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Patterns of Brain Activation Associated With Contextual Conditioning to Methamphetamine in Mice

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Classical conditioning is thought to play a key role in addiction. The authors used c-Fos immunohistochemistry to demonstrate a conditioned physiological response to methamphetamine (meth) in mice. Male outbred mice were placed into an environment where they had previously experienced 2 mg/kg meth or saline. The meth-paired mice displayed increased c-Fos in several brain regions, including the nucleus accumbens, prefrontal cortex, orbitofrontal cortex, basolateral amygdala, and bed nucleus of the stria terminalis. No conditioned locomotor activity was observed, but individual activity levels strongly correlated with c-Fos in many regions. A batch effect among immunohistochemical assays was demonstrated. Results implicate specific brain regions in classical conditioning to meth and demonstrate the importance of considering locomotor activity and batch in a c-Fos study.

Keywords: methamphetamine, mice, classical conditioning, locomotor activity, c-Fos

One of the greatest obstacles to successful treatment of drug addiction is craving and subsequent relapse to drug use. Even after months of abstinence, recovering addicts often experience intense, overpowering urges that renew drug-seeking behavior (Schroeder, Binzak, & Kelley, 2001). It is well known that craving can be triggered in abstinent drug users by exposure to drug-associated cues. For example, detoxified human cocaine users report increased craving when they watch people take cocaine, and cigarette smokers crave cigarettes when they handle cigarettes or watch people smoke (Brody et al., 2002; Childress et al., 1999). These and other studies suggest that Pavlovian learning (i.e., classical conditioning) plays a key role in craving, but the neural substrates and circuitry underlying this process are not known.

Basic research often begins in mice or rats, but this poses a problem for craving research because craving is a subjective human emotion, and it is not clear whether rodents experience craving or, if they do, how to measure it (Littleton, 2000). One strategy that has been adopted by several researchers is to construct partial models, each of which taps into a small part of the larger constructs such as craving or addiction. In this framework, the goal is not to establish a complete animal model of craving but to study certain features relevant to craving in the rodents (Littleton, 2000). For example, Pavlovian learning occurs in rats or mice after drug administration and can be studied in a variety of ways. One approach has been to measure locomotor activity in a drug-free animal placed into an environment where it had previously experienced a drug (Adams, Careri, Efferen, & Rotrosen, 2000; Panlilio & Schindler, 1997). Many drugs are known to stimulate locomotor activity in mice and rats if given at an appropriate dose. These include methamphetamine (meth; Kamens, Burkhart-Kasch, McMinnon, Reed, & Phillips, 2005), cocaine (Tolliver & Carney, 1995), morphine (Morland, Jones, Palomares, & Alkana, 1994), and alcohol (Phillips, Huson, Gwiazdon, Burkhart-Kasch, & Shen, 1995). After repeated administration and repeated pairing of the drug with a particular environment, mere exposure to the drug-paired environment (in a drug-free state) is often sufficient to elicit locomotor stimulation (Adams et al., 2000; Panlilio & Schindler, 1997; Schroeder et al., 2001; Schroeder, Holahan, Landry, & Kelley, 2000). This increased locomotor activity in the drug-paired environment is thought to reflect an altered motivational state of the animal (Panlilio & Schindler, 1997; Schroeder et al., 2001). However, this conditioned locomotor effect is not always seen in mice and depends on the strain or genotype (Meyer & Phillips, 2003; Phillips, Dickinson, & Burkhart-Kasch, 1994; Phillips et al., 1995).

Conditioned locomotor activity is an example of a conditioned behavioral response because the animal's behavior changes in response to the drug-paired cues, but that leaves open the question of which physiological systems underlie the behavior or altered motivational state displayed in the model. Physiological responses conditioned by drugs of abuse have also been documented in mice (Le Foll, Frances, Diaz, Schwartz, & Sokoloff, 2002; Mead, Vasilaki, Spyraki, Duka, & Stephens, 1999), rats (Brown, Robertson, & Fibiger, 1992; Franklin & Druhan, 2000; Neisewander et al., 2000; Schroeder et al., 2001), and humans (Brody et al., 2002; Childress et al., 1999), and in the human studies, some of these responses have been hypothesized to underlie craving. For example, when cocaine users watched people take cocaine and smokers watched people smoke, blood flow increased in several brain

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regions (including the cingulate cortex [Cg], orbitofrontal cortex [OFC], prefrontal cortex [PFC], and amygdala) in response to the video images of drug taking and in conjunction with reports of craving, as indicated by positron emission tomography (using [^{15}O] H_2O tracer). These physiological responses were interpreted as representing patterns of neural activation that underlie the emotional state of craving (Brody et al., 2002; Childress et al., 1999).

Increased neural activation in response to drug-paired cues has also been documented in rats for several different drugs by means of immunohistochemical detection of c-Fos protein (Brown et al., 1992; Franklin & Druhan, 2000; Neisewander et al., 2000; Schroeder et al., 2000, 2001; Topple, Hunt, & McGregor, 1998). This method gives high resolution (up to a single cell) and tracks changes in gene expression that have been suggested to play a role in learning and plasticity (Anokhin & Rose, 1991; Kaczmarek, 1993; Kaczmarek & Nikolajew, 1990; Maleeva, Ivolgina, Anokhin, & Limborskaia, 1989; Swank, Ellis, & Cochran, 1996; Tischmeyer, Kaczmarek, Strauss, Jork, & Matthies, 1990). Occasionally, when a neuron is stimulated, a reaction cascade occurs that affects the expression of genes. One gene that is often immediately induced is *c-fos*. This is followed by an increase in c-Fos protein that reaches peak concentrations approximately 90 min after stimulation (Nestler, Barrot, & Self, 2001; Zangenehpour & Chaudhuri, 2002). Protein complexes containing c-Fos bind to promotor regions of target genes and change their expression (Herdegen & Leah, 1998). In these rat studies, it was inferred that the changes in c-Fos were associated with an altered motivational state. Such studies have been conducted with cocaine (Brown et al., 1992; Ciccocioppo, Sanna, & Weiss, 2001; Franklin & Druhan, 2000; Neisewander et al., 2000), morphine (Schroeder et al., 2000), nicotine (Schroeder et al., 2001), and alcohol (Topple et al., 1998). Collectively, these studies have identified many brain regions that become activated by a drug-paired context, and some of these regions overlap among drugs. For example, the Cg, OFC, and nucleus accumbens shell region (NACS) are consistently activated by the drug-paired context, for nearly all drugs of abuse tested. These results mirror what has been found in human imaging studies (Brody et al., 2002, 2004; Childress et al., 1999; Daglish et al., 2001, 2003; Garavan et al., 2000; Grusser et al., 2004; Heinz et al., 2004; Kilts, Gross, Ely, & Drexler, 2004; Myrick et al., 2004; Tapert, Brown, Baratta, & Brown, 2004; Tapert et al., 2003; Volkow, Fowler, & Wang, 2004; Volkow et al., 1999, 2003; Wilson, Sayette, & Fiez, 2004).

The aim of this study was to develop a mouse model of a classically conditioned physiological response to meth by using immunohistochemical detection of c-Fos protein. More specifically, the goal was to identify brain regions that become activated or inactivated (as indexed by levels of c-Fos protein) when mice are placed into an environment where they had previously experienced meth. Because meth and meth-paired cues can increase locomotor activity in mice (Itzhak, 1997; Kitanaka, Kitanaka, & Takemura, 2003) and physical activity can affect expression of c-Fos (Rhodes, Garland, & Gammie, 2003), we measured locomotor activity during testing to determine the extent to which the c-Fos responses could be explained by physical activity in the model. To the best of our knowledge, it is unknown whether meth-paired cues can elevate c-Fos after effects of locomotor activity have been accounted for, and the basic model, using c-Fos as the conditioned response, has rarely been applied to mice for any drug of abuse (but see Le Foll et al., 2002; Mead et al., 1999).

We chose to study meth because it is known to elicit powerful feelings of craving in humans (Hartz, Frederick-Osborne, & Galloway, 2001) and because meth abuse is currently on the rise in the United States. Mice were chosen as the model organism because our long-term goal is to use a version of this model to identify the genes that contribute to variation in the brain responses. The genetic tools available for this purpose (e.g., inbred strains, gene mapping, knockout, and transgenic technology) are well established for mice. The approach of finding the genes that underlie a trait offers promise for elucidating underlying mechanisms (Crabbe, 2002; Rhodes & Crabbe, 2003). However, before we embark on any genetic studies, we plan to evaluate the specificity of the model by carrying out additional experiments using natural rewards (e.g., food) or aversive stimuli (e.g., electric shock) for the conditioning, instead of meth. This will require many iterations of the immunohistochemical assay and a comparison of c-Fos responses across experiments. Therefore, the final aim of this study was to quantify differences that arise as a result of variation in the immunohistochemical assay itself to determine the extent to which adjustments and/or standards will have to be used when comparing c-Fos responses across experiments.

