Nestler, 1998; Widnell *et al*, 1996). It has been shown that decreased CREB function in the NAc is associated with increased cocaine and morphine reward (Carlezon *et al*, 1998; Barrot *et al*, 2002). Interestingly, increasing PKA activity in the NAc using the PKA activator, Sp-cAMP, leads to a decrease in cocaine reward as measured by cocaine self-administration, whereas the PKA inhibitor, Rp-cAMP, had an opposite effect and increased cocaine reward (Self *et al*, 1998).

Evidence for the direct involvement of CREB in alcohol drinking behaviors comes from CREB α/Δ haplodeficient mice, which have decreased CREB and pCREB protein levels in the NAc, cortex, hippocampus, and amygdala, and consume higher amounts of alcohol compared to littermate wild-type mice (Pandey *et al*, 2004). Some studies have reported that modulation of PKA signaling in the brain alter the alcohol drinking behaviors in animals (Thiele *et al*, 2000; Wand *et al*, 2001; Yao *et al*, 2002). Others and we have found that voluntary ethanol intake leads to decreases in CREB phosphorylation without modulating the CREB protein levels specifically in the NAc shell but not in the core structures of NAc (Misra *et al*, 2001; Li *et al*, 2003). Also, it has been shown that C57BL/6 mice, which innately consume higher amounts of alcohol compared to DBA/2 mice, have decreased CREB, pCREB, and NPY protein levels specifically in the NAc shell compared to DBA/2 mice (Misra and Pandey, 2003). These previous studies suggest that PKA and CREB may be involved in alcohol preference and dependence; however, the direct role of nucleus accumbal PKA signaling in alcohol drinking behaviors is unclear at present. Since NPY is a CREB-inducible gene that is highly abundant in the NAc (Hendry, 1993), shows rewarding-like properties (Josselyn and Beninger, 1993; Brown *et al*, 2000), and is altered in brain areas after administration of alcohol (Roy and Pandey, 2002; Bison and Crews, 2003), it is possible that NPY may play a role in regulating the mesolimbic reward pathway and subsequently alcohol drinking behaviors. We therefore decided to pharmacologically manipulate the phosphorylation status of CREB using PKA inhibitor (Rp-cAMP) and activator (Sp-cAMP) in the NAc shell and then examine the effects on alcohol drinking behaviors. We also examined the effects of NPY infusion in the NAc shell on alcohol drinking behaviors.

Several studies link higher anxiety levels with initiation and maintenance of alcohol drinking behaviors in humans and animals (Cappell and Herman, 1972; Bibb and Chambless, 1986; Spanagel *et al*, 1995; Kushner *et al*, 1990; Koob, 2003a; Pandey, 2003). The CREB signaling in the central nucleus of amygdala (CeA) in particular has been implicated in the association between alcohol drinking behaviors and anxiety (Pandey *et al*, 2003a, b). Studies have shown that the overexpression of the nonfunctional CREB (mutated at serine-133) in the NAc shell leads to increased anxiety-like behaviors in rats (Barrot *et al*, 2002). On the other hand, it has been shown that increasing CREB in the NAc shell produces depression-like effects in rats (Pliakas *et al*, 2001; Newton *et al*, 2002). However, the role of nucleus accumbal PKA signaling in anxiety-like behaviors and their relationship to alcohol preference remains unclear. Therefore, we also examined the effects of pharmacological manipulations of CREB phosphorylation in the shell of NAc on anxiety-like behaviors in rats.

MATERIALS AND METHODS

Surgery for Cannulae Implantation

All experiments were conducted in accordance with the National Institute of Health Guidelines for Care and Use of Laboratory Animals and approved by the Institutional Animal Care Committee. Adult male Sprague–Dawley rats (200–250 g) purchased from Harlan (Indianapolis, IN) were used in this study. All rats were housed under a 12-h light/dark cycle and had free access to water and food. Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and placed in a stereotaxic apparatus. Rats were implanted bilaterally with CMA/11 guide cannulae (CMA microdialysis, MA) targeted 3 mm above the NAc shell. Cannulae were secured to the skull using dental cement and screws. The coordinates for the NAc shell were 1.2 mm anterior and $\pm$1.0 mm lateral to the bregma, and 6.8 mm ventral. Cannulae were covered with guided caps (CMA microdialysis, MA).

Effects of PKA Activator and Inhibitor on Alcohol and Sucrose Preference

Following surgery, rats were allowed to recover for 7 days after which they were subjected to habituation to the two-bottle free choice paradigm for alcohol preference (Pandey *et al*, 2003a; Misra and Pandey, 2003). Artificial cerebrospinal fluid (aCSF), PKA inhibitor (Rp-cAMP) (Rp-adenosine 3′, 5′-cyclic monophosphothioate triethylamine), and/or PKA activator (Sp-cAMP) (Sp-adenosine 3′, 5′-cyclic monophosphothioate triethylamine) (Sigma, St Louis, MO) were infused into the NAc shell. All drugs were dissolved in aCSF and doses were based on doses used in previous studies showing that these concentrations of Rp-cAMP and Sp-cAMP can alter opiate and alcohol withdrawal symptoms and working memory performance (Pandey *et al*, 2003a; Punch *et al*, 1997; Taylor *et al*, 1999). After cannulation, rats were placed in individual cages and habituated to drink water evenly from two bottles. Once rats started drinking water equally, they were infused over a period of 2 min with 0.5 μl/day of aCSF, Rp-cAMP (40 nmol), and/or Sp-cAMP (80 nmol), for 3 days between 1600 and 1800, just prior to the start of the dark cycle. At this time, rats were given either 7 or 9% ethanol in water in one bottle and water in the other under the two-bottle free choice paradigm. Bottle positions were changed daily to avoid the formation of a place preference. Ethanol and water as well as total fluid consumption (ml) was measured daily at this time and fresh solutions were provided every day. After ethanol preference measurements, rats were perfused and brains were collected for the gold-immunolabeling procedure and Nissl staining. A separate group of rats were cannulated to the NAc shell and tested for 4% sucrose preference using the two-bottle free choice paradigm. Rats were habituated to drinking

water evenly from two bottles after which they were infused (over a period of 2 min) with 0.5 μl/day of aCSF or Rp-cAMP (40 nmol), once daily for 3 days. Following each day infusion, rats were provided with fresh 4% sucrose dissolved in water in one bottle and water in the other. Sucrose and water intakes were recorded daily. Following the sucrose preference, brains were collected for Nissl staining. Animal body weights were recorded for both ethanol and sucrose preference tests.

Gold-Immunolabeling of CREB, p-CREB, PKA, NPY, and NeuN in the NAc Shell and Core Structures

