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FOS expression induced by an ethanol-paired conditioned stimulus

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Abstract

To identify brain areas involved in ethanol-induced Pavlovian conditioning, brains of male DBA/2J mice were immunohistochemically analyzed for FOS expression after exposure to a conditioned stimulus (CS) previously paired with ethanol (2 g/kg) in two experiments. Mice were trained with a procedure that normally produces place preference (Before: ethanol before the CS) or one that normally produces place aversion (After: ethanol after the CS). Control groups received unpaired ethanol injections in the home cage (Delay) or saline only (Naïve). On the test day, mice were exposed to the 5-min CS 90 min before sacrifice. Before groups showed a conditioned increase in activity, whereas the After group showed a conditioned decrease in activity. FOS expression after a drug-free CS exposure was significantly higher in Before-group mice than in control mice in the bed nucleus of the stria terminalis (Experiment 1) and anterior ventral tegmental area (Experiments 1–2). Conditioned FOS responses were also seen in areas of the extended amygdala and hippocampus (Experiment 2). However, no conditioned FOS changes were seen in any brain area examined in After-group mice. Overall, these data suggest an important role for the mesolimbic dopamine pathway, extended amygdala and hippocampus in ethanol-induced conditioning.

Keywords: Conditioning; Ethanol; *c-Fos*; FOS; Locomotor activity; Conditioned place preference; Amygdala; Hippocampus; Ventral tegmental area; Bed nucleus of stria terminalis; Inbred mice (DBA/2J)

1. Introduction

Through Pavlovian conditioning processes, conditioned stimuli (CSs) paired with drug effects may acquire the ability to elicit conditioned responses when subsequently presented without the drug (Siegel and Ramos, 2002). Cues associated with addictive drugs such as alcohol, cocaine, morphine, and nicotine trigger craving in some individuals, which may be conducive to relapse (e.g., Flannery et al., 2003; Litt et al., 2000). From the animal literature, it is well documented that stimuli associated with drug administration can reinstate drug-seeking behavior following extinction or prolonged abstinence (e.g., Bienkowski et al., 1999; Gracy et al., 2000; Katner and Weiss, 1999; Weiss et al., 2000, 2001). Thus, drug-paired cues may play an important role in maintaining or re-establishing drug-seeking behavior.

Given that drug cues elicit conditioned responses, these cues presumably trigger neural events underlying these responses. Brain regions activated by CSs associated with rewarding drug effects may influence drug seeking, whereas brain regions activated by CSs associated with negative drug effects may mediate drug avoidance. Identifying the brain regions underlying these responses may be helpful for understanding the general neural, as well as specific neurotransmitter, systems involved in such behaviors. Researchers may then be able to develop appropriate pharmacological treatments that would alter the subject's response to drug-paired stimuli, thereby reducing risk of relapse.

Mapping expression of FOS protein is one method of identifying brain regions activated by CSs (Davidson et al., 1996; Herrera and Robertson, 1996). Studies using the conditioned taste aversion paradigm indicate that tastants paired with negative drug effects elicit particular patterns of FOS induction (Swank et al., 1995; Thiele et al., 1996). Further, these effects can be modulated by the context where conditioning occurs (Swank, 2000). FOS mapping has also been used to investigate

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neural activation evoked by drug cues in reward models, such as oral ethanol self-administration (Topple et al., 1998) and i.v. self-administered cocaine (Neisewander et al., 2000; Ciccocioppo et al., 2001). Environmental cues eliciting conditioned locomotion also elicit FOS patterns in rat brain. Such effects have been found for cocaine (Brown et al., 1992; Franklin and Druhan, 2000), morphine (Schroeder et al., 2000; Schroeder and Kelley, 2002), nicotine (Schroeder et al., 2001), and methamphetamine (Rhodes et al., 2005). Further, discrete CSs (such as a light or tone) may differ from general contexts in modulation of FOS response (Hotsenpiller et al., 2002). More recently, FOS mapping has been used to map neural pathways activated during expression of cocaine-induced conditioned place preference (CPP) in rats (Miller and Marshall, 2005).

Relatively little FOS expression mapping has been done investigating responses to ethanol-paired cues and such data are especially lacking in the mouse. The purpose of the present experiments was to identify candidate brain regions involved in mediating responses conditioned to the positive and negative effects of ethanol in DBA/2J mice using FOS immunohistochemistry. A differential conditioning procedure was used in which ethanol was paired with one stimulus (CS+) and saline with another stimulus (CS−). The basic parameters used for conditioning (i.e., dose, trial duration, number of trials) were selected on the basis of previous studies indicating their efficacy in producing conditioned changes in activity (Cunningham and Noble, 1992) and conditioned changes in preference/aversion for a paired stimulus (e.g., Cunningham et al., 1997). Although the response to CS− was not assessed during these experiments, CS− trials were included during the training phase in order to match the procedures in which we have previously demonstrated conditioned changes in activity and preference/aversion to the CS+.

In Experiment 1, two different temporal relationships between the CS+ and the ethanol unconditioned stimulus (US) were examined. One experimental group (Before-S) received ethanol injection immediately before exposure to the CS+, whereas the other experimental group (After-S) received ethanol immediately after CS+ exposure. These two procedures were compared because previous research has shown that they produce opposite effects on behavior in a place-conditioning task. More specifically, pre-CS ethanol produces CPP whereas post-CS ethanol produces conditioned place aversion (CPA) (Cunningham and Henderson, 2000; Cunningham et al., 1997). Because these motivationally opposite behaviors are thought to be independently mediated (Cunningham et al., 2002), Before-S group mice were expected to have different patterns of FOS activation than After-S group mice, and both paired groups were expected to differ from unpaired-drug (Delay-S group) and drug-naive (Naïve-S group) control mice. On the final day, all mice were first tested for conditioned changes in activity after exposure to the CS+ and saline. Ninety minutes later, their brains were removed for FOS immunohistochemistry.

Based on the outcome of the first experiment, Experiment 2 was designed to replicate the comparison between the Before group and the unpaired-drug control (Delay) group. Thus, in contrast to most previous reports of FOS changes induced by conditioned stimuli, we were able to examine the reliability of the effects produced by our conditioning procedures. Moreover, in order to better understand ethanol's impact on conditioned changes in FOS expression, half of the mice in each group were tested with ethanol (Before-E, Delay-E) and half were tested with saline (Before-S, Delay-S). Thus, differences in expression induced by exposure to the CS+ alone and the CS−US combination could be evaluated.

2. Materials and methods

2.1. Subjects

Forty-eight male DBA/2J mice (The Jackson Laboratory, Bar Harbor, ME) were used in each study. Mice were 6 weeks of age upon arrival and were allowed 2 weeks of acclimation prior to the start of the study. Mice were housed in groups of 4 in polycarbonate cages with cob bedding. A 12 h light/dark cycle was maintained, with lights on at 7:00 a.m. Food and water were available ad libitum. The NIH "Principles of laboratory animal care" were followed in conducting these studies; the protocol was approved by the OHSU IACUC.

2.2. Apparatus

Twelve aluminum and acrylic boxes (30 × 15 × 15 cm) were used (Cunningham et al., 2006). Each box was individually enclosed in a light- and sound-attenuating chamber (Coulbourn Instruments Model E10-20). The floors of the boxes were removable and of two textures: grid floors consisting of 2.3-mm stainless steel rods mounted 6.4-mm apart in acrylic frames, and hole floors made with 16 GA stainless steel perforated with 6.4-mm diameter holes. Previous research has shown that drug-naive mice are not biased in favor of either floor prior to conditioning (e.g., Cunningham, 1995; Cunningham et al., 2003). Six sets of infrared detectors mounted along the side of the conditioning box were used to monitor locomotor activity and left/right position of the animal. Boxes were cleaned between subjects by wiping the walls and floors with a damp sponge and by replacing paper towels beneath the floors.

2.3. Ethanol solution

A 2 g/kg dose of ethanol was used. This dose has previously been shown to produce robust conditioned activation (Cunningham and Noble, 1992), place preference (e.g., Cunningham and Prather, 1992) and place aversion (e.g., Cunningham et al., 1997) in DBA/2J mice. Ethanol was administered IP in a volume of 12.5 ml/kg. The solution was 20% v/v in saline, prepared from 95% ethanol. All saline injections were equal in volume to ethanol injections.