Method

Subjects and Husbandry

Genetically variable WSC-2 male mice were studied at approximately 60 days old. The WSC-2 strain was derived from the outbred HS/Ibg stock (Institute for Behavioral Genetics, Boulder, Colorado), which was established through crossing eight standard inbred strains. A population consisting of nine breeding pairs has been maintained in our laboratory for 92 generations. A rotational breeding scheme was used, in which offspring of common grandparents were prevented from mating to reduce the incidence of inbreeding (see Crabbe, Kosobud, Young, Tam, & McSwigan, 1985). The mice were housed 3–4 per cage in standard polycarbonate or polysulfone shoebox cages with Bed-o-Cob (Maumee, OH) bedding. Twenty mice from nine different families were transferred to individual housing 1 week prior to the start of the experiment. Rooms were controlled for temperature (21 ± 1 °C) and photoperiod (12-hr light–dark cycle). Food (Purina 5001; Ralston-Purina, St. Louis, MO) and water were provided ad libitum except during behavioral testing, when neither was present. All mice were housed and tested in the Veterinary Medical Unit at the Portland Veterans Affairs Medical Center, which is an Association for Assessment and Accreditation of Laboratory Animal Care-approved facility. All procedures were approved by the appropriate Institutional Animal Care and Use Committee and adhered to National Institutes of Health guidelines.

Contextual Conditioning

The experimental design was adapted from Brown et al. (1992), who studied effects of cocaine conditioning in rats. The 20 mice were randomly assigned to two groups: meth-paired ($n = 10$) or saline-paired ($n = 10$). Starting 1 hr after lights had shut off in the animal rooms (a time when nocturnal mice are normally active), meth-paired subjects were weighed; injected intraperitoneally with meth hydrochloride (Sigma, St. Louis, MO; 2 mg/kg in a volume of 0.005 ml 0.9% saline/gram body weight); and then placed into an activity monitor (see section entitled *Activity Monitors* below), where locomotor activity was measured for 30 min. The dose of meth was chosen because, in eight standard inbred strains of mice, it produces a robust stimulation of locomotor activity but does not induce

stereotypy (Phillips, 1999). After the locomotor test, the subjects were returned to their home cages (individually housed), where they were injected with saline 90 min later (2 hr after the meth injection). Control subjects were exposed to the identical procedure, except the order of administration of meth and saline was reversed. Specifically, control mice were injected with saline before being placed into the activity monitor and were later injected with meth in their home cage. This procedure was repeated on 10 consecutive days. These 10 days were considered to be the training phase of the study, in which half the mice were trained to associate the activity monitor with meth and the other half were not. Note that all the mice were treated equivalently with respect to exposure to meth and to the monitors, the only differences being where they experienced meth and the time during the night when meth was administered (a difference of 2 hr). On the test day, 48 hr after the final training session, all mice were given a saline injection and then placed directly into the activity monitors, where locomotor activity was measured for 90 min. Mice were asphyxiated with CO_2 immediately after the 90-min test.

Each day before the training and on the test day, the mice were moved from the colony room into the activity-monitor room immediately after lights had shut off in the colony room. They were allowed to acclimate to the activity-monitor room (where the lights were also shut off) for 1 hr before the injections were given and locomotor tests began. After the locomotor tests, the mice were moved back into the colony room, where they received the second injection 90 min later, except on the test day, when the mice were asphyxiated immediately after the test. Red lights were used so that we could handle the mice in the otherwise dark rooms. The subjects were moved between the rooms, which were adjacent to each other, on a cart with a sheet covering the cages. It took approximately 2 min to inject each mouse, place it into the activity monitor, and turn on the recording device. Thus, a 40-min delay occurred between testing the first and last subjects. On the test day, we used a 4-min delay between each mouse to allow enough time to process the tissue between mice.

Activity Monitors

The activity monitors consisted of clear plastic boxes (40 cm long × 40 cm wide × 30 cm high) covered by clear plastic lids and set inside black acrylic chambers containing foam insulation for the exclusion of outside noise. Lights were turned off in the activity monitors for the locomotor test. Distance traveled (in centimeters) was measured with Accuscan (Columbus, OH) photobeam apparatus and software. Eight pairs of intersecting photo beams were projected 2 cm above the floor. The software interpreted consecutive beam breaks to estimate distance traveled.

Brain Regions

The brain regions listed in Table 1 were chosen prior to data collection. Most were chosen because they have been implicated in motivation or addiction in the literature. The visual cortex (VX), dentate gyrus (DG), lateral habenular nucleus (LHB), Edinger–Westphal nucleus (EW), and superior colliculus (SC) were included as negative controls and were not expected to differ between the treatment groups.

Immunohistochemistry

Analysis of c-Fos expression generally followed a published method (Ryabinin, Wang, Freeman, & Risinger, 1999), with some modifications. Immediately following the test session, the mice were killed by CO_2 asphyxiation. Their brains were removed and placed directly into a solution containing 2% paraformaldehyde in phosphate-buffered saline (PBS), where they remained overnight at 4 °C. The PBS solution (10 mM, pH 7.4) was prepared with 4.05 g NaOH, 16.85 g $NaH_2PO_4 \times H_20$, and 4.25 g NaCl in 1 L water. Twenty-four hours later, the brains were transferred to 20% (wt/vol) sucrose in PBS, and then to 30% (wt/vol) sucrose in PBS the following day. A cryostat was used to cut coronal sections (40 μm), which were placed into in a 24-well plate containing PBS. Alternate (every other) sections were washed in PBS and then incubated, free-floating, in 0.3% (vol/vol) hydrogen peroxide in PBS for 15 min. Blocking was performed with 4.5% (vol/vol) goat serum in a PBS solution containing 0.3% (vol/vol) Triton X-100 (PBS–Triton) for 4 hr at room temperature. The sections were then incubated overnight at room temperature in a solution containing c-Fos primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:10,000 in PBS–Triton containing 0.1% bovine serum albumin. To visualize the antibody, we used the peroxidase method (Vectastain ABC kit; Vector Laboratory, Burlingame, CA), with goat anti-rabbit secondary antibodies (1:200 dilution in PBS–Triton) and metal-enhanced diaminobenzidine as the chromogen (Pierce, Rockford, IL). We stopped the diaminobenzidine reaction by washing the sections several times in PBS. The sections were sorted before being mounted on slides in a solution containing ~60% water, ~40% ethanol, 0.12% acetic acid, and 0.3% gelatin.

Table 1
Brain Regions Chosen Prior to Data Collection

Abbreviation	Brain region
AH	Anterior hypothalamus
BL	Basolateral amygdala
BM	Basomedial amygdala
BNST	Bed nucleus of the stria terminalis
Ce	Central nucleus of the amygdala
Cg	Cingulate cortex
CPU1	Caudate-putamen (anterior)
CPU2	Caudate-putamen (posterior)
DG[a]	Dentate gyrus of the hippocampus
ENT	Entorhinal cortex
EW[a]	Edinger-Westphal nucleus
LH	Lateral hypothalamus
LHB[a]	Lateral habenular nucleus
LSD	Lateral septum (dorsal)
LSI	Lateral septum (intermediate)
LSV	Lateral septum (ventral)
MPA	Medial preoptic area
NACS	Nucleus accumbens shell
NACC	Nucleus accumbens core
OFC	Orbitofrontal or insular cortex
PAG	Periaqueductal gray
PFC	Prefrontal cortex
PIR	Piriform cortex
PV	Paraventricular thalamic nucleus
PVN	Paraventricular hypothalamic nucleus
SC[a]	Superior colliculus
SX	Somatosensory cortex
VP	Ventral pallidum
VX[a]	Visual cortex

Note. Most regions were chosen because they have been implicated in motivation or addiction in the literature.
[a] These regions were included as negative controls that were not expected to vary between groups.

Image Analysis

We took the following steps to ensure that c-Fos immunoreactivity was measured consistently between individual subjects:

1. All sections were exposed to diaminobenzidine for exactly 2.5 min.

2. The light level emitted by the microscope was kept constant for all samples.

3. In the image analysis, a thresholding procedure was applied to distinguish c-Fos-positive nuclei automatically, following Bachtell et al. (2003). All pixels in the image below a threshold level of staining were considered to be background and were eliminated. The particles remaining in the image after the threshold was applied were considered positive for c-Fos and were counted. The same threshold was used for all sections.

4. All slides were coded, and the counting (automated) was performed by one individual (Justin S. Rhodes), who was blind to the experimental conditions.

5. Only particles within a specified size range were counted (between 10 and 100 pixels, under 200× magnification).

The immunohistochemistry was performed in five batches separated by 1 week. Each batch consisted of 2 saline-paired and 2 meth-paired subjects. The same vial of primary c-Fos antibody was used in all five batches, and the same experimenter (Justin S. Rhodes) conducted each batch. For each individual mouse, c-Fos-positive nuclei were counted unilaterally, in three alternate sections per brain region, to obtain an average count per brain region for analysis. An average was used to reduce the noise introduced by variation in immunohistochemical staining among sections, following Rhodes et al. (2003). The remaining alternate sections were kept for reserve purposes in case the assay had to be repeated. Microscopic (Olympus BX51, Melville, NY) images of the sections were captured with a high-resolution digital camera (Olympus Qcolor3) interfaced with a Macintosh personal computer running OS-X. All c-Fos-positive cells were automatically counted (by means of Image J software; Rasband, 2005) within a box (0.70 × 0.52 mm) placed at the locations shown in Figure 1 (following Paxinos & Franklin, 2001). If the brain regions were smaller than the box, as was the case for paraventricular thalamic nucleus (PV), LHB, and EW, then either the particles were counted visually (this was done for EW) or a smaller box was placed individually around the regions (this was done for PV and LHB). For these smaller boxes, dimensions were visually determined for each region.