The cellular protein expression of various signaling proteins was determined by the gold-immunolabeling histochemical procedure as described recently by us (Misra *et al*, 2001; Pandey *et al*, 2001, 2003a; Roy and Pandey, 2002). To examine the neurotoxicity related to PKA activator or inhibitor infusion into NAc shell, we also determined the immunolabeling of neuron-specific neuronal (NeuN) marker proteins. Brain sections (20 μm) were washed with phosphate-buffered saline (PBS) (2×10 min) and then blocked with RPMI 1640 (with L-glutamine) medium (Life Technologies, Grand Island, NY, USA) 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 anti-CREB, pCREB, PKA-Cα, NPY, or NeuN primary antibodies [1 : 500 dilution for CREB, pCREB (Upstate Biotech., Lake Placid, NY), PKA-Cα (Santa Cruz Biotech., Santa Cruz, CA) and NPY (ImmunoStar Inc., Hudson, WI) and a 1 : 200 dilution for NeuN (Chemicon, Temecula, CA) in 1% BSA prepared in PBS containing 0.25% Triton X-100] for 18 h at room temperature. Following 2×10-min washes with PBS and 2×10-min washes with 1% BSA in PBS, sections were incubated with a gold particle (1 nm)-conjugated anti-rabbit secondary antibody (1 : 200 dilution in 1% BSA in PBS) for 1 h at room temperature. Sections are further rinsed several times in 1% BSA in PBS, followed by rinsing in water. The gold-immunolabeling was then silver enhanced (Ted Pella Inc., Redding, CA) between 12 and 25 min depending on the type of antibody and washed several times with water. Sections were then mounted on slides and examined under a light microscope. For negative control brain sections, an identical protocol was used; however, 1% BSA in PBS was substituted for the primary antibody. The antibodies for CREB, p-CREB, PKA-Cα, and NPY are well characterized in our lab (Pandey *et al*, 2001, 2003a, 2004; Roy and Pandey, 2002). The quantification of gold-immunolabeled proteins was performed using an Image Analysis System connected to a light microscope that calculates the number of silver-enhanced immuno-gold particles/100 μm^2 area of a defined brain structure at high magnification ($\times 100$). The threshold of each image was set up in such a way that an area without staining gives a zero count. Under this condition, silver-enhanced immuno-gold particles in the defined areas (minimum of three fields in each section) of three adjacent brain sections of each rat were counted and then values were averaged for each rat.

Effects of NPY Infusions into NAc on Alcohol Preference

Another group of rats were cannulated to the NAc shell as described above. Rats were placed in individual cages and habituated to drink water evenly from two bottles. After rats started drinking the same amount of water from both bottles, rats were infused once daily with 0.5 μl of aCSF, Rp-cAMPs (40 nmol), and/or NPY (100 pmol), for 3 days between 1600 and 1800. At this time, rats were given either 7% ethanol in water in one bottle or water in the other. The ethanol and water intakes were measured daily at this time and fresh solutions were provided. The percent of water and ethanol intake from total fluid intake (ml) were calculated. Animal body weights were recorded. There were no significance differences in body weights between groups. After ethanol preference measurements, rats were perfused and brains were collected for Nissl staining to check the cannula positions.

Measurement of Anxiety-Like Behaviors Using the Elevated Plus Maze

Another group of Sprague–Dawley rats were implanted with cannulae into the NAc shell and once again allowed to recover for 1 week. Animals were then infused with 0.5 μl once daily either with aCSF or Rp-cAMP (0.5 μl of 40 nmol) for 3 days in the NAc shell as mentioned above. At 12 h after infusion (last infusion), animals were used to measure the anxiety-like behaviors using elevated plus maze (EPM) test. The test procedure was the same as that described by our group and others (Pandey *et al*, 1999a, 2001, 2003a; Rassnick *et al*, 1993; Lal *et al*, 1993). The EPM is constructed of white Plexiglas and black metal and consists of two open arms (no Plexiglas walls) and two closed arms (with Plexiglas walls) directly opposite each other and interconnected to a central platform (Lafayette Instruments Company, IN). Each test rat was habituated for 5 min to the testing room and then placed on the central platform facing an open arm. The animal was then observed for a 5-min test period. The number of entries made to each type of arm (open or closed) as well as the amount of time spent in each type of arm was recorded. EPM test results are expressed as the mean ± SEM of the percent of open-arm entries and the mean percent of time spent on the open arms (open-arm activity), and the general activity of the rats was represented by total number of entries (closed plus open arms).

Confirmation of Cannulae Position and Dispersion of Solution

Nissl staining was performed in the brain sections of all animals that were cannulated to check the cannula positions. Methylene blue infusion was performed in some animals in order to verify cannulae positions and dispersions of solutions in the NAc shell. This technique has been shown to act as a marker for the location of infusion of exogenous materials into targeted brain regions (Verner *et al*, 2003). A few rats were infused with aCSF (0.5 μl) and Rp-cAMP (0.5 μl of 40 nmol) once daily for 3 days. On the fourth day morning, 2% methylene blue dye (Sigma, St Louis, MO) in PBS (pH 7.4) was infused once into the NAc

shell of these rats. At 1 h after infusion, brains were dissected out and placed in 4% paraformaldehyde for 3 days for fixation. Brains were then frozen. Sections (50 μm) were cut and placed in 0.1 M phosphate buffer (PB) (pH 7.4) for diaminobenzidine (DAB) staining. Sections were washed in 50% EtOH followed by three 30 min washes in a Tris phosphate buffer solution (TPBS; Tris-HCl 10 mM, PB 10 mM, and 0.9% NaCl, pH 7.4). Sections were then washed in a nickel-DAB solution (DAB 10 mg; 5 ml 0.4 M PB and 10 ml ddH_2O, 200 μl of 0.4% NH_4Cl, 200 μl of 20% glucose, and 800 μl 1% nickel ammonium sulfate) for 10 min. Immunoreactivity was visualized with addition of glucose oxidase 1 μl/ml. This reaction resulted in a black staining at the site of methylene blue infusion. The area of dye diffusion was observed under a light microscope.

Statistics

The differences between two groups were evaluated by Mann–Whitney U test. The differences between more than two groups were evaluated by one-way analysis of variance (ANOVA) test followed by multiple comparisons using the Tukey's test.

RESULTS

Effect of NAc Shell Infusion of PKA Inhibitor/Activator on Ethanol Preference in Rats

We implanted bilateral cannulae targeting the NAc shell. To examine the pattern of diffusion of the solution, methylene blue dye was infused in some rats. The DAB staining of these sections indicates that the dye was diffused only in the shell structures of the NAc but not in the core structures of the NAc (Figure 1). These results suggest that microinjected solutions were diffused only in the NAc shell. For alcohol preference, rats were habituated to drink water from two bottles. When rats started drinking water equally from either bottle, bilateral microinjections of a PKA activator (Sp-cAMP) and/or inhibitor (Rp-cAMP) or aCSF (0.5 μl of aCSF, 0.5 μl of 40 nmol Rp-cAMP, or 80 nmol Sp-cAMP) into the NAc shell were performed once daily for 3 days.

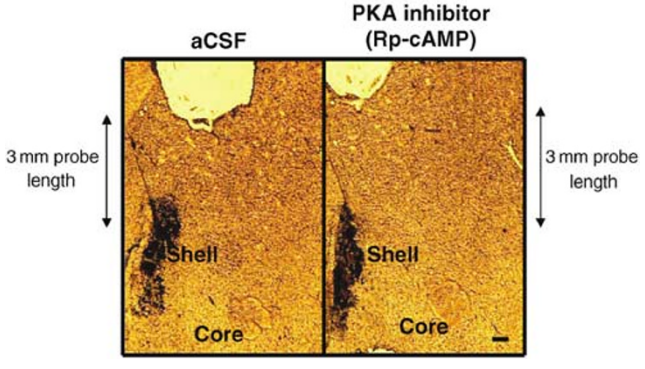

Figure 1 Low magnification view of DAB staining of NAc structures after infusion of methyl blue (dye). As can be seen, DAB has reacted with the blue dye and formed a black complex in the NAc shell. The photomicrograph also indicates the position of the cannula, which is just above the NAc shell (scale bar = 200 μm).

These concentrations of Rp-cAMP and Sp-cAMP have been shown to be effective in manipulating PKA-Cα, p-CREB, and NPY levels in the CeA of rats (Pandey *et al*, 2003a; Zhang and Pandey, 2003). The rats were tested for alcohol preference during these 3 days only and were not exposed to ethanol before. Rats were provided with 7% ethanol in one of the bottles and water in the other bottle during these 3 days. Intake was measured in g/kg/day for the 3 days of infusion (upper panels) as well as mean percent of ethanol intake and percent of water intake of their total fluid intake for 3 days (Figure 2, lower panel). It was found that rats infused with PKA inhibitor consumed significantly larger amounts of 7% alcohol compared to aCSF-infused rats (ANOVA, df = 3, $P<0.001$, Tukey's test for Rp-cAMP infusion *vs* aCSF infusion $P<0.001$) (Figure 2). PKA activator infusion had no effect on 7% alcohol intake; however, when coinfused with PKA inhibitor (15 min before PKA inhibitor infusion), it significantly attenuated the PKA inhibitor-induced increase in 7% alcohol intake. The total fluid intakes (ml/day) were similar between groups in each study (Figure 2, lower panels). Also, there was no significant difference in body weights between the groups. These results clearly demonstrated that decreased PKA function in the NAc shell increases alcohol preference in rats.