2.4. Conditioning and test procedure

2.4.1. Experiment 1

The experimental design and procedure are summarized in Table 1. Mice were randomly assigned to one of four groups: Before-S, After-S, Delay-S, or Naïve-S (n = 12/group). The first

Table 1
Summary of conditioning protocol for mice in Experiment 1 (n = 12/group)

Phase	Group			
	Before-S (place preference)	After-S (place aversion)	Delay-S (unpaired control)	Naïve-S (no-drug control)
Habituation (1 day)	Sal → paper	Paper → sal	Sal → paper	Sal → paper
Homecage (2 h later)	Sal	Sal	Sal	Sal
CS+ trials [a] (6 days)	EtOH → CS+	CS+ → EtOH	Sal → CS+	Sal → CS+
Homecage (2 h later)	Sal	Sal	EtOH	Sal
CS− trials [a] 6 days)	Sal → CS−	CS− → sal	Sal → CS−	Sal → CS−
Homecage (2 h later)	Sal	Sal	Sal	Sal
Test [b] (1 day)	Sal → CS+	CS+ → sal	Sal → CS+	Sal → CS+
90 min later	Brain removed	Brain removed	Brain removed	Brain removed

[a] Only one 5-min conditioning trial was given each day. CS+ and CS− trials were given in alternating order across days (counterbalanced within each group). A 2-day break occurred between the 2nd and 3rd trial of each type and between the 4th and 5th trial of each type. EtOH = ethanol (2 g/kg); Sal = saline. Arrows indicate the order in which mice were exposed to each injection and CS.

[b] The test occurred 24 h after the last conditioning trial. All mice were exposed to the CS+ for 5 min and then returned to the home cage for 90 min before brains were removed.

part of the group label refers to the treatment received during the conditioning phase; the second part refers to the drug condition on the test day (i.e., all groups received saline). All mice were given a 5 min habituation session on the day before conditioning began. On that day, mice received a saline injection either before (Before-S, Delay-S, and Naïve-S groups) or after (After-S group) exposure to a paper floor in the conditioning box. Subsequently, mice were exposed to a series of six CS+ and six CS− trials given in alternating order across days (counterbalanced within each subgroup). On CS+ trials, all mice were exposed to the grid floor (CS+) for 5 min and, depending on group assignment, received an injection of ethanol immediately before CS+ (Before-S group), immediately after CS+ (After-S group), or 2 h later in the home cage (Delay-S group). To match groups for handling and injection, Delay-S and Naïve-S groups received a saline injection on CS+ trials immediately before CS+, and 2 h later Before-S, After-S, and Naïve-S groups received a saline injection in the home cage. The procedure on CS− trials was similar, but each group was exposed to the hole floor (CS−) for 5 min and both injections were saline. Thus, Before-S mice were trained using a procedure known to produce CPP and After-S mice were trained using a procedure known to produce CPA (Cunningham et al., 1997). Delay-S mice were unpaired controls matched for ethanol experience, and Naïve-S mice were no-drug controls (Cunningham, 1993). Based on previous research, the control groups would not be expected to show conditioned changes in activity or preference for the ethanol-paired cue (Cunningham and Noble, 1992).

On the final (test) day, all mice received a saline injection and 5-min exposure to the CS+ floor. A saline injection was given immediately before (Before-S, Delay-S, Naïve-S groups) or after (After-S group) CS+ exposure. Mice were returned to the home cage after CS+ exposure. Ninety minutes later they were sacrificed (via CO_2 inhalation) and their brains removed for FOS processing. Although Before-S and After-S mice received conditioning treatments that were expected to produce CPP and CPA, respectively, they were tested using a procedure that did not assess CS+ preference/aversion to avoid confounding the FOS response to CS+ by concurrent exposure to CS−.

2.4.2. Experiment 2

The experimental design and procedure for Experiment 2 are outlined in Table 2. Mice were randomly assigned to one of four groups: Before-S, Before-E, Delay-S or Delay-E (n = 12/group). Both of the Before groups were exposed to the same (place preference) conditioning procedure given to the Before-S group

Table 2
Summary of conditioning protocol for mice in Experiment 2 (n = 11–12/group)

Phase	Group			
	Before-S (place preference)	Delay-S (unpaired control)	Before-E (place preference)	Delay-E (unpaired control)
Habituation (1 day)	Sal → paper	Sal → paper	Sal → paper	Sal → paper
Homecage (2 h later)	Sal	Sal	Sal	Sal
CS+ trials [a] (6 days)	EtOH → CS+	Sal → CS+	EtOH → CS+	Sal → CS+
Homecage (2 h later)	Sal	EtOH	Sal	EtOH
CS− trials [a] 6 days)	Sal → CS−	Sal → CS−	Sal → CS−	Sal → CS−
Homecage (2 h later)	Sal	Sal	Sal	Sal
Test [b] (1 day)	Sal → CS+	Sal → CS+	EtOH → CS+	EtOH → CS+
90 min later	Brain removed	Brain removed	Brain removed	Brain removed

[a] Only one 5-min conditioning trial was given each day. CS+ and CS− trials were given in alternating order across days (counterbalanced within each group). A 2-day break occurred between the 2nd and 3rd trial of each type and between the 4th and 5th trial of each type. EtOH = ethanol (2 g/kg); Sal = saline. Arrows indicate the order in which mice were exposed to each injection and CS.

[b] The test occurred 24 h after the last conditioning trial. All mice were injected, exposed to the CS+ for 5 min, and then returned to the home cage for 90 min before brains were removed.

in Experiment 1, whereas both of the Delay groups received the same unpaired control treatment given to the Delay-S group in Experiment 1. The test day for the Before-S and Delay-S groups in Experiment 2 was identical to that for the Before-S and Delay-S groups in Experiment 1. In contrast, the Before-E and Delay-E groups were injected with ethanol (2 g/kg) instead of saline before CS+ exposure on the test day. Thus, this procedure allowed for comparisons between experimental mice exposed to both the CS+ and US on test (Before-E), experimental mice exposed to the CS+ only on test (Before-S), control mice receiving their first pairing of the CS+ and US (Delay-E), and control mice that never had the CS+ paired with the US (Delay-S). As in Experiment 1, mice were sacrificed via CO_2 inhalation and their brains removed for FOS processing 90 min after exposure to the CS+ floor.

2.5. Immunohistochemistry

Immunohistochemistry was done according to previously described protocols yielding consistent results in previous studies (Bachtell et al., 1999, 2003; Ryabinin et al., 2000). Brains were fixed overnight in 2% paraformaldehyde in phosphate-buffered saline (PBS) and cryoprotected in 30% sucrose in PBS. Frozen coronal slices (40 μm) were cut on a microtome, beginning at the nucleus accumbens (Bregma level + 2.0) and continuing to the ventral tegmental area (Bregma level − 3.08). Serial sections were collected in PBS. Every fifth section was processed for immunohistochemistry. Endogenous peroxidase activity was inhibited with 0.3% hydrogen peroxide, and 3% goat serum in PBS was used for blocking. A rabbit polyclonal primary antibody directed toward the N-terminal of the FOS protein (Santa Cruz, Biotechnology Inc., Santa Cruz, CA), and not cross-reactive with other members of the FOS family of proteins (Ryabinin et al., 1999) was incubated with slides at dilution 1:10,000 overnight at 4 °C. A goat anti-rabbit antibody in a 1:200 dilution was used as a secondary antibody (Vector Laboratories Inc., Burlingame, CA). The reaction with the avidin–biotin–horseradish peroxidase complex was performed using the Vectastain ABC kit (Vector Laboratories Inc.). Enzymatic development was done using the Metal-Enhanced DAB kit (Pierce, Rockford, IL).