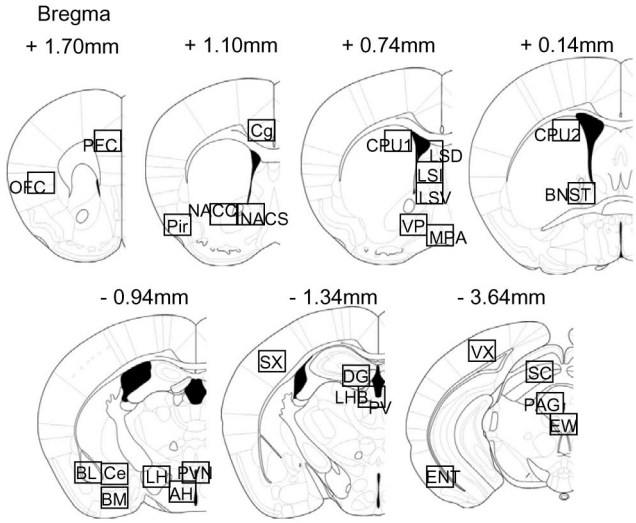

Figure 1. Locations where c-Fos-positive nuclei were counted (boxes, shown roughly to scale, were 0.70 × 0.52 mm). As noted, smaller boxes (not shown) were used for counting paraventricular thalamic nucleus (PV) and lateral habenular nucleus (LHB), and Edinger–Westphal nucleus (EW) was hand-counted. Reprinted from *The Mouse Brain in Stereotaxic Coordinates*, 2nd edition, G. Paxinos and K. Franklin, Figures 17, 22, 25, 30, 39, 42, 61, Copyright, 2001, with permission from Elsevier. PFC = prefrontal cortex; OFC = orbitofrontal (or insular) cortex; Cg = cingulate cortex; Pir = piriform cortex; NACC = nucleus accumbens core; NACS = nucleus accumbens shell; CPU1 = anterior caudate-putamen; LSD = dorsal lateral septum; LSI = intermediate LS; LSV = ventral LS; VP = ventral palladium; MPA = medial preoptic area; CPU2 = posterior caudate-putamen; BNST = bed nucleus of the stria terminalis; BL = basolateral amygdala nucleus; Ce = central amygdala nucleus; LH = lateral hypothalamus; PVN = paraventricular nucleus of the hypothalamus; BM = basomedial amygdala nucleus; AH = anterior hypothalamus; SX = somatosensory cortex; DG = dentate gyrus of the hippocampus; VX = visual cortex; SC = superior colliculus; PAG = periaqueductal gray; ENT = entorhinal cortex.

Statistics

We analyzed c-Fos data first to determine treatment effects without considering contributions of batch or locomotor activity. In subsequent analyses, the effects of batch and locomotor activity were explicitly determined. First, the data were analyzed by means of simple *t* tests to compare numbers of c-Fos-positive nuclei between the two groups for each brain region. Second, the data were analyzed with a two-way analysis of variance (ANOVA), with batch of immunohistochemical assay entered as a blocking factor and group entered as the treatment factor. Even though steps were taken to measure c-Fos immunoreactivity consistently among individual subjects (see above), variation was expected to occur among the five batches because of subtle and uncontrollable differences between immunohistochemical assays. Because the two treatment groups were equally represented per batch, this factor did not need to be considered in the statistical analyses (and was not for the *t* tests or for the regression analyses described below). Without batch entered as a factor, though, the variability attributed to batch is combined into the mean square error, resulting in inflated Type II error rates and *p* values. Thus, the two-way ANOVA was used explicitly to determine the contribution of batch to the error and to obtain uninflated *p* value estimates. Third, data were analyzed with simple linear regression and analysis of covariance (ANCOVA) with locomotor activity as the covariate. This was done to determine the correlation between the numbers of c-Fos-positive nuclei and locomotor activity and the effect of group (meth-paired vs. saline-paired) after adjustment for variation explained by locomotor activity.

Because we conducted multiple tests to compare c-Fos counts between the groups for 27 brain regions, we determined the global, experiment-wise, false discovery rate that would occur for our data if we were to apply the standard .05 *p* value cut-off to determine positive results. This was done with an open source software called Q-value (Dabney & Storey, 2002). Our data yielded many small *p* values (e.g., 11 out of 27 total *t* tests yielded a p value $< .05$). Applying the standard .05 *p* value cut-off would yield a global false discovery rate of 1% (i.e., 1 out of 100 positive results would be false positives). Thus, even though multiple tests were conducted, for these data, the application of the standard *p* value cut-off of .05 is appropriate (Storey, 2002).

The significance level for the acute behavioral response to meth was determined with a repeated measures ANOVA, with day (1–10) as the within-subjects factor and group (meth-paired vs. saline-paired) as the between-subjects factor. A simple *t* test was used to compare locomotor activity between the two groups on the test day (Day 12).

Results

Behavior

As expected, the acute administration of meth had a profound effect on locomotor activity (see Figure 2). Mice that were given a meth injection (2 mg/kg) immediately prior to the activity tests (Days 1–10) traveled, on average, 3.5 times as far in the activity

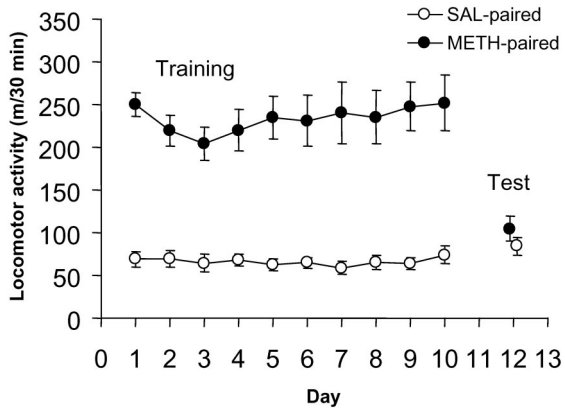

Figure 2. Acute administration of methamphetamine (METH) increased locomotor activity, but no sensitization or conditioned effect on locomotor activity occurred. Days 1–10: Mean (± *SEM*) distance traveled in 30 min by male mice (from the WSC-2 outbred strain) immediately after an intraperitoneal injection of 2 mg/kg methamphetamine or saline (SAL). The same mice were measured each day ($n = 10$ per group). The mice were left undisturbed in their home cages on Day 11. On the test day (Day 12), all mice were given a saline injection before being placed into the activity monitors for 90 min. Day 12: Mean (± *SEM*) distance traveled in the first 30 min of the 90-min test for mice that had previously experienced methamphetamine or saline in the activity monitors on Days 1–10.

monitors compared with those given a saline injection, $F(1, 177) = 355.70$, $p < .0001$. An unexpected result was that no sensitization to the locomotor activating effects of meth was observed. The stimulant effect of meth did not increase over days but instead remained relatively constant. Day, $F(9, 177) = 0.35$, $p = .96$, and Day × Group interaction, $F(9, 177) = 0.29$, $p = .99$, were not significant.

On the test day (Day 12), when all mice were given a saline injection prior to being placed into the activity monitors, the meth-paired mice traveled a distance similar to that of the saline-paired mice, $t(18) = 1.16$, $p = .26$ (i.e., there was no conditioned effect on locomotor activity). Figure 2 (Day 12) shows results for the first 30 min of the 90-min test to facilitate comparison with the previous 10 days of data, which were from 30-min tests. When data were analyzed for the entire 90 min or broken down into 10-min increments, results were the same (i.e., no difference in locomotor activity between meth-paired and saline-paired mice).

Patterns of Brain Activation

Meth-paired mice displayed significantly increased numbers of c-Fos-positive nuclei in several brain regions (see Table 2). The cortex, in particular, was strongly activated (see Figure 3). This included not only regions traditionally emphasized for their role in motivation and addiction such as the PFC, OFC, and Cg, but also nontraditional regions such as somatosensory cortex (SX), piriform cortex (Pir), and entorhinal cortex (ENT). In addition, several subcortical regions were activated, including the NACS, basolateral amygdala (BL), basomedial amygdala (BM), bed nucleus of the stria terminalis (BNST), and PV. Increased activation was not a general phenomena occurring everywhere in the brain, though. Several brain regions remained unchanged. In particular, anterior hypothalamus (AH), lateral hypothalamus (LH), LHB, and SC showed nonsignificant differences between meth-paired and saline-paired mice, and the trend was in the opposite direction. The LHB is of particular interest because this region lies immediately adjacent to PV and was examined in the same sections as PV (see Figure 4). Finally, no c-Fos-positive nuclei were observed in any mouse in the paraventricular hypothalamic nucleus (PVN) and central amygdala nucleus (Ce). Taken together, these results demonstrate that specific brain regions become activated when mice are placed into a meth-paired environment.