In a separate group of animals, we investigated the effect of PKA inhibitor infusion on 9% alcohol preference using the two-bottle free choice paradigm. Intake was measured in g/kg/day for the 3 days of infusion (upper panels) as well as

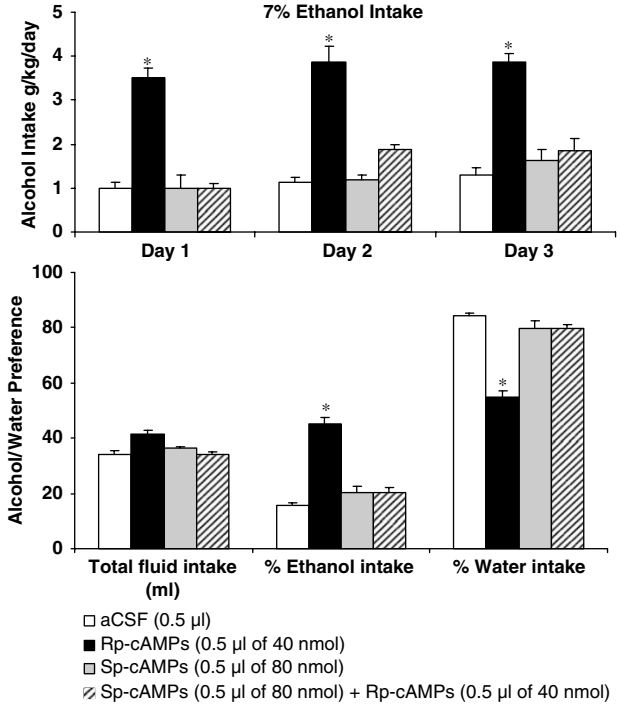

Figure 2 Effect of NAc shell infusion (3 days of once daily) of PKA activator (Sp-cAMP) and/or PKA inhibitor (Rp-cAMP) on 7% alcohol preference as measured by the two-bottle free choice paradigm. Results represent daily ethanol intake (g/kg/day; upper panel) and the mean percentage of 7% (ethanol in water solution) ethanol intake and percentage of water intake of total fluid intake of 3 days (lower panel). Values are the mean ± SEM of five rats in each group. *Significantly ($P<0.001$) different from aCSF-infused rats.

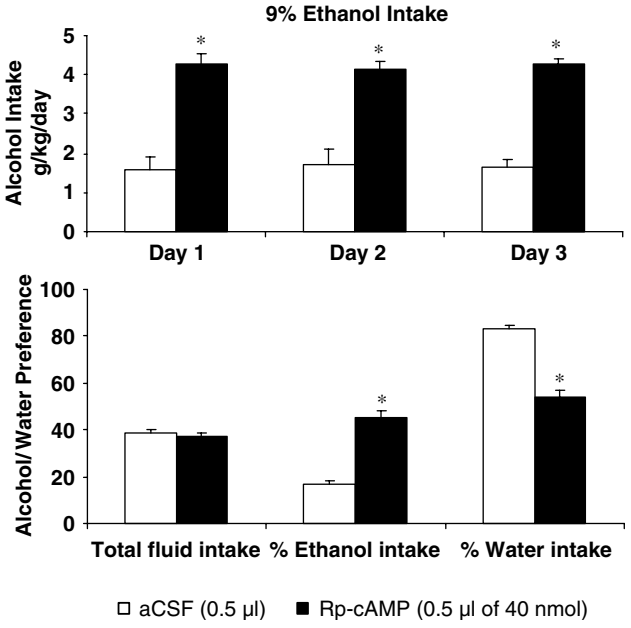

Figure 3 Effect of NAc shell infusion of PKA inhibitor once daily for 3 days on 9% alcohol as measured by the two-bottle free choice paradigm. Results represent the alcohol intake (g/kg/day) for the 3 days of infusion (upper panel), and the mean percentage of ethanol intake and water intake of total fluid intake (ml) of 3 days (lower panel). Values are the mean ± SEM of five rats in each group. *Significantly ($P<0.001$) different from aCSF-infused rats.

mean percent of ethanol intake and percent of water intake of their total fluid intake for 3 days (Figure 3). It was found that rats infused with 0.5 µl of 40 nmol Rp-cAMP had a significantly higher preference for 9% ethanol than those infused with 0.5 µl of aCSF (Rp-cAMP infusion *vs* aCSF infusion $P<0.001$) (Figure 3). We measured the mean blood alcohol levels of Rp-cAMP-infused rats on the morning of the 4th day after the rats had consumed ethanol for 3 days and found it to be 108 ± 12 mg% [mean ± SEM ($n=5$ rats)].

Effect of NAc Shell Infusion of PKA Inhibitor/Activator on the Cellular Protein Expression of CREB, pCREB, PKA-Cα, NPY, and NeuN in the NAc

After NAc shell infusion of aCSF, Rp-cAMP, Sp-cAMP, or Sp-cAMP + Rp-cAMP and measurement of 7% alcohol preference, brains were collected for immunohistochemistry on the 4th day. The CREB- and pCREB protein-positive nuclei are shown in Figures 5a and 6a, respectively. The PKA-Cα- and NPY-positive cell bodies are shown in Figures 4a and 7a, respectively. It was found that there were no differences in CREB protein levels in the NAc shell or core between rats infused with aCSF, Rp-cAMP, Sp-cAMP, or Sp-cAMP + Rp-cAMP (Figure 5a and b). However, Rp-cAMP infusion into the NAc shell caused significantly lower levels of PKA-Cα, pCREB, and NPY in the NAc shell (ANOVA, df = 3, $P<0.001$, Tukey's test for Rp-cAMP infusion *vs* aCSF infusion $P<0.001$) but not in the core compared to aCSF-infused rats (Figures 4a and b, 6a and b, and 7a and b, respectively). Interestingly, coinfusion of a PKA activator (Sp-cAMP, 15 min prior to Rp-cAMP infusion) blocked the decreases in protein levels of PKA-Cα, pCREB, and NPY associated with Rp-cAMP infusion alone in the NAc shell (Figures 4a and b, 6a and b, and 7a and b). PKA activator infusion alone led to significant increases in protein levels of PKA-Cα in the NAc shell (ANOVA, df = 3, $P<0.001$, Tukey's test for Sp-cAMP infusion *vs* aCSF infusion $P<0.001$) (Figure 4a and b), but no significant difference was observed in pCREB or NPY protein levels compared to aCSF-infused rats (Figures 6a and b, and 7a and b). There was no difference in PKA-Cα, pCREB, or NPY levels found in the NAc core after infusion of aCSF, Rp-cAMPs, and/or Sp-cAMPs into NAc shell, indicating that the changes are specific to the NAc shell (Figures 4a and b, 6a and b, and 7a and b). These results suggest that inhibition of PKA in the NAc shell decreases the CREB phosphorylation and NPY expression in the shell but not the NAc core in rats. Since PKA activator antagonized the PKA inhibitor-induced decreases in CREB phosphorylation and NPY expression, these results suggest that PKA inhibitor-induced decreases in CREB phosphorylation were mediated by PKA but not other protein kinases.