2.6. FOS cell quantitation

Cell counts were done blind to group membership of the subjects. Selection of specific brain areas for examination (see Table 3) was based on previous data showing their activation by exposure to ethanol or potential involvement in ethanol's rewarding or conditioned rewarding effects. Slides were selected to match structure location based on the mouse brain atlas (Franklin and Paxinos, 1997) and were viewed on an Olympus microscope (BX40), and a Sony CCD-IRIS/RGB videocamera relayed the image to a Macintosh Power PC. It is believed that this sampling methodology is adequate for FOS counts and is an unbiased method (Saper, 1996). The digitized video images were analyzed using NIH Image 1.63 software. The image of each region was adjusted using a threshold procedure so that no positive signals were contained in adjacent areas; then, within each region, only signals of > 10 pixels were counted. Although selection of threshold is a subjective procedure, a previous study showed that independent observers tend to choose the same threshold levels (Rieux et al., 2002). A single section from each brain region was counted for each animal and included in the statistical analysis.

2.7. Statistical analyses

Locomotor activity from the conditioning trials in both experiments was analyzed using a three-way (Group × Trial type × Trial) ANOVA. Test activity from Experiment 1 was analyzed by a one-way ANOVA; test activity from Experiment 2 was analyzed with a two-way (Conditioning group × Test drug) ANOVA. The level of significance was set at $p < .05$, and post-hoc tests (Fishers PLSD) were performed when statistical differences were found.

FOS counts were analyzed statistically to compare the mean number of FOS-positive cells in each brain region across groups. One-way ANOVAs were used for Experiment 1 and two-way (Conditioning group × Test drug) ANOVAs for Experiment 2. Significant outcomes were followed by pair-wise post-hoc comparisons (Fishers PLSD). Brain regions showing mean counts of zero in one or more groups were not subjected to analysis. A 0.05 level of significance (two-tailed) was chosen, despite the possibility of Type I errors, to avoid missing regions with quantitative but small changes in FOS expression. Replication of Experiment 1 findings was therefore sought in Experiment 2. However, it should be noted that, based on differences in experimental design, some effects could not be examined in both studies. For instance, After-S mice were used only in Experiment 1, so differences between these mice and other groups could not be reexamined. Also, no mice were tested under ethanol in Experiment 1, so the effects of ethanol on test or interactions of ethanol with conditioning were assessed only in Experiment 2.

3. Results

3.1. Locomotor activity data

3.1.1. Experiment 1: conditioning phase

Locomotor activity rates during each 5-min CS+ and CS− conditioning trial are shown in Fig. 1. Mean activity rates (± SEM) averaged across all CS+ trials were 180.5 ± 6.9, 30.5 ± 2.7, 46.3 ± 3.1 and 45.0 ± 1.9 for groups Before-S, After-S, Delay-S and Naïve-S, respectively. Across all CS− trials, mean activity rates were 48.0 ± 2.1, 27.7 ± 3.0, 44.4 ± 2.7 and 41.7 ± 2.0, respectively. The highest activity was seen on CS+ trials in the Before-S group, the only condition in which activity was recorded immediately after ethanol injection. On CS− (saline) trials, the Before-S group showed a lower level of activity comparable to that seen in both the Delay-S and Naïve-S control groups after saline injection on both types of trials. Across trials, the After-S group displayed an even lower level of activity on both trial types, consistent with previous studies involving post-CS ethanol injection (Cunningham et al., 1997).

Conditioning activity data analysis revealed significant main effects of Group [$F(3, 44) = 173.1$, $p < .0001$], Trial type [$F(1, 44) = 395.2$, $p < .0001$] and Trials [$F(5, 220) = 13.1$, $p < .0001$]. In addition, all two-way interactions (Group × Trial type [$F(3, 44) = 337.0$, $p < .0001$], Group × Trials [$F(15, 220) = 5.2$, $p < .0001$)], Trial type × Trials [$F(5, 220) = 2.9$, $p < .02$]) and the three-way interaction [$F(15, 220) = 6.4$, $p < .0001$] were significant. The three-way interaction was due to group differences in the pattern of changes across trials in the response on CS+ and CS− trials. That is, the Before-S group showed a large difference between CS+ and CS− trials that increased across conditioning trials, whereas all of the other groups showed little difference between trial types or a small difference that decreased over trials. Follow-up analyses supported this interpretation, showing significant Trial type × Trials interactions in the Before-S [$F(5, 55) = 8.0$, $p < .001$] and After-S [$F(5, 55) = 2.5$, $p < .05$] groups, but no interaction in either control group. Repeated-measures follow-up ANOVAs were used to evaluate the Trials effect across CS+ trials separately for each group. These analyses revealed a significant decrease in activity across CS+ trials in the After-S, Delay-S and Naïve-S groups [all Fs$(5, 55) > 3.8$, $p < .01$]. In contrast, the Before-S group showed a significant increase in activity across CS+ trials [$F(5, 55) = 4.1$, $p < .01$], consistent with previous studies showing sensitization to ethanol-induced locomotor activation in DBA/2J mice (Cunningham and Noble, 1992). Pairwise comparisons indicated that the After-S group showed significantly lower activity on CS+ trials than all other groups [all Fs$(1, 22) > 15.1$, $p < .001$].

Table 3
Mean (±SEM) number of FOS-positive cells per optical field in Experiment 1

Brain region[a]	Bregma level[b]	Before-S	After-S	Delay-S	Naïve-S
Hipocampus					
CA1	−1.70	0.2±0.1	0.3±0.1	0.7±0.5	0.0±0.0
CA3	−1.70	2.3±0.7	1.8±0.5	1.3±0.5	11±0.4
CA4	−1.70	1.0±0.4	0.3±0.3	1.2±0.5	0.7±0.3
DO	−1.70	16.7±2.0	16.3±1.8	15.3±1.7	14.7±1.0
Amygdala					
ACo	−1.46	11.5±2.2	11.1±2.4	12.4±3.8	13.0±3.5
BLA	−1.46	17.8±3.5	12.5±3.5	10.6±2.9	6.7±2.2
CeL	−1.46	1.6±0.5	3.8±2.2	2.8±2.1	2.3±0.7
CeM	−1.46	1.3±0.4	1.8±0.8	0.8±0.4	1.8±0.7
La	−1.46	0.4±0.3	0.8±0.4	1.3±0.5	0.0±0.0
Me	−1.46	25.9±3.2	23.8±3.1	20.4±3.6	16.7±4.0
Striatum					
AcbC	+1.10	1.3±0.4	1.5±0.6	1.8±0.6	1.3±0.4
AcbS	+1.10	7.7±1.2	7.8±2.9	10.8±2.5	5.1±1.0
CPuL	+1.10	0.5±0.4	0.0±0.0	0.1±0.1	0.0±0.0
CPuM	+1.10	0.6±0.2	0.7±0.4	0.6±0.3	0.8±0.4
VP	+0.50	5.5±1.1	3.6±0.7	2.9±0.7	3.0±0.7
Thalamus					
CM	−1.70	1.8±0.5	3.2±0.9	1.8±0.6	0.9±0.3
PV	−0.94	50.3±4.7	40.5±5.5	45.8±4.2	35.3±5.0
VLO	−2.80	39.8±4.4	33.4±7.0	26.6±4.5	25.7±4.3
VPL	−1.70	0.1±0.1	0.3±0.2	0.0±0.0	0.0±0.0
Hypothalamus					
AH	−0.58	20.4±3.8	15.7±2.6	28.6±4.4	25.8±2.7
Arc	−1.70	21.8±4.4	18.8±4.0	15.4±3.2	11.6±1.2
DMH[c]	−1.70	50.7±5.1[d]	35.2±4.3	28.4±4.6	31.3±3.7
LH	−0.94	18.8±2.6	13.7±1.8	15.7±2.2	13.7±21
MPO[c]	+0.50	29.0±2.4	27.0±3.8	24.5±4.9	14.1±1.6[e]
Pa	−0.94	15.8±3.4	20.4±2.8	16.6±2.4	13.3±1.7
VMH	−1.70	23.7±5.8	32.7±5.9	22.4±5.2	25.1±7.6
Cortex					
cg	+1.10	4.1±1.1	6.7±2.2	5.5±2.3	3.1±1.6
M	+1.10	7.2±1.6	9.2±3.0	7.6±2.6	3.8±0.9
Pir	+1.10	26.8±4.0	24.3±4.9	19.9±2.3	16.7±2.9
S	+1.10	0.3±0.2	0.8±0.5	0.4±0.2	0.4±0.2
Other Regions					
BST[c]	+0.10	10.1±2.4[d]	5.1±1.3	4.0±1.2	5.1±1.2
BSTIA	−1.46	3.9±0.8	2.3±0.8	3.3±1.2	2.7±0.5
BSTLP	+0.14	2.9±0.9	2.3±0.8	2.3±0.6	2.3±0.9
EW	−2.92	0.0±0.0	0.2±0.2	0.2±0.1	0.6±0.5
LSV	+0.50	30.3±3.3	29.6±3.8	21.8±3.3	29.4±5.6
PAG	−2.92	29.3±4.2	28.2±4.3	23.4±3.2	23.7±2.9
SNC[c]	−2.92	11.0±2.9[f]	6.4±1.2	3.6±0.8	4.2±1.0
SNR	−2.92	9.1±3.4	14.4±4.5	11.9±5.3	5.3±1.6
Tu	+1.10	5.5±1.1	5.8±1.0	6.7±31	3.9±1.1
VTAant[c]	−2.92	16.9±1.6[f]	12.2±1.8	10.3±1.7	9.0±2.0