Correlation: Brain and Behavior

Remarkably, every brain region examined except BNST, LHB, and EW showed a significant ($p < .05$) correlation between the number of c-Fos-positive nuclei and locomotor activity (see Table 3). These analyses were conducted with all subjects considered together ($n = 20$; 10 meth-paired and 10 saline-paired). In several regions, the correlation was very strong ($R^2 \geq .50$ for SX, ENT, nucleus accumbens core [NACC], DG, lateral hypothalamus [LH], dorsal lateral septum [LSD], and SC; see Figure 5). Recall that locomotor activity did not differ between meth-paired and saline-paired mice on the test day. After we used ANCOVA to adjust for the variation in c-Fos explained by locomotor activity, meth-paired mice still showed significantly increased levels of c-Fos compared with saline-paired mice in the same brain regions that showed positive results when locomotor activity was not considered in the analysis (the group effects in Table 2 were similar to those in Table 3). Figure 5C shows an example of this. In NACS, c-Fos levels were correlated with locomotor activity in both the meth-paired and saline-paired groups, but for a given level of locomotor activity, meth-paired mice showed relatively greater numbers of c-Fos-positive nuclei (Figure 5C). Similar results occurred for all the other regions that showed positive results in Table 2, except BNST, where no relationship between locomotor activity and c-Fos levels existed (see Table 3).

Although LH showed negative results when locomotor activity was not included in the analysis (see Table 2), it emerged as showing positive results after correction for locomotor activity by ANCOVA (the effect of group was not significant in Table 2, whereas it was in Table 3). ANCOVA revealed that the numbers of c-Fos-positive nuclei were significantly reduced in meth-paired mice compared with saline-paired mice in LH for a given level of locomotor activity (see Figure 5D; least-square adjusted means [± *SE*] were 54 ± 8.0 vs. 85 ± 8.4).

Batch Effects on Immunohistochemistry

The immunohistochemistry was conducted in five separate batches, and despite taking measures to apply the procedures consistently across the batches (see the *Image Analysis* section), we detected different levels of c-Fos staining among the batches for many of the brain regions examined (see Table 2). In several regions, the batch effects were very strong, accounting for more than 50% of the variation in the numbers of c-Fos-positive nuclei among individuals (e.g., VX, $R^2 = .61$; AH, $R^2 = .62$; LH, $R^2 = .65$; LSD, $R^2 = .53$; intermediate lateral septum [LSI], $R^2 = .71$; medial preoptic area [MPA], $R^2 = .66$; periaqueductal gray [PAG], $R^2 = .61$). As an example of the magnitude of this effect, consider

Table 2
Mean (± SEM) Number of c-Fos-Positive Nuclei in 29 Brain Regions of Saline (Sal)-Paired and Methamphetamine (Meth)-Paired Mice

Brain region	Sal-paired	Meth-paired	*t* test	2-way ANOVA: Batch	2-way ANOVA: Group
Cortex					
PFC	67 ± 11.7	138 ± 17.9	$t(17) = 3.3,\ p = .004$	$F(4, 13) = 3.0,\ p = .058$	$F(1, 13) = 14.7,\ p = .002$
OFC	24 ± 7.3	68 ± 13.6	$t(17) = 2.9,\ p = .010$	$F(4, 13) = 3.0,\ p = .061$	$F(1, 13) = 10.7,\ p = .006$
Cg	112 ± 19.5	199 ± 28.8	$t(18) = 2.5,\ p = .023$	$F(4, 14) = 2.1,\ p = .136$	$F(1, 14) = 7.8,\ p = .015$
PIR	37 ± 5.3	71 ± 8.1	$t(18) = 3.5,\ p = .003$	$F(4, 14) = 1.4,\ p = .299$	$F(1, 14) = 13.1,\ p = .003$
SX	31 ± 6.7	64 ± 9.0	$t(16) = 3.0,\ p = .009$	$F(4, 12) = 2.0,\ p = .160$	$F(1, 12) = 10.9,\ p = .006$
ENT	66 ± 9.5	129 ± 14.7	$t(16) = 3.6,\ p = .003$	$F(4, 12) = 1.9,\ p = .181$	$F(1, 12) = 15.6,\ p = .002$
VX	366 ± 50.3	311 ± 59.6	$t(15) = 0.71,\ p = .487$	$F(4, 11) = 4.8,\ p = .017$	$F(1, 11) = 1.3,\ p = .280$
Basal ganglia and septum					
NACS	24 ± 5.0	63 ± 9.7	$t(18) = 3.6,\ p = .002$	$F(4, 14) = 5.1,\ p = .010$	$F(1, 14) = 24.9,\ p < .001$
NACC	10 ± 2.9	18 ± 5.9	$t(18) = 1.3,\ p = .220$	$F(4, 14) = 2.3,\ p = .113$	$F(1, 14) = 2.1,\ p = .172$
BNST	5 ± 0.1	19 ± 5.1	$t(18) = 2.6,\ p = .019$	$F(4, 14) = 1.4,\ p = .296$	$F(1, 14) = 7.1,\ p = .018$
CPU1	56 ± 10.8	89 ± 29.6	$t(17) = 1.1,\ p = .285$	$F(4, 13) = 1.1,\ p = .386$	$F(1, 13) = 1.3,\ p = .283$
CPU2	51 ± 9.5	83 ± 37.8	$t(17) = 0.87,\ p = .399$	$F(4, 13) = 1.2,\ p = .361$	$F(1, 13) = 0.70,\ p = .419$
LSD	62 ± 10.2	77 ± 13.6	$t(17) = 0.91,\ p = .377$	$F(4, 13) = 4.0,\ p = .025$	$F(1, 13) = 1.3,\ p = .277$
LSI	107 ± 16.0	134 ± 22.0	$t(17) = 1.0,\ p = .315$	$F(4, 13) = 9.6,\ p < .001$	$F(1, 13) = 3.0,\ p = .105$
LSV	88 ± 11.6	138 ± 25.0	$t(17) = 1.9,\ p = .077$	$F(4, 13) = 2.8,\ p = .071$	$F(1, 13) = 4.7,\ p = .050$
VP	42 ± 5.9	63 ± 12.3	$t(17) = 1.6,\ p = .121$	$F(4, 13) = 2.1,\ p = .140$	$F(1, 13) = 3.2,\ p = .099$
Hippocampus and thalamus					
DG	10 ± 1.7	13 ± 2.3	$t(18) = 1.0,\ p = .313$	$F(4, 14) = 2.9,\ p = .061$	$F(1, 14) = 1.5,\ p = .236$
PV	60 ± 4.2	93 ± 7.1	$t(18) = 4.0,\ p < .001$	$F(4, 14) = 10.9,\ p < .001$	$F(1, 14) = 51.9,\ p < .001$
LHB	37 ± 5.7	28 ± 4.4	$t(18) = 1.2,\ p = .229$	$F(4, 14) = 1.9,\ p = .172$	$F(1, 14) = 1.9,\ p = .195$
Amygdala					
BL	22 ± 4.4	53 ± 7.3	$t(17) = 3.7,\ p = .002$	$F(4, 13) = 7.9,\ p = .002$	$F(1, 13) = 32.2,\ p < .001$
BM	59 ± 10.4	133 ± 14.0	$t(17) = 4.3,\ p < .001$	$F(4, 13) = 2.8,\ p = .070$	$F(1, 13) = 26.7,\ p < .001$
Ce	0	0	—	—	—
Hypothalamus					
AH	115 ± 14.6	96 ± 13.3	$t(18) = 0.95,\ p = .357$	$F(4, 14) = 6.5,\ p = .004$	$F(1, 14) = 2.0,\ p = .180$
LH	78 ± 11.8	60 ± 13.7	$t(17) = 1.0,\ p = .324$	$F(4, 13) = 6.9,\ p = .003$	$F(1, 13) = 1.9,\ p = .196$
MPA	15 ± 4.3	20 ± 7.2	$t(17) = 0.54,\ p = .599$	$F(4, 13) = 6.6,\ p = .004$	$F(1, 13) = 0.42,\ p = .526$
PVN	0	0	—	—	—
Midbrain					
EW	6 ± 0.87	4 ± 0.65	$t(17) = 1.37,\ p = .186$	$F(4, 13) = 2.0,\ p = .159$	$F(1, 13) = 1.8,\ p = .198$
PAG	58 ± 5.8	60 ± 7.0	$t(17) = 0.14,\ p = .121$	$F(4, 13) = 5.18,\ p = .010$	$F(1, 13) = 0.07,\ p = .795$
SC	73 ± 12.6	72 ± 13.9	$t(16) = 0.10,\ p = .930$	$F(4, 12) = 2.8,\ p = .077$	$F(1, 12) = 0.01,\ p = .917$

Note. Groups were compared by means of simple *t* tests and by two-way analyses of variance (ANOVAs) with batch of immunohistochemical assay entered as a blocking factor. Regions in bold showed significantly elevated ($p < .05$) numbers of c-Fos-positive nuclei in meth-paired versus saline-paired mice. Sample sizes were 10 per group, but several brain regions had 1 or more missing values because of lost or damaged sections (see degrees of freedom for the *t* tests, which ranged from 15 to 18).

that the average (± *SE*) numbers of c-Fos-positive nuclei in LH per batch ($n = 4$ per batch; 2 meth-paired and 2 saline-paired mice) were 46 ± 22.2, 37 ± 5.3, 47 ± 8.9, 90 ± 21.4, and 117 ± 4.7 for Batches 1–5, respectively. For LSI, the respective averages were 110 ± 10.7, 73 ± 18.3, 81 ± 11.6, 128 ± 20.1, and 204 ± 24.2, and for MPA they were 9 ± 3.6, 8.5 ± 2.4, 7 ± 0.58, 17 ± 3.0, and 43 ± 11.5. For all brain regions except posterior caudate-putamen, EW, and PAG, the *p* value for the effect of group was smaller in the two-way ANOVA, after adjusting for the effect of batch, than in the *t* test, in which batch effects were ignored (see Table 2). Interactions between batch and treatment group were never significant.