In order to confirm that there was no tissue damage or neuronal loss, Nissl staining was performed (data not shown) and neuron-specific nuclear (NeuN) protein levels were measured. The NeuN protein-positive nuclei are shown in Figure 8a. It was found that there was no neuronal loss in the NAc shell, as NeuN protein levels were similar in all groups in both the NAc shell and core (Figure 8a and b). These results suggest that infusion of Rp-cAMP, Sp-cAMP, or NPY does not produce any toxicity or neuronal damage in the target brain region.

Effect of NAc Shell Infusion of NPY on PKA Inhibitor-Induced Ethanol Intake in Rats

If PKA inhibitor increases the alcohol intake via decreasing the NPY expression in the NAc shell, then NPY coinfusion would prevent the PKA inhibitor-induced increases in alcohol intake. We tested this possibility. For this purpose, a separate group of rats were cannulated to the NAc shell and infused with aCSF (0.5 µl), Rp-cAMP (0.5 µl of 40 nmol), and/or NPY (0.5 µl of 100 pmol). Alcohol preference (7% ethanol) was measured using the two-bottle free choice paradigm. After the rats were habituated to drinking water evenly from two bottles, they were infused with aCSF, Rp-cAMP, and/or NPY. NPY infusion was performed 15 min before PKA inhibitor infusion. Rats were provided with a 7% ethanol in water solution in one bottle and water in the other. It was found that rats infused with Rp-cAMP consumed significantly more ethanol solution compared to aCSF-infused rats (ANOVA, df = 3, $P<0.001$, Tukey's test for Rp-cAMP infusion *vs* aCSF infusion $P<0.001$) (Figure 9). Also, it was found that NPY coinfusion with Rp-cAMP was able to attenuate the PKA inhibitor-induced increases in ethanol intake (Figure 9). NPY infusion alone had no significant effect on 7% ethanol intake. The total fluid intake (ml/day) between all groups was similar (Figure 9). These data indicate that decreasing pCREB and NPY via infusion of Rp-cAMP in the NAc shell leads to increases in alcohol preference; however, coinfusion with NPY is able to attenuate this increased preference to alcohol. Taken together, these results suggest that decreased function of

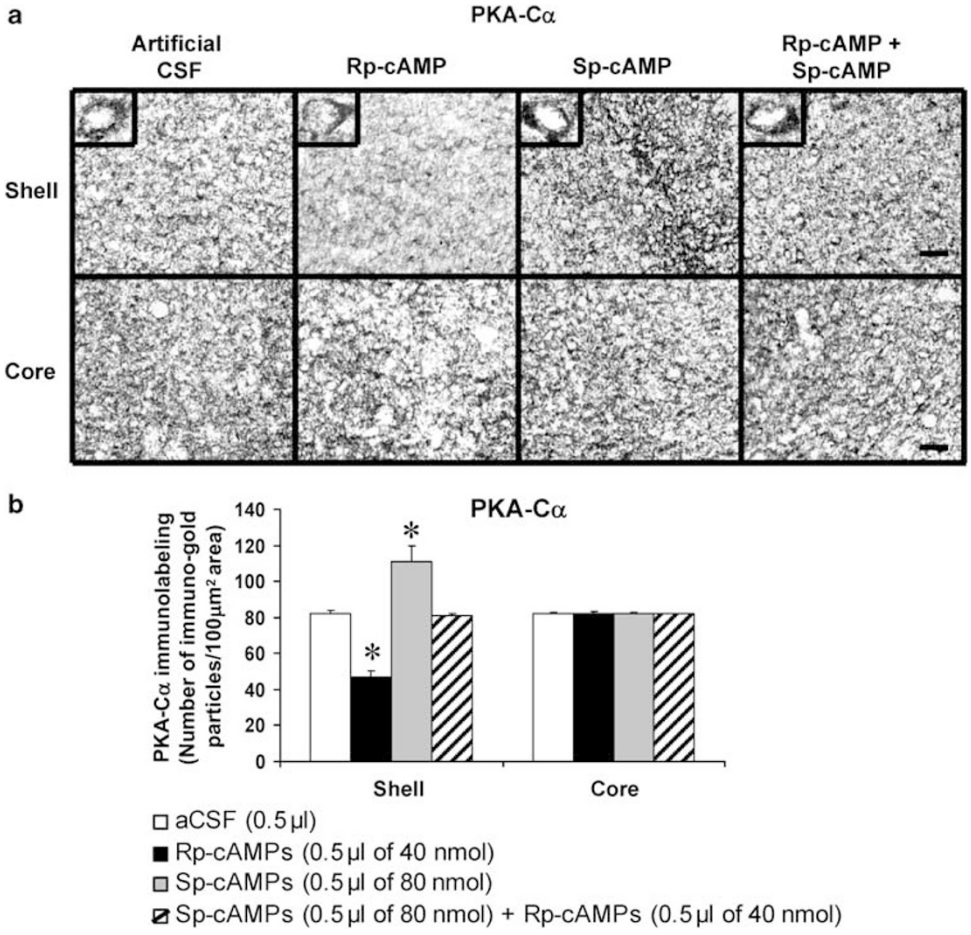

Figure 4 (a) Photomicrographs at low magnification showing PKA-Cα gold-immunolabeling in the NAc structures (shell and core), after 3 days of once daily NAc shell infusion of aCSF, Sp-cAMP, and/or Rp-cAMP, and subsequent alcohol preference testing (scale bar = 30 μm). Inset areas in the upper panel indicate immuno-gold particles within a single cell body at high magnification (× 100). (b) Quantitation of PKA-Cα gold-immunolabeling (number of immuno-gold particles/100 μm^2 area) in the NAc shell and core after 3 days of once daily NAc shell infusion of aCSF, PKA activator (Sp-cAMP), and/or PKA inhibitor (Rp-cAMP), and subsequent alcohol preference testing. Infusion of PKA inhibitor (Rp-cAMP) leads to decreases, while PKA activator (Sp-cAMP) leads to increases in PKA-Cα protein levels in the NAc shell. Interestingly, coinfusion of PKA activator with PKA inhibitor attenuates the decreases in PKA-Cα associated with PKA inhibitor alone in the NAc shell. Values are the mean ± SEM of five rats in each group. *Significantly ($P < 0.001$) different from aCSF-infused rats.

PKA → CREB → NPY signaling in the NAc shell is involved in promoting alcohol drinking behaviors in rats.

Effect of NAc Shell Infusion of PKA Inhibitor on Sucrose Preference in Rats

It is possible that Rp-cAMP-induced increases in alcohol consumption may be associated with a taste preference or increased fluid/food intake. Therefore, a separate group of rats were bilaterally cannulated targeting the NAc shell and, 1 week after surgery, these rats were habituated to drink water evenly from two bottles following the two-bottle free choice paradigm. When the rats were drinking water evenly from two bottles, they were infused with either aCSF (0.5 μl) or Rp-cAMP (0.5 μl of 40 nmol) once daily for 3 days and subsequently given fresh 4% sucrose in one bottle and water in the other daily. It was found that there were no significant differences in sucrose intake (ml/day) in rats infused with aCSF (0.5 μl) compared to those infused with Rp-cAMP (0.5 μl of 40 nmol) (Figure 10, upper panel). Further, there was no difference in the total fluid intake (ml/day) as well as the percent of water and sucrose intakes between the two groups (data represent mean of 3 days) (Figure 10, lower panel). These data indicate that rats infused with PKA inhibitor display no significant differences in preference for sucrose but have an increased preference for alcohol compared to aCSF-infused rats.