Notes to Table 3
n=9–12 mice/group.
[a] AH=anterior hypothalamus; Arc=arcuate hypothalamic nucleus; AcbC=core of nucleus accumbens; AcbS=shell of nucleus accumbens; ACo=anterior cortical amygdaloid nucleus; BLA=basolateral amygdaloid nucleus, anterior part; BST=bed nucleus of stria terminalis; BSTIA=BST, intraamygdaloid division; BSTLP=BST, lateral division, posterior part; CeL=lateral central nucleus of amgydala; CeM=medial central nucleus of amygdala; cg=cingulum; CM=central medial thalamic nucleus; CPuL=lateral caudate putamen; CPuM=medial caudate putamen DG=dentate gyrus; EW=Edinger-Westphal nucleus; DMH=dorsomedial hypothalamus; La=lateral amygdala; LH=lateral hypothalamus; LSV=ventral lateral septum; M=motor cortex; Me=medial amygdala; MPO=medial preoptic nucleus; Pa=paraventricular hypothalamic nucleus; PAG=periaqueductal gray; Pir=piriform cortex; PV=paraventricular nucleus of thalamus; S=somatosensory cortex; VLG=ventral lateral geniculate nucleus; SNC=compact part of substantia nigra; SNR=reticular part of substantia nigra; Tu=olfactory tubercle; VP=ventral pallidum; VMH=ventromedial hypothalamus; VPL=ventral posterolateral thalamic nucleus; VTAant=anterior ventral tegmental area.
[b] Locations (in mm) relative to bregma based on Franklin and Paxinos (1997) mouse atlas.
[c] Significant main effect, one-way ANOVA, $p<.05$.
[d] Significantly higher than all other groups ($p<.05$, Fisher's PLSD).
[e] Significantly lower than all other groups ($p<.05$, Fisher's PLSD).
[f] Significantly higher than Delay-S and Naïve-S groups ($p<.05$, Fisher's PLSD).

3.1.2. Experiment 1: test session

Locomotor activity rates during the test exposure to CS+ are shown in Fig. 2. As can be seen, activity rate in the Before-S group was higher than that in the two control groups, which were similar. In contrast, activity rate in the After-S group was less than half that seen in the control groups. One-way ANOVA yielded a significant main effect of Conditioning group [$F(3, 44) = 20.36$, $p < .001$] and post-hoc (Tukey HSD) comparisons confirmed that the After-S group was significantly less active ($p < .001$) than all other groups. Based on previous findings (Cunningham and Noble, 1992), a direct planned comparison was conducted between the Before-S and Delay-S groups. This

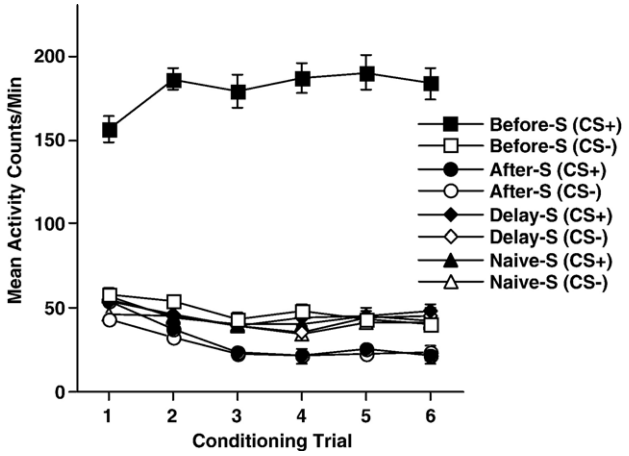

Fig. 1. Mean activity counts per minute (±SEM) during each 5-min ethanol (CS+) and saline (CS−) conditioning trial in Experiment 1. Each group contained 12 mice.

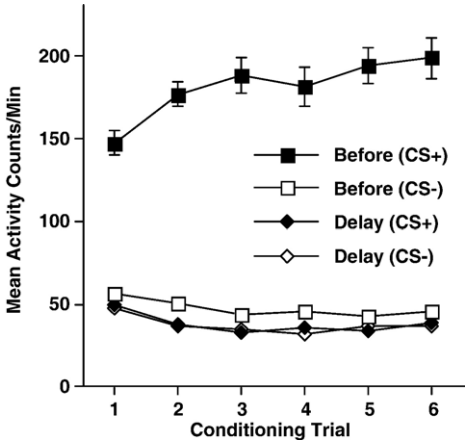

Fig. 3. Mean activity counts per minute (±SEM) during each 5-min ethanol (CS+) and saline (CS−) conditioning trial in Experiment 2. Data are collapsed across test drug within the Before and Delay conditions because this factor was irrelevant during the conditioning phase and because there were no differences between mice assigned to the saline or ethanol test groups. Each group contained 23–24 mice.

analysis showed that Before-S group mice were significantly more active than Delay-S group mice [$F(1, 22) = 5.7$, $p < .03$], confirming development of a conditioned activity response to CS+ in the Before-S group. A similar comparison also indicated a significant difference between Groups Before-S and Naïve-S [$F(1, 22) = 7.7$, $p < .02$].

3.1.3. Experiment 2: conditioning phase

One mouse in the Delay-E group was eliminated during the conditioning phase due to an injection injury; its data were removed from all of the analyses described below. Locomotor activity rates on both types of conditioning trials are shown in Fig. 3. These data are collapsed across test drug (ethanol vs. saline), which was an irrelevant factor during the conditioning phase. Mean activity rates (± SEM) averaged across all CS+ trials were 170.4 ± 11.8, 191.8 ± 10.8, 35.5 ± 4.0 and 40.1 ± 2.4 for groups Before-E, Before-S, Delay-E and Delay-S, respectively. Across all CS− trials, mean activity rates were 42.0 ± 3.5, 53.2 ± 3.1, 36.0 ± 3.8 and 38.6 ± 3.2, respectively. Because preliminary analyses showed no effect of test drug, this factor was excluded from the following analyses to simplify presentation of the conditioning phase data. As can be seen, conditioning trial activity in Experiment 2 was generally quite similar to that seen in the Before-S and Delay-S groups in Experiment 1 (Fig. 1). Statistical analysis yielded significant main effects of Conditioning group [$F(1, 45) = 203.9$, $p < .0001$] and Trial type [$F(1, 45) = 310.0$, $p < .0001$]. There were also significant two-way interactions for Conditioning group × Trial type [$F(1, 45) = 304.9$, $p < .0001$], Conditioning group × Trials [$F(5, 225) = 7.8$, $p < .0001$] and Trial type × Trials [$F(5, 225) = 9.1$, $p < .0001$], as well as a three-way interaction [$F(5, 225) = 10.6$, $p < .0001$]. Follow-up analyses indicated that the three-way interaction was due to a significant Trial type × Trials interaction in the Before groups [$F(5, 115) = 11.6$, $p < .0001$], but not in the Delay groups [$F < 1$], reflecting the large increasing difference between CS+ and CS− trials in the Before groups and the absence of trial type differences in the Delay groups. Repeated-measures follow-up ANOVAs conducted

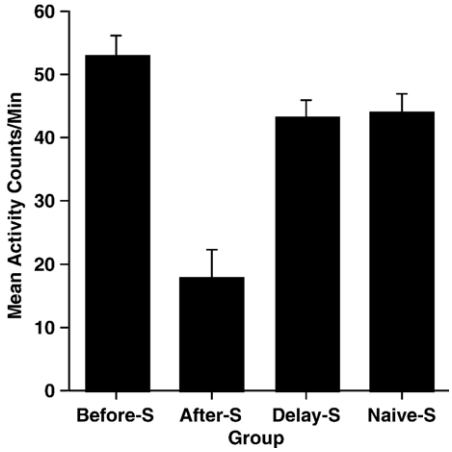

Fig. 2. Mean activity counts per minute (±SEM) during the final 5-min test session in Experiment 1. Each group contained 12 mice.