Discussion

It has been established that classical conditioning plays a role in addiction because drug-associated cues trigger craving and relapse in humans, but the underlying mechanisms responsible for this phenomenon are not known (Brody et al., 2002; Childress et al., 1999; Daglish et al., 2001; Garavan et al., 2000; Volkow et al., 1999). Here we present a mouse model of a classically conditioned physiological response to meth that has the potential to shed light on underlying mechanisms in future genetic studies, pending demonstration that the responses are heritable. The main finding was that specific (not all) brain regions become activated, as indexed by levels of c-Fos protein, when mice are placed into an environment where they had previously experienced meth. Whether any of the responses reflect an altered motivational state in the mice relevant to craving or motivation is not known and will require future investigation. However, many of the brain regions that were activated in this model were also activated in rat and human imaging studies, and in these other studies, the brain responses were correlated with behaviors indicative of motivation (e.g., lever

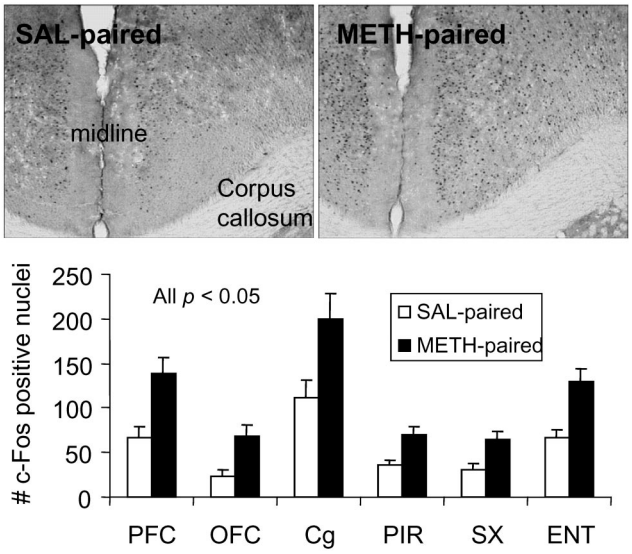

Figure 3. Expression of c-Fos protein increased in many cortical regions when mice were placed into an environment where they had previously experienced methamphetamine (METH). The top panel is a representative example of the cingulate cortex of a meth-paired versus a saline (SAL)-paired mouse, showing increased numbers of c-Fos-positive nuclei in the meth-paired subject. The bottom panel shows mean (± *SEM*) number of c-Fos-positive nuclei in cortical regions of meth-paired and saline-paired mice. PFC = prefrontal cortex; OFC = orbitofrontal cortex; Cg = cingulate cortex; PIR = piriform cortex; SX = somatosensory cortex; ENT = entorhinal cortex.

pressing for intravenous cocaine in rats; Neisewander et al., 2000) and self-reports of craving in humans (Childress et al., 1999). In this study, no changes in locomotor activity were observed, but that does not exclude the possibility that the mice were in an altered motivational state.

Three of the cortical regions that were strongly activated in the meth-paired mice (PFC, OFC, and Cg) have previously been implicated in craving for cocaine (Childress et al., 1999), nicotine (Brody et al., 2002), and heroin (Daglish et al., 2001) in human imaging studies. These same regions have also been implicated in the incentive motivational effects of cocaine (Brown et al., 1992; Ciccocioppo et al., 2001; Franklin & Druhan, 2000; Le Foll et al., 2002; Neisewander et al., 2000), nicotine (Schroeder et al., 2001), amphetamine (Mead et al., 1999), morphine (Schroeder et al., 2000), alcohol (Topple et al., 1998), food (Schroeder et al., 2001), and wheel running (Rhodes et al., 2003) in mice and rats. However, a variety of stimuli, not only cues paired with drugs or natural rewards, appear to activate these regions in humans and rats. For example, the PFC, OFC, and Cg are also activated when human (Fischer, Andersson, Furmark, & Fredrikson, 2000) or rat (Beck & Fibiger, 1995) subjects are exposed to cues paired with a shock (i.e., in a model of fear conditioning). Moreover, Cg is one of the regions most commonly reported to display increased levels of activation in human and rat imaging studies, regardless of the experimental question. Taken together, this suggests that activation of the PFC, OFC, and Cg could play a general role in attention, arousal, anxiety, or learning, rather than a specific role in craving or incentive motivation in our model. Thus, future work will be needed to sort out the functional significance of activation of these regions in the context of this model.

Other cortical regions that are not traditionally associated with motivation were also strongly activated in the meth-paired mice, including SX, Pir, and ENT. The PV, which is known to play a role in the functional integration of limbic cortical and striatal circuitry (Pinto, Jankowski, & Sesack, 2003), was also activated. These regions might also reflect a general role in attention, arousal, or anxiety. These same brain regions were also activated in response to cues paired with a shock in the rat model of fear conditioning described above (Beck & Fibiger, 1995). Nonetheless, some degree of specificity can be seen for these responses relative to other drugs of abuse and natural rewards. In SX, levels of c-Fos protein did not change in response to nicotine-paired cues in a study of male Sprague–Dawley rats (Schroeder et al., 2001). Thus, activation of SX may represent a drug-specific effect (meth vs. nicotine) or a species difference (mouse vs. rat). To the best of our knowledge, no other study besides ours has measured c-Fos expression in Pir or ENT in mice or rats in response to drug-paired cues, so it is not known whether the effects of other drugs of abuse would be similar to those of meth in these regions. However, levels of c-Fos protein strongly increased in Pir and SX in mice prevented from their daily exercise routine on running wheels compared with mice allowed to run, suggesting that these regions become strongly activated when animals are expecting a reward or are frustrated about not receiving a reward (Rhodes et al., 2003). Different results were obtained for ENT in this wheel-running model, though. The ENT was one of the few regions that was strongly activated by the running itself and was relatively silent in the mice prevented from running (Rhodes et al., 2003). Thus, some degree of specificity appears to occur in ENT regarding expectation of natural versus drug rewards because it is strongly activated by meth-paired cues but not by expectation to run.

The NACS was another region that was strongly activated in the meth-paired mice. This nucleus is well known for its role in incentive motivation, and the c-Fos response identified here could be a reflection of such a role (Kelley & Berridge, 2002). However, NACS is also known to play a role in stress and anxiety. For

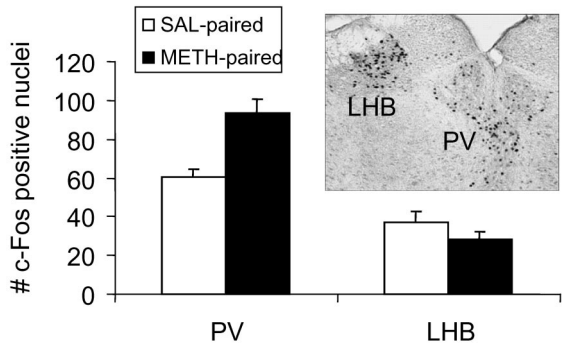

Figure 4. Specific brain regions become activated by a methamphetamine (METH)-paired environment. Mean (± *SEM*) number of c-Fos-positive nuclei in the paraventricular thalamic nucleus (PV) and lateral habenular nucleus (LHB) of meth-paired and saline (SAL)-paired mice. In PV, a strong difference was observed between groups ($p < .001$), whereas in LHB, which is immediately adjacent to PV, no difference was found, and the trend was in the opposite direction.

Table 3
Correlation Between Locomotor Activity and Number of c-Fos-Positive Nuclei and Results of ANCOVA Examining the Effect of Group After Adjusting for the Variation Explained by Locomotor Activity