Effect of NAc Shell Infusion of PKA Inhibitor on Anxiety-Like Behaviors in Rats

A separate group of rats were implanted with cannulae targeted to the NAc shell. After a 1-week postsurgery recovery, they were infused with either aCSF (0.5 μl) or Rp-cAMP (0.5 μl of 40 nmol), once daily for 3 days. Approximately 12 h after last infusion, rats were subjected to the EPM test in order to measure anxiety-like behaviors (Figure 11). As shown above, there is a decrease in PKA → CREB → NPY signaling in the NAc shell under this condition. It was found that the infusion of Rp-cAMP into the NAc shell had no significant effect on total arm entries, as well as on percent open-arm entries and percent time

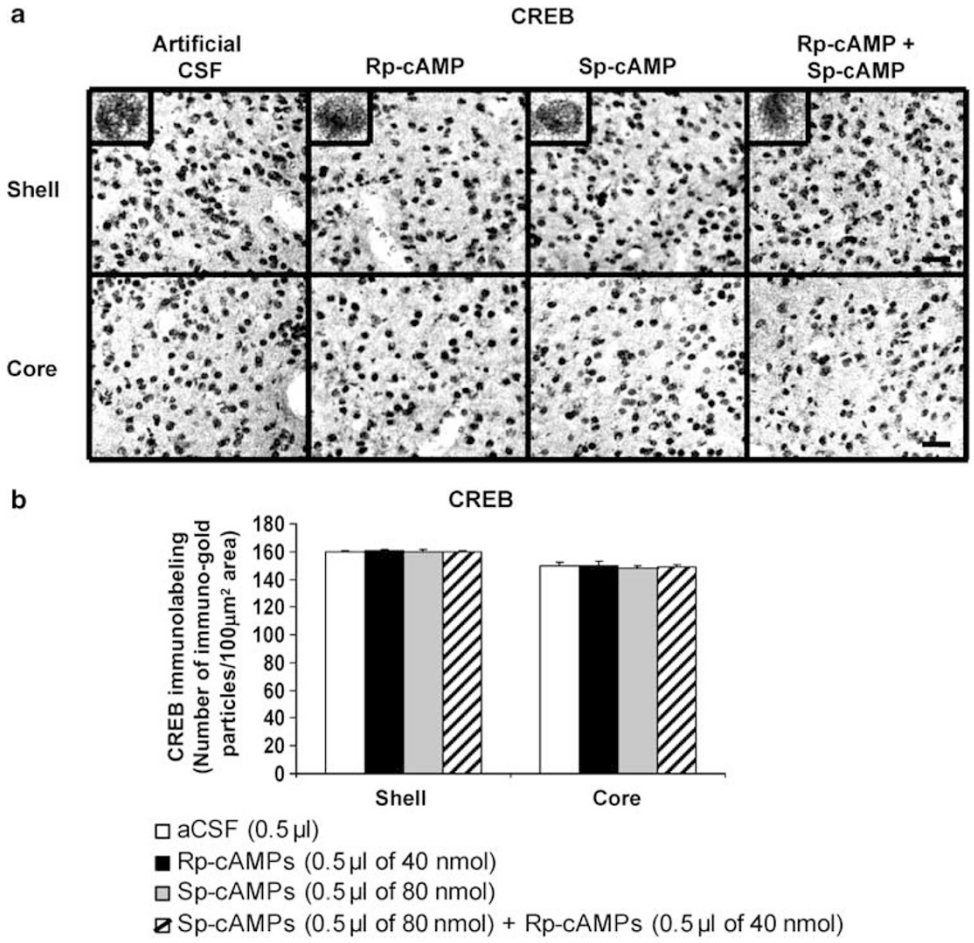

Figure 5 (a) Photomicrographs at low magnification showing CREB gold-immunolabeling in the NAc structures (shell and core), after 3 days of once daily NAc shell infusion of aCSF, Sp-cAMP, and/or Rp-cAMP, and subsequent alcohol preference testing (scale bar = 30 μm). Inset areas in the upper panel indicate immuno-gold particles within a single nucleus at high magnification (× 100). (b) Quantitation of CREB gold-immunolabeling (number of immuno-gold particles/100 μm^2 area) in the NAc shell and core after 3 days of once daily NAc shell infusion of aCSF, PKA activator (Sp-cAMP), and/or PKA inhibitor (Rp-cAMP), and subsequent alcohol preference testing. There is no difference in total CREB protein levels between groups in the NAc shell or core. Values are the mean ± SEM of five rats in each group. *Significantly ($P < 0.001$) different from aCSF-infused rats.

spent on the open arm. These results indicate that decreased NPY levels through decreased PKA-dependent CREB phosphorylation in the NAc shell do not modulate anxiety-like behaviors in rats and these animals have higher alcohol preference independent of anxiety behaviors.

DISCUSSION

The major findings of the present investigation are as follows. (1) Infusion of Rp-cAMP (PKA inhibitor) into the NAc shell leads to decreased pCREB and NPY protein levels in this brain region and behaviorally rats have a higher alcohol preference. Furthermore, coinfusion of Sp-cAMP (PKA activator) prior to Rp-cAMP (PKA inhibitor) infusion prevented the decreases in pCREB and NPY, and also blocked the PKA inhibitor-induced increase in alcohol preference. (2) Coinfusion of NPY prior to PKA inhibitor infusion into the NAc shell also attenuates increases in alcohol preference. (3) Infusion of PKA inhibitor has no significant effect on sucrose preference. (4) Infusion of PKA inhibitor (Rp-cAMPs) into NAc shell has no effect on anxiety-like behaviors in rats. These results suggest that decreased PKA-dependent CREB function in the shell of NAc is involved in alcohol drinking behaviors independent of taste preferences, consummatory behaviors, or anxiety-like behaviors in rats. Therefore, it is possible that these rats may be drinking alcohol due to its pharmacological effects on the brain specifically in the NAc shell.

Several lines of evidence indicate that CREB phosphorylation is crucial in maintaining normal synaptic plasticity in the brain (Frank and Greenberg, 1994; Glazewski *et al*, 1999; Duman *et al*, 2000; Josselyn *et al*, 2001). Previous studies have found that pCREB protein levels are decreased in the NAc shell, but not altered in the frontal cortex, CeA, as well as the NAc core during voluntary ethanol exposure (Misra *et al*, 2001; Li *et al*, 2003). The NAc appears to have a major role in the integration of brain reward mechanisms in that its shell structures are involved in limbic and emotional responses toward stimuli, whereas the core structures are engaged in motor-associated outputs (Self and Nestler, 1998; Ikemoto and Panksepp, 1999; Carelli and Ijames, 2000; Kalivas *et al*, 2001). A substantial amount of data indicates that there is an anatomical and functional dichotomy