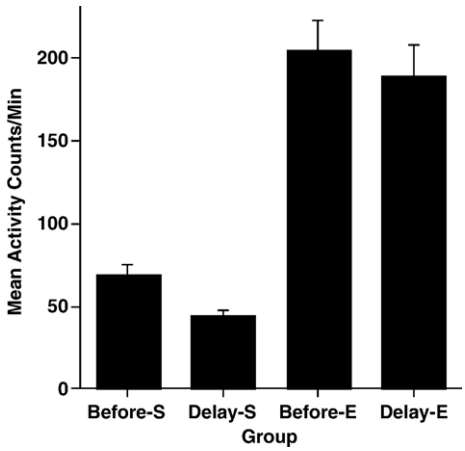

Fig. 4. Mean activity counts per minute (±SEM) during the final 5-min test session in Experiment 2. Each group contained 11–12 mice.

separately for each group showed a significant decrease in activity across CS+ trials in the Delay groups [$F(5, 110) = 7.7$, $p < .0001$], but a significant increase in the Before groups [$F(5, 115) = 7.2$, $p < .0001$]. Thus, as in Experiment 1, repeated exposure to ethanol produced sensitization to ethanol-induced activation in the Before group.

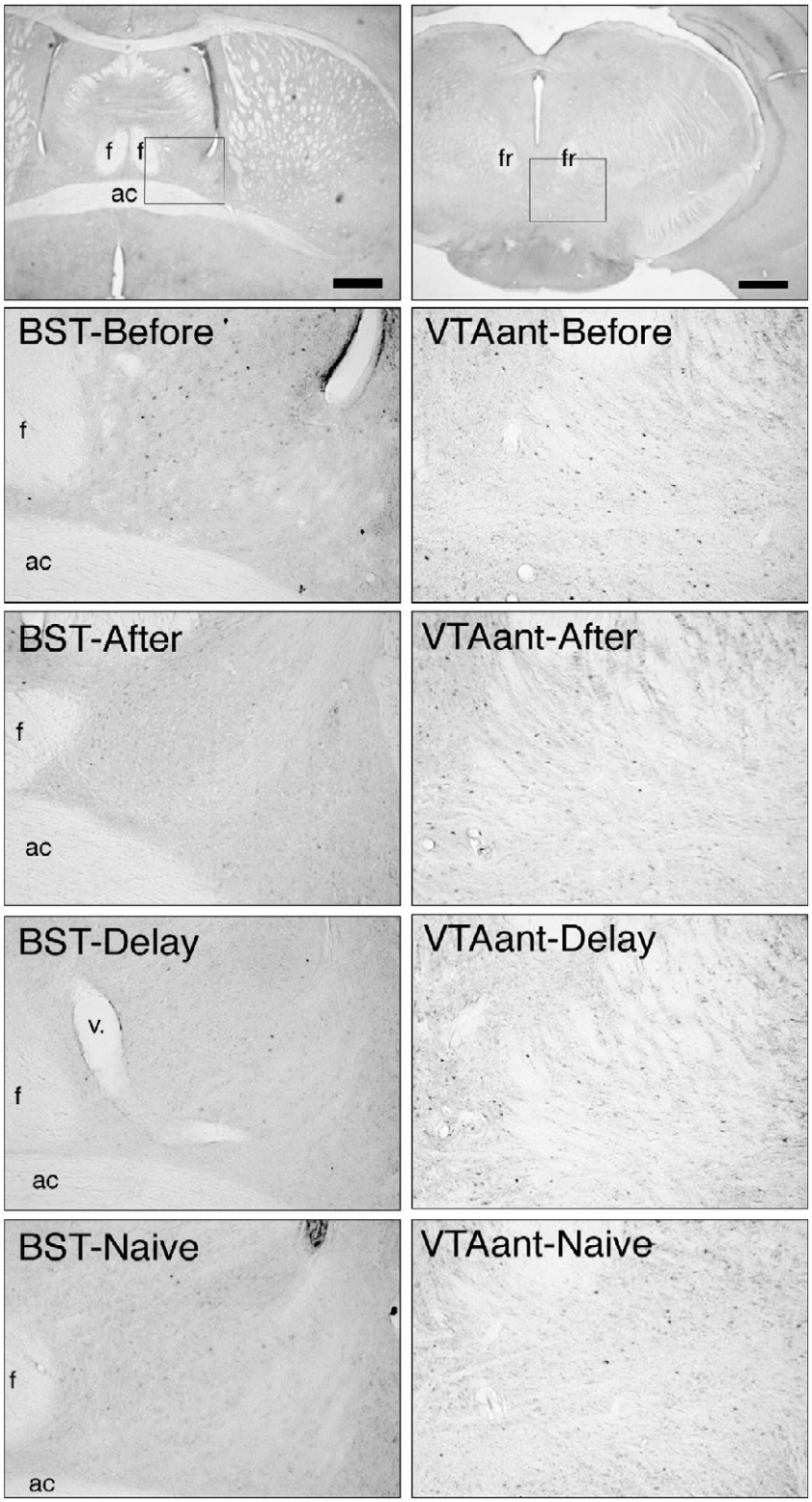

Fig. 5. Photomicrographs showing FOS immunostaining in the BST (left panels) and VTAant (right panels) from representative animals in each group in Experiment 1. In the BST, Before-S (CPP-trained) mice had higher FOS counts than all other groups. In the VTAant, Before-S mice had higher FOS counts than Delay-S and Naïve-S control animals. Abbreviations for neuroanatomical landmarks: ac = anterior commissure; f = fornix; fr = fasciculus retroflexus; v = blood vessel. Scale bar corresponds to 500 μm.

3.1.4. Experiment 2: test session

As in Experiment 1, mice in the Before-S group showed higher test session activity than mice in the Delay-S group (see Fig. 4). Mice tested after ethanol injection showed much higher activity rates similar to those seen on the last trial of conditioning, but little difference between the Before-E and Delay-E groups. The ANOVA showed a significant main effect of Test drug [$F(1, 43) = 118.6$, $p < .001$], reflecting the large stimulant effect of ethanol. However, there was no main effect of Conditioning group or interaction. As in Experiment 1, a direct planned comparison between the Before-S and Delay-S groups revealed significantly greater activity in the Before-S group [$F(1, 22) = 10.7$, $p < .005$], indicating development of a conditioned activity response.

3.2. FOS data

3.2.1. Experiment 1

Cell-count data for all brain regions examined are listed in Table 3. Photomicrographs from representative animals of each group showing FOS staining in the bed nucleus of the stria terminalis (BST) and the anterior ventral tegmental area (VTAant) are shown in Fig. 5.