	Correlation			Analysis of covariance	
Brain region	R^2	n	p	Group	Interaction
Cortex					
PFC	.35	19	.008	$F(1, 15) = 8.20$, $p = .012$	$F(1, 15) = 0.08$, $p = .778$
OFC	.25	19	.031	$F(1, 15) = 5.80$, $p = .030$	$F(1, 15) = 0.60$, $p = .452$
Cg	.21	20	.041	$F(1, 16) = 4.30$, $p = .055$	$F(1, 16) = 0.77$, $p = .392$
PIR	.47	20	< .001	$F(1, 16) = 11.40$, $p = .004$	$F(1, 16) = 0.01$, $p = .927$
SX	.52	18	< .001	$F(1, 14) = 9.70$, $p = .008$	$F(1, 14) = 0.90$, $p = .360$
ENT	.51	18	< .001	$F(1, 14) = 16.00$, $p = .001$	$F(1, 14) = 0.04$, $p = .844$
VX	.46	17	.003	$F(1, 13) = 3.80$, $p = .074$	$F(1, 13) = 0.00$, $p = 1.00$
Basal ganglia and septum					
NACS	.43	20	.002	$F(1, 16) = 12.00$, $p = .003$	$F(1, 16) = 0.15$, $p = .707$
NACC	.61	20	< .001	$F(1, 16) = 0.30$, $p = .589$	$F(1, 16) = 0.53$, $p = .478$
BNST	.14	20	.106	$F(1, 16) = 4.50$, $p = .049$	$F(1, 16) = 0.02$, $p = .888$
CPU1	.33	19	.011	$F(1, 15) = 0.22$, $p = .645$	$F(1, 15) = 1.70$, $p = .217$
CPU2	.43	19	.002	$F(1, 15) = 0.00$, $p = .948$	$F(1, 15) = 3.80$, $p = .069$
LSD	.52	19	< .001	$F(1, 15) = 0.00$, $p = .996$	$F(1, 15) = 0.03$, $p = .860$
LSI	.38	19	.005	$F(1, 15) = 0.10$, $p = .756$	$F(1, 15) = 0.01$, $p = .917$
LSV	.27	19	.022	$F(1, 15) = 1.70$, $p = .210$	$F(1, 15) = 0.00$, $p = .976$
VP	.23	19	.036	$F(1, 15) = 1.20$, $p = .289$	$F(1, 15) = 0.45$, $p = .511$
Hippocampus and thalamus					
DG	.65	20	< .001	$F(1, 16) = 0.03$, $p = .859$	$F(1, 16) = 0.33$, $p = .571$
PV	.42	20	.002	$F(1, 16) = 15.70$, $p = .001$	$F(1, 16) = 0.04$, $p = .835$
LHB	.19	20	.057	$F(1, 16) = 4.50$, $p = .050$	$F(1, 16) = 1.20$, $p = .294$
Amygdala					
BL	.24	19	.033	$F(1, 15) = 10.80$, $p = .005$	$F(1, 15) = 1.30$, $p = .275$
BM	.23	19	.038	$F(1, 15) = 15.30$, $p = .001$	$F(1, 15) = 2.00$, $p = .181$
Hypothalamus					
AH	.24	20	.028	$F(1, 16) = 4.00$, $p = .064$	$F(1, 16) = 3.30$, $p = .089$
LH	.50	19	< .001	$F(1, 15) = 6.70$, $p = .021$	$F(1, 15) = 0.02$, $p = .877$
MPA	.27	19	.022	$F(1, 15) = 0.01$, $p = .907$	$F(1, 15) = 0.45$, $p = .514$
Midbrain					
EW	.01	19	.72	$F(1, 15) = 0.04$, $p = .841$	$F(1, 15) = 0.54$, $p = .472$
PAG	.30	19	.015	$F(1, 15) = 0.38$, $p = .547$	$F(1, 15) = 0.10$, $p = .751$
SC	.55	18	< .001	$F(1, 14) = 1.50$, $p = .245$	$F(1, 14) = 0.05$, $p = .831$

Note. The distance traveled in the first 30 min of the 90-min test on Day 12 was used as the measure of locomotor activity for these analyses. The R^2 statistics and associated p values were obtained from a simple linear regression of the number of c-Fos-positive nuclei against distance traveled, with both methamphetamine (meth)-paired and saline-paired mice combined together. Regions in bold showed a significant ($p < .05$) correlation. The statistics for group refer to a comparison of number of c-Fos-positive nuclei between meth-paired and saline-paired mice after adjustment for the variation explained by locomotor activity. This was done by means of analysis of covariance, with locomotor activity as the covariate and group as the treatment factor. The statistics for interaction refer to a comparison of the slopes of the relationship between locomotor activity and number of c-Fos-positive nuclei between meth-paired and saline-paired mice. Nonsignificant results suggest that the slopes are equal or that the linear relationships are parallel (see Figure 5). ANCOVA = analysis of covariance.

example, when rats were exposed to cues paired with a shock in the rat fear conditioning model described earlier, levels of c-Fos in the NACS increased (Beck & Fibiger, 1995). Moreover, dopamine is released into the NACS in response to certain types of stress (Wu, Yoshida, Emoto, & Tanaka, 1999), and so it is not possible to say with certainty that the changes in c-Fos in NACS reflected any specific role in an altered motivational state in this model without future investigation.

Other regions that were strongly activated in meth-paired mice included the BL and BM. These regions play critical roles in classical conditioning to either aversive or rewarding stimuli (See, Fuchs, Ledford, & McLaughlin, 2003; Walker, Toufexis, & Davis, 2003). They were activated by cues paired with a shock in the rat model of fear conditioning (Beck & Fibiger, 1995) but are also necessary for expression of conditioned responses to drug rewards (See et al., 2003). For example, excitotoxic lesions of the BL have recently been shown to abolish the ability of cocaine-paired cues to reinstate extinguished lever pressing (See et al., 2003). Thus, it is likely that c-Fos levels in BL and BM reflected a generalized activation of circuitry involved in Pavlovian learning in this model.

The strong activation of the BNST in the meth-paired mice together with lack of activation of the PVN and Ce is consistent with animal models of anxiety, but not necessarily stress or fear (Walker et al., 2003). The PVN contains the corticotropin releasing hormone neurons that initiate the hypothalamic–pituitary–adrenal axis stress response. Increased levels of c-Fos in the PVN have been demonstrated in response to numerous different types of stressors in rodents (Campeau & Watson, 1997; Del Bel, Silveira,

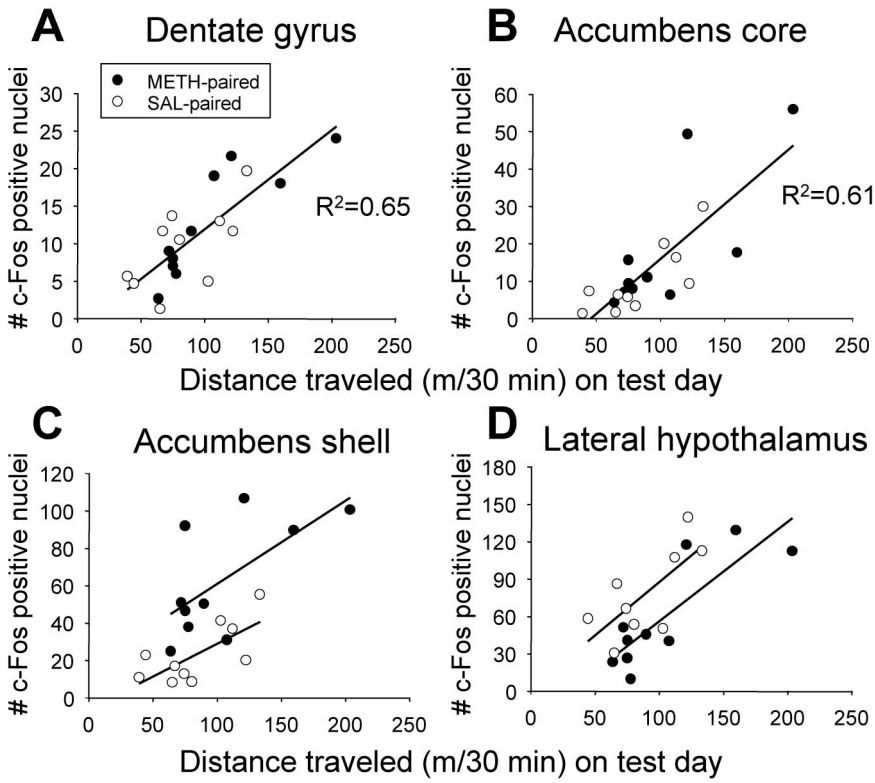

Figure 5. Numbers of c-Fos-positive nuclei were strongly correlated with locomotor activity but do not account for differences in c-Fos between methamphetamine (METH)-paired and saline (SAL)-paired mice. A: Numbers of c-Fos-positive nuclei in the dentate gyrus of individual mice plotted against the distance they traveled in the first 30 min of the 90-min test after receiving a saline injection. The least-square regression line and R^2 are shown. B: Same plot for the nucleus accumbens core. C: Same plot for nucleus accumbens shell, with separate regression lines fit to the meth-paired and saline-paired groups. D: Same plot for lateral hypothalamus, with separate regression lines fit to the meth-paired and saline-paired groups.

Graeff, Garcia-Cairasco, & Guimaraes, 1998; Helmreich, Cullinan, & Watson, 1996; Martinez, Phillips, & Herbert, 1998; Rivest & Rivier, 1994; Steciuk, Kram, Kramer, & Petty, 1999; Tan & Nagata, 2002; Windle et al., 2004). The fact that the PVN was silent here suggests a limited involvement of the hypothalamic–pituitary–adrenal axis in this model. The BNST and Ce are thought to play a role in fear and anxiety in animal models, but with different roles as indicated by lesion studies (as reviewed in Walker et al., 2003). For example, rats normally freeze and show increased contractions of their head, neck, trunk, and leg muscles, in what is termed a *potentiated startle response*, when exposed to cues that have previously been paired with a footshock. Excitotoxic or electrolytic lesions of the Ce completely block this fear-potentiated startle, whereas lesions of the BNST have no effect. This is a robust finding that has been replicated numerous times (Walker et al., 2003). Moreover, in rats, c-Fos levels increased in the Ce in response to shock-paired cues, whereas they did not in the BNST (Beck & Fibiger, 1995). These and many other studies have led Walker et al. (2003) to conclude that the BNST plays a role in anxiety, but not necessarily fear, in rodents. Fear, according to these authors, occurs immediately in response to a threat, versus anxiety, which is a sustained state of apprehension unrelated to an immediate threat. Thus, we believe that this pattern of increased c-Fos in the BNST but not in the PVN and Ce, taken together, may reflect increased anxiety related to the expectation of experiencing meth or frustration about not experiencing meth, but not necessarily fear or stress of an aversive or painful experience, though we recognize the inherent problems of attributing feelings of stress, anxiety, or fear to mice. These data also document an important difference between the classically conditioned c-Fos response to meth versus footshock. The BNST was activated by meth-paired cues but not shock-paired cues, whereas the opposite was true for Ce, which was activated by shock-paired cues but not meth-paired cues (Beck & Fibiger, 1995). Herein lies a hint of specificity, but future work will be needed to explore this in more detail.