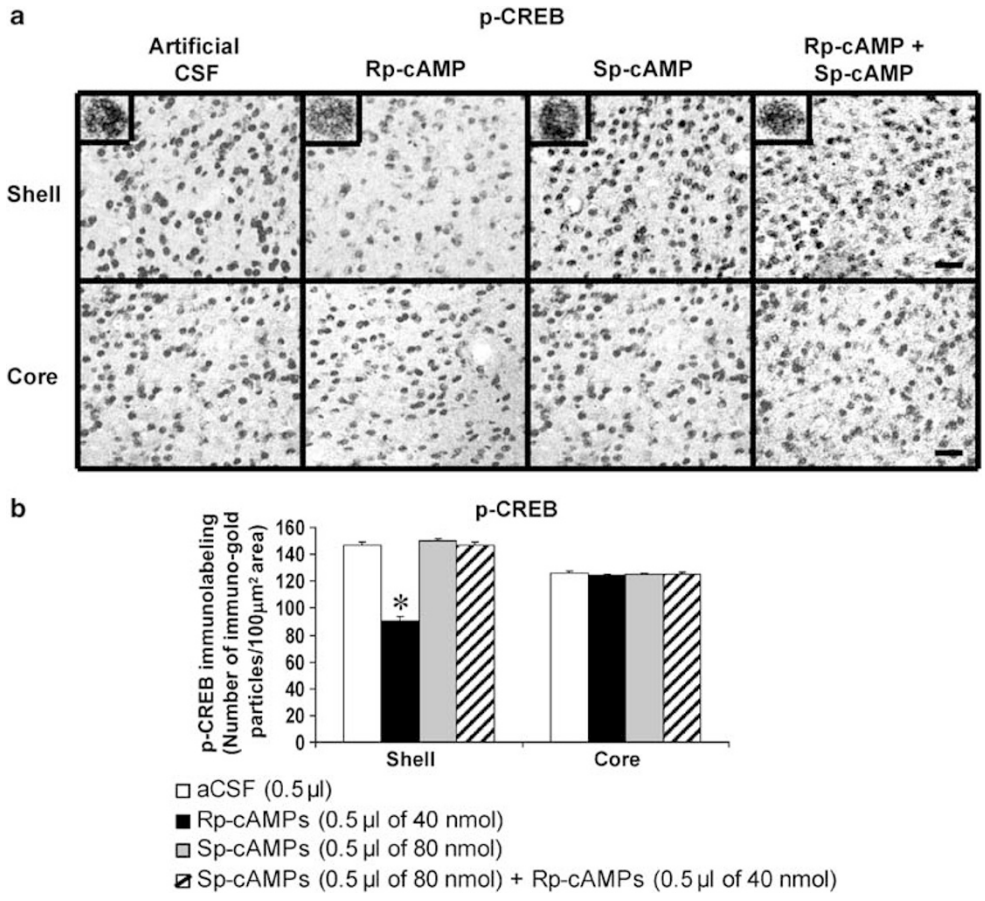

Figure 6 (a) Photomicrographs at low magnification showing pCREB gold-immunolabeling in the NAc structures (shell and core), after 3 days of once daily NAc shell infusion of aCSF, Sp-cAMP, and/or Rp-cAMP, and subsequent alcohol preference testing (scale bar = 30 μm). Inset areas in the upper panel indicate immuno-gold particles within a single nucleus at high magnification (× 100). (b) Quantitation of pCREB gold-immunolabeling (number of immuno-gold particles/100 μm^2 area) in the NAc shell and core after 3 days of once daily NAc shell infusion of aCSF, PKA activator (Sp-cAMP), and/or PKA inhibitor (Rp-cAMP), and subsequent alcohol preference testing. The pCREB protein levels are decreased after infusion of PKA inhibitor, while these changes are attenuated when PKA activator is coinfused with PKA inhibitor in the NAc shell. Values are the mean ± SEM of five rats in each group. *Significantly ($P < 0.001$) different from aCSF-infused rats.

between the NAc shell and core, and also differences in relation to ethanol's effects on cellular function in the shell and core (Di Chiara, 2002; Groenewegen *et al*, 1999; Heimer *et al*, 1997; Zocchi *et al*, 2003). It has been shown that voluntary alcohol intake increases glucose metabolism in the shell but not in the core region of NAc (Porrino *et al*, 1998). Rats also self-administer a variety of drugs of abuse into the shell region of NAc (Rodd-Henricks *et al*, 2002; Carlezon *et al*, 1995; Wise, 1996; McBride *et al*, 1999; Liao *et al*, 2000). Furthermore, systemic administrations of several drugs of abuse including alcohol have the property of releasing dopamine in the shell but not in the core of NAc (Imperato and Di Chiara, 1986; Di Chiara, 2002; Zocchi *et al*, 2003). The present studies provide new evidence that decreased PKA-linked CREB phosphorylation in the NAc shell promotes alcohol drinking behaviors. Other studies also suggest that CREB in the NAc is involved in preference for drugs of abuse. For example, overexpression of CREB using a viral-mediated vector in the NAc shell results in decreased cocaine and morphine reward, whereas decreasing functional CREB with the overexpression of mutated CREB (mCREB) at serine-133 leads to increases in reward and intake of cocaine and morphine (Carlezon *et al*, 1998; Barrot *et al*, 2002). Further, infusion of the phosphodiesterase inhibitor, rolipram, in the NAc or intraperitoneal injection results in increased cAMP, an upstream component of the CREB signaling, and results in the loss of the initiation of cocaine self-administration (Knapp *et al*, 1999, 2001). CREB α/Δ homozygous (−/−) mice have been shown to have an increased preference for cocaine (Walters and Blendy, 2001). Further, CREB α/Δ haplodeficient (+/−) mice have been shown to consume large amounts of alcohol as compared to their wild-type littermates (Pandey *et al*, 2004). Taken together, these studies suggest that decreased CREB in the NAc shell not only promotes cocaine and morphine preference but also alcohol preference as investigated in the present work.

Here, we also observed that decreased CREB phosphorylation after inhibition of PKA is associated with decreased expression of NPY protein in the shell structures of the NAc of rats. Interestingly, lower basal CREB expression in the NAc shell of C57BL/6J (alcohol preferring) mice is also associated with lower protein levels of NPY compared to DBA/2J (alcohol nonpreferring) mice. This difference was not seen in the core region of the NAc or in other brain regions (cortical, amygdaloid, hippocampal, and striatal

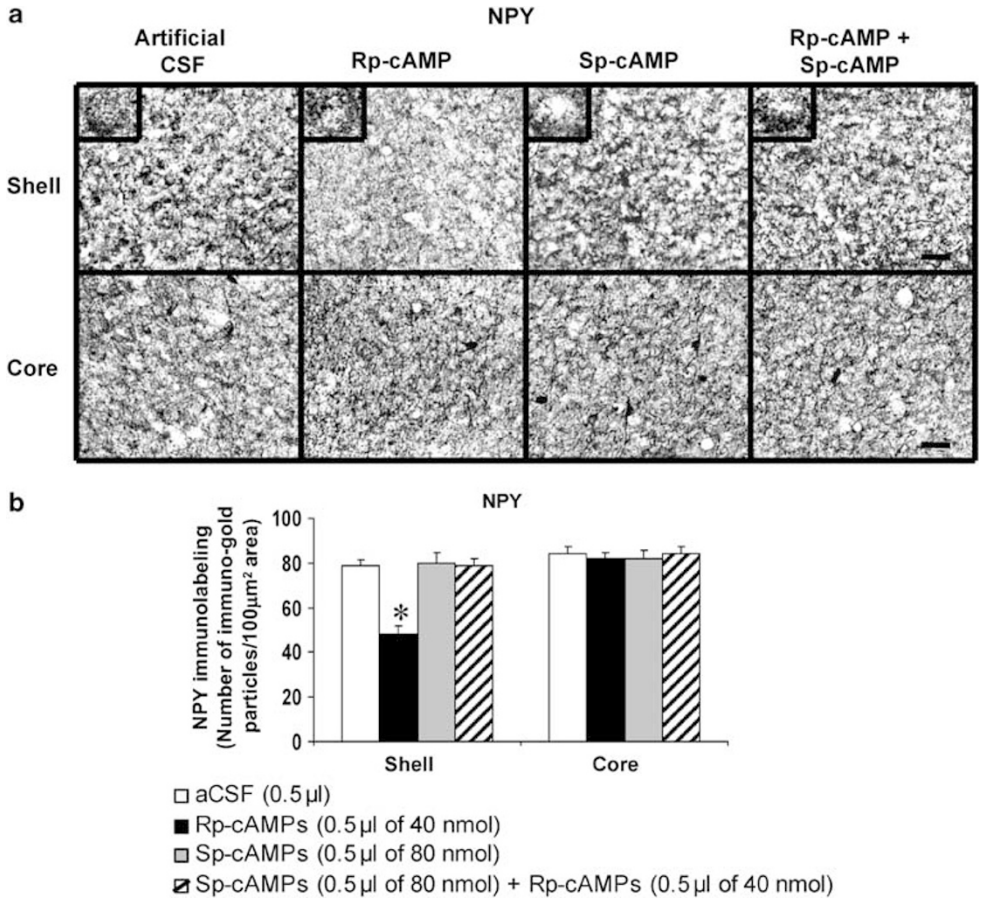

Figure 7 (a) Photomicrographs at low magnification showing NPY gold-immunolabeling in the NAc structures (shell and core), after 3 days of once daily NAc shell infusion of aCSF, Sp-cAMP, and/or Rp-cAMP, and subsequent alcohol preference testing (scale bar = 30 μm). Inset areas in the upper panel indicate immuno-gold particles within a single cell body at high magnification (× 100). (b) Quantitation of NPY gold-immunolabeling (number of immuno-gold particles/100 μm^2 area) in the NAc shell and core after 3 days of once daily NAc shell infusion of aCSF, PKA activator (Sp-cAMP), and/or PKA inhibitor (Rp-cAMP), and subsequent alcohol preference testing. NPY levels are decreased after infusion of PKA inhibitor, while these changes are attenuated when PKA activator is coinfused with PKA inhibitor in the NAc shell. Values are the mean ± SEM of five rats in each group. *Significantly ($P < 0.001$) different from aCSF-infused rats.