Significant group differences were observed in five regions: BST [$F(3, 44) = 2.9$, $p < .05$], dorsomedial hypothalamus (DMH) [$F(3, 43) = 4.9$, $p < .005$], medial preoptic area (MPO) [$F(3, 43) = 3.9$, $p < .02$], substantia nigra pars compacta (SNC) [$F(3, 38) = 3.8$, $p < .02$] and VTAant [$F(3, 43) = 3.9$, $p < .02$]. Post-hoc comparisons revealed that the Before-S group had significantly higher FOS expression in the BST and DMH than all other groups (see Table 3). Moreover, the Before-S group had higher FOS expression than the Delay-S and Naïve-S groups in the SNC and VTAant. In both of the latter brain regions, differences between the Before-S and After-S groups approached, but failed to reach significance ($.05 < p < .07$). Finally, all groups that had received ethanol during the conditioning phase (Before-S, After-S, and Delay-S groups) showed higher numbers of FOS-positive cells in the MPO than the Naïve-S control group, which received only saline injections on all trials.

3.2.2. Experiment 2

Based on findings from Experiment 1, fewer brain regions were examined in Experiment 2 (see Table 4). Three general patterns of results were found: (a) regions showing a main effect of Conditioning Group [CA3, dentate gyrus (DG), medial central nucleus of amygdala (CeM), lateral amygdala (La)] (b) regions showing a main effect of Test drug [lateral central nucleus of amygdala (CeL), paraventricular nucleus of thalamus (PV), and DMH, Edinger-Westphal nucleus (EW)], and (c) regions where significant Conditioning group × Test drug interactions were found [CA1 and CA3 regions of hippocampus, BST and VTAant].

In all four regions showing significant effects of Conditioning group, FOS-positive cell counts were higher in the combined Before condition than in the combined Delay condition [CA3: $F(1, 42) = 7.5$, $p < .01$; DG: $F(1, 42) = 8.3$, $p < .01$; CeM: $F(1, 42) = 5.3$, $p < .03$; La: $F(1, 42) = 7.0$, $p < .02$]. In three of the brain regions showing Test drug effects, mice tested with ethanol had higher FOS-positive cell counts than mice tested with saline [EW: $F(1, 43) = 60.2$, $p < .0001$; PV: $F(1, 43) = 4.3$, $p < .05$; CeL: $F(1, 42) = 8.8$, $p < .005$]. However, this pattern was reversed in DMH, with saline-tested mice showing higher FOS counts than ethanol-tested mice [$F(1, 42) = 4.6$, $p < .04$].

Four brain areas showed a significant Conditioning group × Test drug interaction [BST: $F(1, 38) = 4.9$; VTAant: $F(1, 40) = 5.9$; CA1: $F(1, 42) = 4.6$; CA3: $F(1, 42) = 6.6$; all $ps < .05$]. In three cases, post-hoc pair-wise group comparisons indicated that FOS-positive cell counts were significantly higher in Before group mice than in Delay group mice when tested after saline injection (i.e., Before-S > Delay-S), whereas ethanol-tested mice showed no group difference or a trend in the opposite direction (see Table 4). In three of these brain areas (BST, CA1, CA3), the number of FOS-positive cells in Before group mice was lower after ethanol pretreatment (i.e., Before-E < Before-S).

Table 4
Mean (±SEM) number of FOS-positive cells per optical field in Experiment 2

Brain region [a]	Before-S	Delay-S	Before-E	Delay-E
Hippocampus				
CA1 [b]	6.7±2.0 [c]	2.7±0.5	2.7±0.6 [g]	3.8±0.9
CA3 [eb]	7.2±1.6 [c]	1.8±0.4	3.0±0.7 [g]	2.8±0.9 [g]
DG [e]	15.3±2.6	6.8±1.1	9.6±1.2	7.5±2.0
Amygdala				
BLA	12.4±0.9	9.8±1.7	13.6±1.9	11.9±1.6
CeL [f]	8.2±1.3	4.7±0.9	13.8±2.2	20.1±6.8
CeM [e]	4.9±1.1	2.6±0.5	5.6±0.8	4.0±0.9
La [e]	1.9±0.5	0.9±0.3	2.6±0.3	1.5±0.5
Thalamus				
PV [f]	16.2±2.6	18.8±3.1	19.8±4.1	30.0±4.3
Hypothalamus				
DMH [f]	30.9±2.7	36.4±5.1	22.3±4.4	27.5±3.8
MPO	29.1±3.8	23.8±2.5	18.3±3.9	22.1±3.7
Other Regions				
BST [b]	10.8±3.2 [d]	5.8±1.5	2.2±1.3 [g]	6.3±2.0
EW [f]	1.6±0.5	1.2±0.5	21.1±2.4	20.4±4.6
LSV	9.2±2.7	4.8±1.4	3.9±1.8	5.2±1.6
SNC	0.3±0.1	0.3±0.1	0.8±0.4	2.6±1.7
SNR	0.0±0.0	0.2±0.2	0.6±0.3	0.5±0.3
VTAant [b]	23.4±3.7 [c]	11.9±2.8	13.8±3.0	20.6±5.8

$n = 9$–12 mice/group.
[a] See Table 3 for key to abbreviations and locations for each brain region.
[b] Significant interaction, ANOVA, $p < .05$ (note: results of pair-wise follow-up comparisons indicated below).
[c] Significantly higher than Delay-S ($p < .05$, Fisher's PLSD).
[d] Non-significant trend toward higher response than Delay-S ($p = .09$, Fisher's PLSD).
[e] Significant main effect of conditioning group, ANOVA, $p < .05$.
[f] Significant main effect of test drug, ANOVA, $p < .05$.
[g] Significantly lower than Before-S ($p < .05$, Fisher's PLSD).

4. Discussion

These studies are the first to show conditioned changes in FOS expression to a CS previously paired with ethanol in mice. In two independent studies, FOS expression during a drug-free test exposure to CS+ was significantly higher in experimental

mice that had previously received ethanol coincident with the *onset* of CS exposure (Before-S) than in unpaired (Delay-S) or naïve (Naïve-S) control mice in the VTAant (see Tables 3 and 4). Several other brain areas that showed conditioned increases in FOS were also identified in Experiments 1 (BST, SNC, DMH) and 2 (CeM, La, CA1, CA2, and DG). However, FOS expression was no different from control levels in experimental mice that had previously received ethanol coincident with *termination* of CS exposure (After-S). Measurement of locomotor activity confirmed that our conditioning procedures were effective in producing behavioral differences between groups of mice (Figs. 2 and 4). Before-S mice in both experiments showed locomotor sensitization across ethanol trials and conditioned increases in activity when tested under saline in the presence of CS+. Conversely, After-S mice showed suppressed locomotor activity across conditioning days and on test. Although place preference data were not obtained, these conditioned locomotor effects are consistent with previous studies in DBA/2J mice in which the Before procedure produced CPP and the After procedure produced CPA (Cunningham and Noble, 1992; Cunningham et al., 1997; Cunningham and Henderson, 2000).

4.1. Conditioned changes in FOS

Differences between experimental (Before, After) and control (Delay, Naïve) groups in FOS expression elicited by CS+ presumably reflect the effects of prior conditioning. Moreover, because these differences were measured after a relatively large number of conditioning trials (six), it is reasonable to assume that they reflect neural processes involved in the *expression* of the ethanol-induced conditioned response rather than processes involved in the *acquisition* (i.e., learning) of that conditioned response. In Experiment 1, Before-S mice had higher levels of FOS expression in the BST and DMH compared to all other groups (Table 3). Moreover, Before-S mice had significantly higher levels in the SNC and VTAant compared to Delay-S and Naïve-S controls. (The difference between Before-S and After-S mice for the VTAant region approached significance, $p < .06$). Experiment 2 replicated the finding of higher FOS activation in VTAant in Before-S-mice compared to Delay-S-mice, but did not replicate the finding of group differences in SNC or DMH (Table 4). Although the group difference in BST fell short of the criterion for significance in Experiment 2 ($p = .09$, two-tailed), the direction of the effect was identical to that seen in Experiment 1. In addition, Experiment 2 yielded several other brain areas in which experimental mice had higher levels of FOS expression than control mice, including the CeM and La portions of the amygdala and the CA1, CA3, and DG regions of the hippocampus. In two cases (CA1, CA3), the group difference occurred only when mice were tested with saline (i.e., Before-S vs. Delay-S), which was also true for the effects seen in BST and VTAant In the other three cases (DG, CeM, La), however, the conditioning effect was apparent in both saline- and ethanol-tested mice, suggesting that the ability of CS+ to increase FOS expression did not depend on drug state.