Locomotor Activity

The positive correlation between the number of c-Fos-positive nuclei and distance traveled in the activity monitors observed in nearly every brain region demonstrates that the c-Fos responses are sensitive to locomotor behavior. Several of the same brain regions showed a similar correlation when the number of rotations on running wheels was used as the index of locomotor activity (see below; Rhodes et al., 2003). Depending on the region, the correlation might reflect neural activation involved in the control of the

locomotor behavior, sensory responses to the locomotion, general physiological responses to the locomotion (e.g., effects of brain temperature, blood flow), or neural activation in response to the general arousal that accompanies locomotion. The implication is that the effects of locomotor activity should be considered in c-Fos studies, especially if groups differ in physical activity (which is typical in rodent drug studies).

In the present experiment, it was fortuitous that locomotor activity on the test day was similar between the two main groups (meth-paired and saline-paired), but this is not always the case. Indeed, we had predicted a conditioned increase in locomotor activity and sensitization in the meth-paired group, as this was observed for 1 mg/kg meth in male outbred ICR (Kitanaka et al., 2003) and Swiss Webster mice (Itzhak, 1997) and for 2 mg/kg meth in female inbred C57BL/6J mice (Phillips et al., 1994). The disparity is most likely attributed to methodological differences. Procedures for inducing sensitization and locomotor conditioning vary depending on the drug and the laboratory. In most studies, tests are conducted during the day, which is the normal inactive period of nocturnal mice or rats. In this study, mice were tested 1 hr into the dark phase, when nocturnal mice are normally active (Rhodes et al., 2003). We believe that this could make the difference, as circadian period is known to affect drug-related physiology and behavior (Rhodes, Best, Belknap, Finn, & Crabbe, 2005), and circadian genes have been implicated in drug responses (Uz, Akhisaroglu, Ahmed, & Manev, 2003). In Phillips et al. (1994), drug injections were given every other day, but this does not seem to be necessary to demonstrate sensitization because in Kitanaka et al. (2003), daily injections elicited sensitization. However, the test for conditioned locomotion in Kitanaka et al. (2003) occurred after a 3-day abstinence period. In Itzhak (1997), daily injections were given, but the mice were intermittently exposed to the activity monitors, and the test for conditioned locomotion occurred after a 2-day abstinence period.

In some regions where the positive correlation between c-Fos and locomotor activity was extremely strong, such as DG, ENT, NACC, and SC, the c-Fos responses may have reflected neural activation directly related to the control of the locomotor activity. In another report of wheel running in outbred mice (derived from CD1), c-Fos levels in DG, ENT, and SC were strongly correlated with the distance run (Rhodes et al., 2003). In that study, the subjects were moving much faster and covering distances between 100 and 600 m per 30 min, whereas in this study the mice were moving between 50 and 200 m per 30 min. Thus, in these regions the correlation is robust, occurring for both low and high amounts of physical activity both in cages and on wheels. Arguments against a role for the hippocampal formation (including ENT and DG) in motor function are based on evidence that its destruction does not prevent locomotion (as reviewed in Oddie & Bland, 1998). However, destruction of the hippocampus is known to change quantitative aspects of movement execution, such as the intensity at which a motor act can be carried out (Oddie & Bland, 1998). For example, although hippocampal lesions do not prevent rats from jumping (Myhrer, 1975), they reduce the height to which rats are capable of jumping (Oddie & Bland, 1998). Moreover, the frequency of theta waves in the hippocampus increases immediately ($\sim$ 100 ms) preceding a jump, and the length of the period of the theta wave coinciding with take-off is strongly and inversely correlated with the height, velocity, and peak force parameters of the jump (Morris & Hagan, 1983).

An interesting result emerged for LH in the ANCOVA with locomotor activity as the covariate. In this region, without considering effects of locomotor activity, we found that meth-paired mice had slightly lower levels of c-Fos than saline-paired mice, but this effect was not statistically significant (see Table 2). However, approximately 50% of the variation in c-Fos among individual subjects could be explained by differences in locomotor activity in LH (see Figure 5D and Table 3), and after adjusting for differences explained by locomotor activity, we saw significantly fewer c-Fos-positive nuclei in meth-paired mice than in saline-paired mice. Thus, the negative result became a positive result when effects of locomotor activity were considered in the analysis. This is important because LH is the only region where lower levels of c-Fos were found in the meth-paired mice compared with saline-paired mice. In all other regions showing positive results, levels of c-Fos were higher in meth-paired mice than in saline-paired mice. The result is also important because it provides another hint of specificity. Levels of c-Fos strongly increased in LH in the mice prevented from their daily exercise routine (Rhodes et al., 2003), and they also increased in rats exposed to a cue paired with a shock (Beck & Fibiger, 1995) and in rats exposed to an environment paired with access to beer (Topple et al., 1998), whereas here, c-Fos levels were reduced in mice exposed to an environment paired with meth.

The main result shown in Table 2, giving the list of brain regions that were activated by contextual conditioning to meth, is robust. The same list of brain regions emerged as significant in the analysis that corrected for effects of locomotor activity. All the regions that showed positive results for group in Table 2 (without locomotor activity as a covariate) also showed positive results for group in Table 3 (with locomotor activity as a covariate). This result was expected given that the meth-paired and saline-paired mice did not differ in locomotor activity on the test day. The implication is that the increased c-Fos in the meth-paired mice occurs over and above any correlation with locomotor activity in these brain regions (see Figure 5C, where data for NACS are shown as an example).

Methodological Considerations

In this experiment, we administered meth to all mice in order to keep that variable constant and thus compare differences explicitly attributable to the environment paired with the drug. Although all mice received meth, a 2-hr delay differentiated the two groups, so that half the mice received meth in their home cage (saline-paired) 90 min after a 30-min exposure to an activity monitor, and half received meth immediately before the 30-min activity monitor exposure (meth-paired). This design was borrowed from Brown et al. (1992), who studied cocaine conditioning in rats. It is possible that on the test day, when the drug-free, saline-paired mice were placed in the activity monitor, they were in a state of anticipation or expectation of receiving meth later in the day. However, it seems likely that whatever expectation occurred in saline-paired mice, it would have been weaker than what the meth-paired mice were experiencing because meth was administered in closer temporal association with the activity monitor in meth-paired versus saline-paired mice. This view is supported empirically by the

observed differences in c-Fos between the two groups in many brain regions and by the relatively low levels of c-Fos staining in saline-paired mice. An alternative experimental design has been used in rats to control explicitly for delayed expectation, but this design consists of four groups (Schroeder et al., 2001). In this design, each rat receives only one injection per day during the training phase (drug or saline). Group 1 receives drug before the activity monitor each day. Group 2 receives saline before the activity monitor each day. Group 3 receives saline in the home cage each day, and Group 4 receives drug in the home cage each day. The primary comparison is between Groups 1 and 2. Groups 3 and 4 are used only to determine whether any of the differences between Groups 1 and 2 might be due to chronic drug rather than drug-paired context. However, it is not clear how responses in 1 and 2 should be corrected if differences exist between 3 and 4. Moreover, it is important that sample sizes are equivalent in all four groups so that power is the same for all of the tests. We opted for our design because it requires half the number of animals. Our long-term goal is to use this procedure to compare responses among different drugs and natural rewards and in gene mapping studies, and the simpler the design, the more amenable it is to high throughput.

Immunohistochemical Batch Effects

Despite taking measures to apply the immunohistochemistry consistently between batches (see *Method*), we found strong differences in levels of staining between batches for many brain regions. This did not affect the main results, because each batch included an equal number of meth-paired and saline-paired subjects, and the effect sizes for the group difference (meth-paired vs. saline-paired) were large enough to overcome the batch effects. However, it should be noted that p values were reduced in nearly every brain region when results were adjusted for batch effects (see Table 2). The implication is that batch effects should be considered in c-Fos studies to reduce Type II error. Moreover, results demonstrate that adjustments will have to be made for differences in the immunohistochemical assay before c-Fos responses can be compared between experiments. We plan to compare c-Fos responses in this experiment to those of future experiments to identify the specificity of the model (see below).

Conclusions and Future Directions

Here we demonstrate a classically conditioned physiological response to meth. Expression of c-Fos protein increased in specific brain regions when mice were placed into an environment where they had previously experienced meth. We showed that this effect was not explained by variation in locomotor activity. The functional explanation for the increased c-Fos is unclear at this time, and probably differs for the different brain regions. In some regions, the increase might reflect a general heightened state of arousal. In others, it might represent a role in Pavlovian learning, anxiety, expectation, or incentive motivation for meth. One strategy that may be useful in sorting between these possibilities is to compare the responses between experiments that use an aversive stimulus (e.g., a shock or a lithium chloride injection) or a natural reward (e.g., food or wheel running) instead of meth for the conditioning. This will require careful control over variation induced by the immunohistochemical assays. If successful, the strategy might identify specific conditioned physiological responses that occur for meth but do not occur for natural rewards or aversive stimuli. Researchers could then use inbred strains and quantitative trait loci analysis to target these responses for genetic exploration and identification of the genes that underlie variation in these brain responses.