structures) investigated (Misra and Pandey, 2003). Also, CREB α/Δ haplodeficient (+/−) mice have been shown to have low NPY protein and mRNA levels and consume more alcohol than their wild-type littermates (Pandey *et al*, 2004). Here, we found that normalizing the CREB phosphorylation and NPY levels in the NAc shell, by coinfusion of PKA activator with inhibitor, resulted in attenuation of a higher alcohol preference. Further, we found that coinfusion of NPY prior to PKA inhibitor infusion led to the attenuation of increased alcohol drinking behaviors induced by PKA inhibitor infusion alone into NAc shell of rats. It was found that NAc shell infusion of either PKA activator or NPY alone did not produce changes in alcohol drinking behaviors as compared to aCSF-infused rats. Interestingly, NAc shell infusion of PKA activator alone did increase protein levels of PKA-Cα but produced no significant changes in pCREB or NPY protein levels. This may be related to the pharmacological phenomenon of a ceiling effect, in that this dose of the drug leads to increases in PKA activity but is unable to produce any effect on CREB function due to already high basal levels of pCREB and subsequently NPY protein. Further, it has been shown that NPY mutant mice drink a large amount of alcohol, where as transgenic mice overexpressing NPY have a decreased alcohol preference (Thiele *et al*, 1998). Infusion of NPY into the NAc has been shown to induce a conditioned place preference, indicating that NPY may produce a reinforcing effect in this brain region (Brown *et al*, 2000; Josselyn and Beninger, 1993). Therefore, it is reasonable to speculate that NPY mimics the effects of alcohol and, consequently, upregulating NPY blocks the need for ethanol-induced euphoria and reward. Thus, the coinfusion of NPY with PKA inhibitor into the NAc shell is able to attenuate the increased alcohol intake associated with the PKA inhibitor-induced decreases in CREB phosphorylation in this brain structure, indicating that perhaps CREB's actions on alcohol intake may be mediated through its effect on the NPY system in the NAc shell.

Another brain region implicated in the motivational aspects of alcohol abuse is the central nucleus of the amygdala (McBride, 2002; Koob, 2003a; Pandey, 2004). The NAc and amygdaloid nuclei are all part of a macrostructure referred to as the extended amygdala, which also consists of the bed nucleus of the stria terminalis (BNST) and the sublenticular substantia inominata, first discovered by Johnston (1923). The interaction between various extended

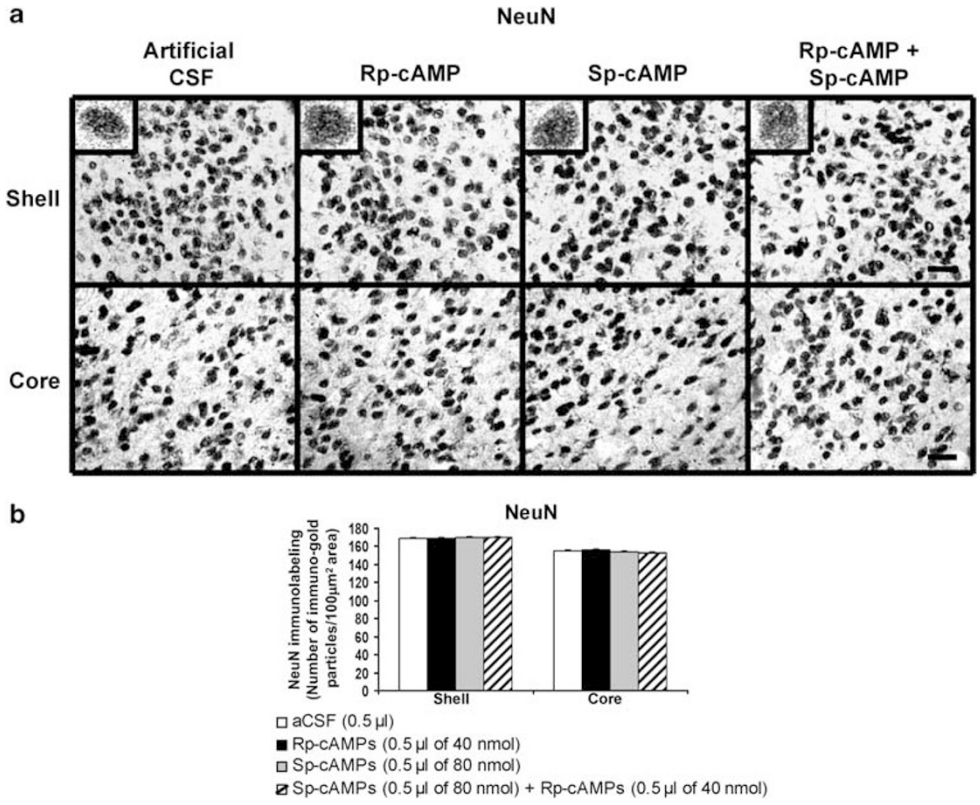

Figure 8 (a) Photomicrographs at low magnification showing NeuN gold-immunolabeling in the NAc structures (shell and core), after 3 days of once daily NAc shell infusion of aCSF, Sp-cAMP, and/or Rp-cAMP, and subsequent alcohol preference testing (scale bar = 30 μm). Inset areas in the upper panel indicate immuno-gold particles within a single nucleus at high magnification (× 100). (b) Quantitation of NeuN gold-immunolabeling (number of immuno-gold particles/100 μm^2 area) in the NAc shell and core after 3 days of once daily NAc shell infusion of aCSF, PKA activator (Sp-cAMP), and/or PKA inhibitor (Rp-cAMP), and subsequent alcohol preference testing. There are no significant differences in NeuN protein levels between groups in the NAc shell or core. Values are the mean ± SEM of five rats in each group.