In aggregate, these experiments implicate portions of the mesolimbic dopamine pathway (VTAant), extended amygdala (CeM, La, BST), hypothalamus (DMH), and hippocampus (CA1, CA2, DG) as potential mediators of conditioned responses to ethanol-paired cues. Acute ethanol exposure has previously been shown in mice to increase FOS expression in two of these brain areas, both of which are part of the extended amygdala. More specifically, injection of ethanol (1.5–4 g/kg) has been reported to induce FOS in the central nucleus of the amygdala (CeA: Demarest et al., 1999; Hitzemann and Hitzemann, 1997; Ryabinin and Wang, 1998) and BST (Demarest et al., 1999; Ryabinin and Wang, 1998) in mice. Thus, the present studies show that an ethanol-paired CS+ is able to induce FOS in brain areas that are directly activated by ethanol exposure. However, ethanol-induced induction of FOS in a particular brain area does not necessarily endow CS+ with the ability to produce conditioned increases in FOS in that same area. For example, although acute injection of ethanol at 2 g/kg has been reported to increase FOS expression in AcbC of DBA/2J mice (Hitzemann and Hitzemann, 1997), we found no evidence of a conditioned FOS response in this brain area.

The present studies also found that an ethanol-paired CS+ acquired the ability to induce FOS in several brain areas that have previously been reported to be unaffected by acute or chronic ethanol exposure in DBA/2J mice (VTA: Hitzemann and Hitzemann, 1997; La, CA1, CA2, DG: Ryabinin and Wang, 1998). The conditioned FOS response in VTAant, which was significant in both experiments, is of particular interest given the previously reported sensitivity of VTA dopamine neurons to direct activation by ethanol as measured by extracellular single unit recordings in a mouse brain slice preparation (Brodie, 2002; Brodie and Appel, 2000). As part of the mesolimbic dopamine system, the VTA has been widely implicated in the neurocircuitry involved in mediating the reinforcing effects of ethanol and other abused drugs (Koob et al., 1998; McBride et al., 1999). The finding that rats will self-administer ethanol directly into VTA provides compelling evidence that this brain site is importantly involved in mediating ethanol's reinforcing effects (Gatto et al., 1994; Rodd et al., 2004). Thus, despite the lack of a direct ethanol effect on FOS expression in the VTA of DBA/2J mice (Hitzemann and Hitzemann, 1997), our finding of conditioned FOS increases in this brain area appears consistent with many other findings implicating this area in the mediation of ethanol's primary reinforcing effects. Involvement of the VTA in ethanol-induced conditioning is also consistent with findings from several studies suggesting that this brain area plays an important role in place preference conditioning induced by other abused drugs (Gholami et al., 2003; Harris and Aston-Jones, 2003; Neumaier et al., 2002; Popik and Kolasiewicz, 1999). In all of these studies, effects on CPP were believed to be due to modulation of VTA dopamine cells. It is not known, however, whether the VTA neurons activated in the present studies are dopaminergic.

Conditioned increases in FOS were also observed in two structures within the extended amygdala: BST (Experiment 1) and CeM (Experiment 2). Both of these areas receive projections from the VTA (Swanson, 1982) and both show increases in FOS expression after acute ethanol exposure (Demarest et al., 1999; Hitzemann and Hitzemann, 1997; Ryabinin and Wang, 1998).

Moreover, ethanol (like morphine, nicotine and cocaine) produces dose-dependent increases in extracellular dopamine in the BST (Carboni et al., 2000). The extended amygdala has been implicated in the primary reinforcing effects of ethanol (Koob, 2003; McBride, 2002). For example, infusion of a $GABA_A$ antagonist into either the CeA or BST has been reported to decrease ethanol self-administration in rats (Hyytiä and Koob, 1995). Moreover, infusion of a dopamine D_1 antagonist into the BST has been found to reduce ethanol self-administration (Eiler et al., 2003) and to interfere with the acquisition and expression of morphine-induced CPP (Zarrindast et al., 2003). The extended amygdala has also been more broadly implicated in associative learning processes (Balleine and Killcross, 2006; Fanselow and Poulos, 2005). Thus, development of an ethanol-induced conditioned FOS response within the extended amygdala appears to be consistent with the larger role this brain area plays in learning and drug reinforcement.

Because several previous studies have shown that acute ethanol exposure increases FOS in BST (Demarest et al., 1999; Ryabinin and Wang, 1998), the finding that test session exposure to ethanol reduced the FOS response in this brain area was somewhat unexpected (Before-S vs. Before-E, Table 4). However, because there was no difference in the FOS response in BST between the Delay-S and Delay-E groups (Table 4), it appears that repeated exposure to ethanol (during the conditioning phase) reduced the FOS response in BST to acute ethanol, much the same as repeated ethanol exposure has been reported to reduce the FOS response in CeA to acute ethanol (Ryabinin and Wang, 1998). Nevertheless, despite losing its ability to directly induce FOS in BST, test exposure to ethanol was able to suppress the FOS increase in BST. One potential interpretation of this finding is that the FOS increase in BST in the Before-S group may have been triggered by the omission of an expected ethanol injection (see Section 4.4 for further discussion of this possibility).

The finding of conditioned FOS increases in hippocampus (CA1, CA3, DG) in Experiment 2 was somewhat surprising given that acute ethanol exposure has previously been shown either to have no effect (Ryabinin and Wang, 1998) or to reduce FOS expression (Ryabinin, 1998) in hippocampus. Nevertheless, the finding of ethanol-induced conditioned increases in the hippocampus appears to be consistent with previous reports of increased FOS in hippocampal areas of rats exposed to an environment in which they had previously self-administered ethanol (CA3: Topple et al., 1998) or cocaine (CA1, DG: Neisewander et al., 2000). Increased FOS in hippocampal areas has also been reported for rats (CA1, CA3, DG: Wisłowska-Stanek et al., 2005) and mice (Milanovic et al., 1998) exposed to a context previously paired with electric shock in a fear conditioning procedure. Thus, it appears that activation of FOS in the hippocampus may develop as a conditioned response to cues previously paired with a variety of biologically important events.

Our finding of a conditioned FOS increase in the amygdala but no conditioned change in the nucleus accumbens is consistent with the results of one early study that examined cocaine-induced conditioning in rats (Brown et al., 1992). However, these outcomes are opposite to those reported in several subsequent studies of conditioning induced by cocaine (Franklin and Druhan, 2000; Hotsenpiller et al., 2002; Miller and Marshall, 2005) or morphine (Schroeder and Kelley, 2002) in rats. In all of these other studies, a conditioned increase in FOS was observed in the nucleus accumbens (core), but not in the amygdala. In a recent study of methamphetamine-induced conditioning in outbred mice, conditioned increases in FOS were found in the nucleus accumbens shell and in both the basolateral and basomedial amygdala (Rhodes et al., 2005). In that study, which appears to be the only previous study to examine BST, conditioned FOS increases were also found in that brain area, consistent with the outcome of our studies. Unfortunately, none of these other studies examined FOS changes in the brain area showing the most robust conditioned effect in both of our studies (VTAant). It is not clear whether the discrepancies between our studies and these other studies are due to differences in species (i.e., rats vs. mice), drug (cocaine vs. morphine vs. methamphetamine vs. ethanol) or methodology.

It is important to consider whether increased FOS seen in several brain areas in the Before-S mice was due to their conditioned locomotor behavior. Previous studies have shown inconsistent results on this issue. Thus, on one hand, several studies have demonstrated dissociation of conditioned locomotion and conditioned FOS induction (Hotsenpiller et al., 2002; Mead et al., 1999; Schroeder et al., 2000). On the other hand, Rhodes et al. (2005) found a positive correlation between locomotion and FOS expression in several areas. Importantly, however, the latter investigators found no correlation between locomotor activity and expression of FOS in BST. Moreover, locomotor activity was not significantly correlated with FOS-positive cell counts in either BST or VTAant in our study (data not shown). Thus, it is unlikely that the ethanol-induced conditioned increases in FOS in the present studies were simply a byproduct of the conditioned increase in activity.