amygdaloid brain regions is thought to mediate the positive and negative affective states associated with the alcohol addictive process (Koob, 2003a; Pandey, 2004). It has been proposed that CREB may play a role in both affective states through a different neurocircuitry in the extended amygdala (Pandey, 2004). It has been shown that decreased CREB function in the CeA is associated with anxiety and alcohol drinking behaviors, as well as anxiety related to alcohol withdrawal in rats (Pandey *et al*, 2003a; Pandey, 2003). We reported earlier that infusion of PKA inhibitor into CeA but not in basolateral amygdala provoked anxiety-like behaviors and increased the alcohol preference in rats (Pandey *et al*, 2003a). Thus, CREB function in the CeA may represent a brain area important in the negative affective state of addiction. In the current study, it was found that PKA inhibitor infusion in the NAc shell of rats had no effect on anxiety-like behaviors; however, these rats had a higher preference for alcohol compared to aCSF-infused rats. Previous studies have shown that decreased CREB expression in the shell of NAc using viral vector containing mutated CREB causes increased anxiety-like behaviors (Barrot *et al*, 2002) and decreased depression-like behaviors (Newton *et al*, 2002). The reasons for the differences between the present study in terms of anxiety behaviors and the study by Barrot *et al* (2002) are not clear but may be related to differences in the techniques used to modulate CREB function. However, other findings indicate that C57BL/6J mice are not anxious, consume large amounts of ethanol, and have lower basal levels of CREB, p-CREB, and NPY in the NAc shell but not in the amygdaloid structures compared with DBA/2J mice (Podhorna and Brown, 2002; Belknap *et al*, 1993; Misra and Pandey, 2003). On the other hand, alcohol preferring (P) rats consume large amounts of alcohol, are highly anxious, and have lower levels of CREB, p-CREB, and NPY in the central and medial amygdaloid but not in the NAc structures compared to alcohol non-preferring (NP) rats (Stewart *et al*, 1993; Li *et al*, 1993; Pandey *et al*, 1999b, 2003b, 2005; Hwang *et al*, 1999). Interestingly, when PKA and CREB are lower in both NAc and amygdala such as in PKA (RII-β subunit) mutant and CREB haplodeficient mice, these mice have higher alcohol preference and display higher baseline anxiety levels as compared with littermate wild-type mice (Fee *et al*, 2003; Thiele *et al*, 2000; Pandey *et al*, 2004). Taken together, these data suggest a dichotomy in the function of NAc and amygdaloid structures in terms of their roles in mediating anxiety and alcohol drinking behaviors. It appears that the NAc may be related to positive affective/reinforcing states of alcohol addiction and thus play a role in the rewarding aspects of alcohol, where as the amygdala may represent a center for negative affective states such as anxiety associated with alcohol withdrawal or pre-existing conditions of anxiety (Pandey, 2004; Koob, 2003a).

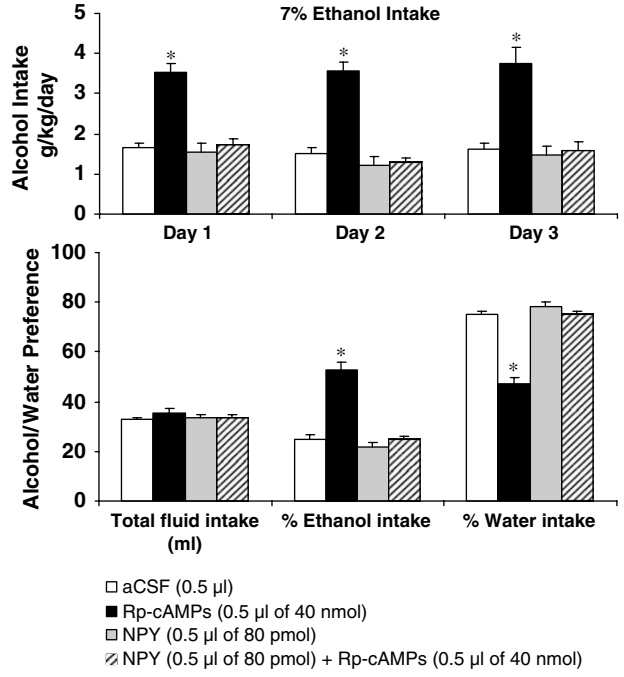

Figure 9 Effect of 3 days of once daily NAc shell infusion of aCSF, NPY, and/or PKA inhibitor (Rp-cAMP) on alcohol preference as measured by the two-bottle free choice paradigm. Results represent daily ethanol intake (g/kg/day; upper panel) and the mean percentage of 7% (ethanol in water solution) ethanol intake and percentage of water intake of total fluid intake of 3 days (lower panel). Values are the mean ± SEM of 8–9 rats in each group. *Significantly ($P<0.001$) different from aCSF-infused rats.

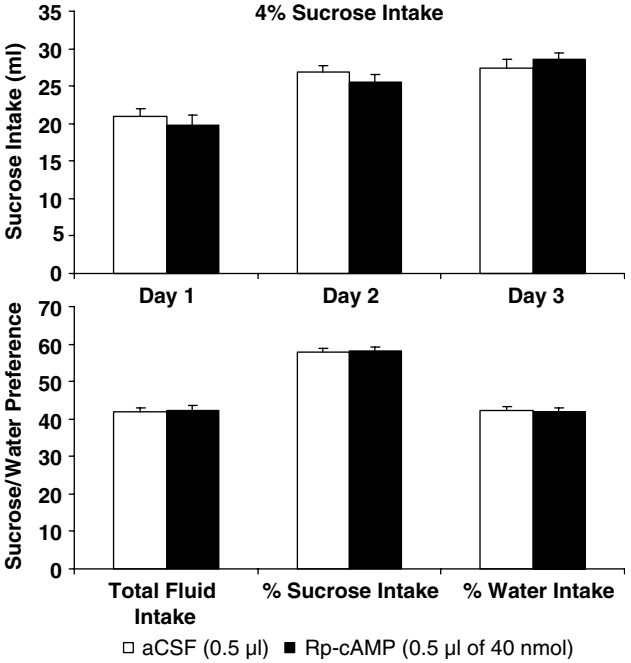

Figure 10 Effect of PKA inhibitor infusion once daily for 3 days on 4% sucrose preference as measured by the two-bottle free choice paradigm in rats. Results represent the sucrose intake (ml/day) for the 3 days of infusion of PKA inhibitor or aCSF (upper panel), as well as the mean percent of sucrose and water intake of total fluid intake (ml) of 3 days (lower panel). Values are the mean ± SEM of five rats in each group. *Significantly ($P<0.001$) different from aCSF-infused rats.

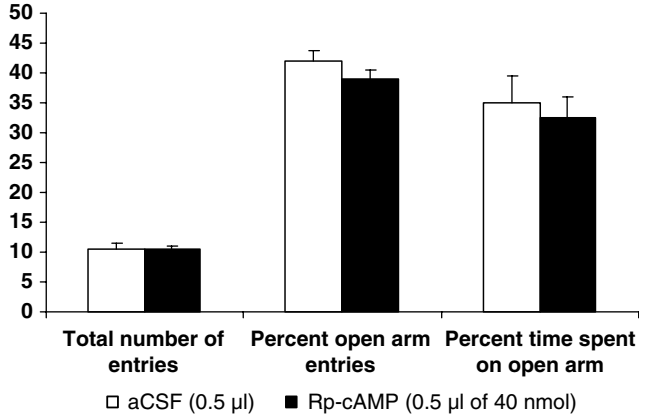

Figure 11 Effect of NAc shell infusion (3 days of once daily) of aCSF and PKA inhibitor (Rp-cAMP) on open- and closed-arm activities of the elevated plus maze as a measure of anxiety-like behaviors. Values are the mean ± SEM of 11–13 rats in each group.

In summary, the novel findings of the present investigation indicate that decreased PKA function in the NAc shell is associated with higher alcohol drinking behaviors. The results also suggest the possibility that PKA may mediate alcohol drinking behaviors independently of taste preferences or consummatory behaviors. Interestingly, decreased PKA-dependent CREB phosphorylation in the NAc shell may not be involved in anxiety-like behaviors. Thus, it appears that the decreased function of CREB in the NAc shell is an important factor in promoting and possibly maintaining alcohol drinking behaviors due to abnormal reward mechanisms. Future studies will investigate the role of other nucleus accumbal CREB-related genes (McClung and Nestler, 2003; Lonze and Ginty, 2002) in alcohol drinking behaviors.

ACKNOWLEDGEMENTS

This study was supported by grants from the National Institute on Alcohol Abuse and Alcoholism (AA-10005; AA13341) and the Department of Veterans Affairs (Merit Review Grant; VA Career Scientist award) to SCP.

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