4.2. Ethanol effects on FOS expression

In several brain regions, FOS induction was affected primarily by acute exposure to ethanol or a history of ethanol exposure rather than by conditioning. For example, test session exposure to ethanol elevated FOS levels in the CeL, PV and EW, but suppressed FOS expression in the DMH (Experiment 2, Table 4). These data are consistent with previous findings showing ethanol-induced increases in the CeL and PV (Chang et al., 1995; Hitzemann and Hitzemann, 1997; Ryabinin et al., 1997). Increased FOS expression in the EW of ethanol-tested mice was expected on the basis of several previous reports in both rats (Chang et al., 1995; Ryabinin et al., 1997; Topple et al., 1998; Weitemier et al., 2001) and mice (Bachtell et al., 1999, 2002; Ryabinin et al., 2001, 2003). It is important to note that the mouse EW (identified based on morphological criteria) does not contain preganglionic parasympathetic neurons and is involved in regulation of non-ocular functions (Bachtell et al., 2003, 2004; Ryabinin and Weitemier, 2006; Weitemier et al., 2005). However, CeL, PV, and EW did not show greater FOS induction in the Before groups, suggesting that these regions may not be involved in conditioned responses induced by ethanol.

Surprisingly, ethanol experience during conditioning altered FOS expression in the MPO during a subsequent saline test (Experiment 1, Table 3). That is, Before-S, After-S, and Delay-S mice all had higher levels of FOS in this region than Naïve-S controls. Because Delay-S mice never had ethanol paired with the apparatus, increased FOS in the MPO in the ethanol-experienced groups can be attributed to prior ethanol history rather than to ethanol-induced conditioning to the CS+. Although elevated FOS protein in the MPO suggests this region serves as a historical marker for ethanol experience, additional analyses showed that the level of FOS induction in the MPO was not related to the recency (either 24 or 48 h) of last ethanol exposure (data not shown). This nonspecific effect of ethanol exposure could not be reexamined in Experiment 2 because there were no ethanol-naive mice in that study. The role of the MPO in ethanol's effects is not well characterized, but microinjections of ethanol into this region induce sleep, which appears to be GABA-mediated (Mendelson, 2001).

4.3. Comparison of before and after groups

The results of Experiment 1 showing different patterns of neural activation for Before-S and After-S mice are consistent with the hypothesis that ethanol-induced CPP and CPA are mediated by different underlying processes (Cunningham et al., 2002). In general, the FOS profiles for these two groups differ in that Before-S mice had more activation in the BST and DMH compared to After-S mice (as well as both control groups), and FOS differences in the VTAant approached significance at $p < .06$. However, no After-S mice were tested in Experiment 2, so these differences could not be reexamined. The activation of reward circuitry (VTAant and BST) in Before-S mice but not in After-S mice fits with the notion that CPP reflects rewarding effects of ethanol whereas CPA does not.

Somewhat surprisingly, there were no significant differences in FOS expression between the After-S group and either control group (Delayed-S, Naïve-S). In other words, FOS expression in the brain areas we examined was not sensitive to the neural changes that presumably underlie the conditioned activity suppressing and aversive effects of a CS that immediately precedes injection of ethanol (Cunningham et al., 1997). This unexpected outcome may be due to the exclusion of brain stem regions from the FOS analysis. Ethanol-induced taste aversion conditioning has previously been shown by others to increase FOS expression in the nucleus of the solitary tract, the parabrachial nucleus, and the area postrema in subjects subsequently presented with an ethanol-paired tastant (Thiele et al., 1996). In light of data suggesting a genetic correlation between ethanol-induced conditioned taste aversion and ethanol-induced CPA (Cunningham and Ignatoff, 2000), it is possible that brain regions activated by the CS+ in the After-S group may have been missed by not examining FOS changes in the brain stem.

4.4. Novelty

In Experiment 2, conditioned FOS effects were seen in three brain areas (CA1, CA2, VTAant) only during the saline test, but not during the ethanol test (Table 4). There are at least two explanations for this outcome. One possibility is that acute ethanol exposure may have suppressed expression of the conditioned increase in FOS in those brain areas. This explanation seems quite plausible for the two hippocampal areas in light of previous studies showing that ethanol has a general suppressive effect on FOS expression in hippocampus (Ryabinin, 1998). However, as noted earlier (Section 4.1), it is also possible that the apparent conditioned increases in FOS observed in saline-tested mice were caused by a novelty response to omission of the expected ethanol injection. Given the specific brain areas involved, this explanation fits with the recent suggestion that the hippocampus and VTA comprise a functional loop that detects novelty (Lisman and Grace, 2005). However, if CS− evoked FOS increases in VTA and hippocampus were caused by the novelty of ethanol omission in saline-tested mice, there should have been similar changes in After-S mice because they also failed to receive an expected ethanol injection following test exposure to the CS+. Thus, the fact that After-S mice did not differ from control mice in these brain areas (Table 3) argues against the novelty-response interpretation. Moreover, the novelty interpretation cannot explain the findings of conditioned increases in FOS that were not affected by drug state during testing (DG, CeM, La). Although the design and outcomes of the present studies allowed us to address the novelty interpretation, this issue has largely been ignored in previous studies of drug-induced conditioned changes in FOS in which subjects were exposed to a similar type of novelty on test (e.g., Brown et al., 1992; Franklin and Druhan, 2000; Miller and Marshall, 2005; Rhodes et al., 2005; Schroeder and Kelley, 2002).

4.5. Final caveats

Given the discrepancies between studies in the brain areas showing conditioned FOS responses, primary emphasis should be placed on VTAant, which showed clear and consistent differences between the Before and Delay groups in both experiments. Based on the significant group difference in Experiment 1 and the strong trend in Experiment 2, BST should also receive strong consideration as a candidate brain area involved in ethanol-induced Pavlovian conditioning. The reasons behind the discrepancies in other brain areas are not clear, but they may be the result of batch effects in FOS immunohistochemistry (e.g., Rhodes et al., 2005). Although the degree of concordance between experiments was lower than expected, it should be noted that the present study is among the first in which drug-induced conditioned changes in FOS expression are reported from two independent experiments involving the same comparison groups. In most previously published studies (Brown et al., 1992; Franklin and Druhan, 2000; Hotsenpiller et al., 2002; Miller and Marshall, 2005; Neisewander et al., 2000; Rhodes et al., 2005; Schroeder et al., 2001; Schroeder and Kelley, 2002; Topple et al., 1998), effects of the drug conditioning procedure on FOS expression were examined in only one experiment, providing no opportunity to assess the reliability of observed group differences in specific brain areas.

Because the conditioning procedure used for Before mice has previously been shown to produce CPP, there is a strong likelihood that the brain regions showing conditioned changes in FOS in these studies are normally involved in the expression of CPP. However, the correlational nature of this mapping technique encourages caution when interpreting these results. For example, although these studies consistently showed increased FOS induction in the VTAant in response to an ethanol-paired CS+, they do not provide direct evidence that this region mediates or modulates expression of CPP. Experimental manipulations that target this candidate structure (e.g., lesions or microinjections) are required to more completely determine their influence on approach and contact with ethanol-paired stimuli. Recently, the importance of the VTA in modulating expression of ethanol-induced CPP was confirmed in a study (Bechtholt and Cunningham, 2005) that showed a reduction in expression of CPP following intra-VTA infusion of an opioid antagonist (methylnaloxonium) or $GABA_B$ agonist (baclofen).

Finally, the present studies do not address whether regions involved in the *acquisition* (initial learning) of an ethanol conditioned response differ from those mediating the *expression* of that response. It is likely that patterns of neural activity elicited by a drug-paired stimulus shift across learning trials (Phillips et al., 2003). Because the present studies examined tissue collected after a relatively large number of conditioning trials (six), they are probably most relevant to understanding brain systems involved in the *expression* of ethanol-induced conditioning. Future studies must address their possible role in the acquisition of ethanol-induced conditioned responses.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pbb.2007.04.017.

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