It is widely believed that the neurobiological mechanisms involved in motivation for drugs also play a role in natural forms of learning and motivation, and a wide degree of overlap is expected and indeed has been documented (Kelley & Berridge, 2002). But if drugs have a high potential to elicit a compulsive, pathological form of motivation (i.e., craving) in response to drug-paired cues in humans, then there must be some distinguishing physiological response (e.g., difference in intensity and/or signal) that specifically makes them strong triggers of the pathology. To date, few studies have identified this specificity. It remains to be determined whether the c-Fos responses are sensitive enough to be used in this capacity. This will be the topic of future investigation.

References

Adams, J. U., Careri, J. M., Efferen, T. R., & Rotrosen, J. (2000). Conditioned locomotor stimulant effects of cocaine in rats do not result from interference with habituation. *Psychopharmacology, 151,* 13–18.

Anokhin, K. V., & Rose, S. P. (1991). Learning-induced increase of immediate early gene messenger RNA in the chick forebrain. *European Journal of Neuroscience, 3,* 162–167.

Bachtell, R. K., Weitemier, A. Z., Galvan-Rosas, A., Tsivkovskaia, N. O., Risinger, F. O., Phillips, T. J., et al. (2003). The Edinger-Westphal-lateral septum urocortin pathway and its relationship to alcohol consumption. *Journal of Neuroscience, 23,* 2477–2487.

Beck, C. H., & Fibiger, H. C. (1995). Conditioned fear-induced changes in behavior and in the expression of the immediate early gene c-fos: With and without diazepam pretreatment. *Journal of Neuroscience, 15,* 709–720.

Brody, A. L., Mandelkern, M. A., Lee, G., Smith, E., Sadeghi, M., Saxena, S., et al. (2004). Attenuation of cue-induced cigarette craving and anterior cingulate cortex activation in bupropion-treated smokers: A preliminary study. *Psychiatry Research, 130,* 269–281.

Brody, A. L., Mandelkern, M. A., London, E. D., Childress, A. R., Lee, G. S., Bota, R. G., et al. (2002). Brain metabolic changes during cigarette craving. *Archives of General Psychiatry, 59,* 1162–1172.

Brown, E. E., Robertson, G. S., & Fibiger, H. C. (1992). Evidence for conditional neuronal activation following exposure to a cocaine-paired environment: Role of forebrain limbic structures. *Journal of Neuroscience, 12,* 4112–4121.

Campeau, S., & Watson, S. J. (1997). Neuroendocrine and behavioral responses and brain pattern of c-fos induction associated with audiogenic stress. *Journal of Neuroendocrinology, 9,* 577–588.

Childress, A. R., Mozley, P. D., McElgin, W., Fitzgerald, J., Reivich, M., & O'Brien, C. P. (1999). Limbic activation during cue-induced cocaine craving. *American Journal of Psychiatry, 156,* 11–18.

Ciccocioppo, R., Sanna, P. P., & Weiss, F. (2001). Cocaine-predictive stimulus induces drug-seeking behavior and neural activation in limbic brain regions after multiple months of abstinence: Reversal by D(1) antagonists. *Proceedings of the National Academy of Sciences, USA, 98,* 1976–1981.

Crabbe, J. C. (2002). Genetic contributions to addiction. *Annual Review of Psychology, 53,* 435–462.

Crabbe, J. C., Kosobud, A., Young, E. R., Tam, B. R., & McSwigan, J. D. (1985). Bidirectional selection for susceptibility to ethanol withdrawal seizures in *Mus musculus. Behavior Genetics, 15,* 521–536.

Dabney, A., & Storey, J. (2002). Q-value [Computer software]. Retrieved from http://faculty.washington.edu/~jstorey/qvalue/

Daglish, M. R., Weinstein, A., Malizia, A. L., Wilson, S., Melichar, J. K., Britten, S., et al. (2001). Changes in regional cerebral blood flow elicited by craving memories in abstinent opiate-dependent subjects. *American Journal of Psychiatry, 158,* 1680–1686.

Daglish, M. R., Weinstein, A., Malizia, A. L., Wilson, S., Melichar, J. K., Lingford-Hughes, A., et al. (2003). Functional connectivity analysis of the neural circuits of opiate craving: "More" rather than "different"? *NeuroImage, 20,* 1964–1970.

Del Bel, E. A., Silveira, M. C., Graeff, F. G., Garcia-Cairasco, N., & Guimaraes, F. S. (1998). Differential expression of c-fos mRNA and Fos protein in the rat brain after restraint stress or pentylenetetrazol-induced seizures. *Cellular and Molecular Neurobiology, 18,* 339–346.

Fischer, H., Andersson, J. L., Furmark, T., & Fredrikson, M. (2000). Fear conditioning and brain activity: A positron emission tomography study in humans. *Behavioral Neuroscience, 114,* 671–680.

Franklin, T. R., & Druhan, J. P. (2000). Expression of Fos-related antigens in the nucleus accumbens and associated regions following exposure to a cocaine-paired environment. *European Journal of Neuroscience, 12,* 2097–2106.

Garavan, H., Pankiewicz, J., Bloom, A., Cho, J. K., Sperry, L., Ross, T. J., et al. (2000). Cue-induced cocaine craving: Neuroanatomical specificity for drug users and drug stimuli. *American Journal of Psychiatry, 157,* 1789–1798.

Grusser, S. M., Wrase, J., Klein, S., Hermann, D., Smolka, M. N., Ruf, M., et al. (2004). Cue-induced activation of the striatum and medial prefrontal cortex is associated with subsequent relapse in abstinent alcoholics. *Psychopharmacology, 175,* 296–302.

Hartz, D. T., Frederick-Osborne, S. L., & Galloway, G. P. (2001). Craving predicts use during treatment for methamphetamine dependence: A prospective, repeated-measures, within-subject analysis. *Drug and Alcohol Dependence, 63,* 269–276.

Heinz, A., Siessmeier, T., Wrase, J., Hermann, D., Klein, S., Grusser-Sinopoli, S. M., et al. (2004). Correlation between dopamine D(2) receptors in the ventral striatum and central processing of alcohol cues and craving. *American Journal of Psychiatry, 161,* 1783–1789.

Helmreich, D. L., Cullinan, W. E., & Watson, S. J. (1996). The effect of adrenalectomy on stress-induced c-fos mRNA expression in the rat brain. *Brain Research, 706,* 137–144.

Herdegen, T., & Leah, J. D. (1998). Inducible and constitutive transcription factors in the mammalian nervous system: Control of gene expression by *Jun, Fos* and *Krox,* and CREB/ATF proteins. *Brain Research. Brain Research Reviews, 28,* 370–490.

Itzhak, Y. (1997). Modulation of cocaine- and methamphetamine-induced behavioral sensitization by inhibition of brain nitric oxide synthase. *Journal of Pharmacology and Experimental Therapeutics, 282,* 521–527.

Kaczmarek, L. (1993). Molecular biology of vertebrate learning: Is c-fos a new beginning? *Journal of Neuroscience Research, 34,* 377–381.

Kaczmarek, L., & Nikolajew, E. (1990). c-fos protooncogene expression and neuronal plasticity. *Acta Neurobiologiae Experimentalis (Warsaw), 50,* 173–179.

Kamens, H., Burkhart-Kasch, S., McMinnon, C., Reed, C., & Phillips, T. (2005). Sensitivity to psychostimulants in mice bred for high and low stimulation to methamphetamine. *Genes, Brain and Behavior, 4,* 110–125.

Kelley, A. E., & Berridge, K. C. (2002). The neuroscience of natural rewards: Relevance to addictive drugs. *Journal of Neuroscience, 22,* 3306–3311.

Kilts, C. D., Gross, R. E., Ely, T. D., & Drexler, K. P. (2004). The neural correlates of cue-induced craving in cocaine-dependent women. *American Journal of Psychiatry, 161,* 233–241.

Kitanaka, N., Kitanaka, J., & Takemura, M. (2003). Behavioral sensitization and alteration in monoamine metabolism in mice after single versus repeated methamphetamine administration. *European Journal of Pharmacology, 474,* 63–70.

Le Foll, B., Frances, H., Diaz, J., Schwartz, J. C., & Sokoloff, P. (2002). Role of the dopamine D3 receptor in reactivity to cocaine-associated cues in mice. *European Journal of Neuroscience, 15,* 2016–2026.

Littleton, J. (2000). Can craving be modeled in animals? The relapse prevention perspective. *Addiction, 95*(Suppl. 2), S83–S90.

Maleeva, N. E., Ivolgina, G. L., Anokhin, K. V., & Limborskaia, S. A. (1989). Analysis of the expression of the c-fos proto-oncogene in the rat cerebral cortex during learning. *Genetika, 25,* 1119–1121.

Martinez, M., Phillips, P. J., & Herbert, J. (1998). Adaptation in patterns of c-fos expression in the brain associated with exposure to either single or repeated social stress in male rats. *European Journal of Neuroscience, 10,* 20–33.

Mead, A. N., Vasilaki, A., Spyraki, C., Duka, T., & Stephens, D. N. (1999). AMPA-receptor involvement in c-fos expression in the medial prefrontal cortex and amygdala dissociates neural substrates of conditioned activity and conditioned reward. *European Journal of Neuroscience, 11,* 4089–4098.

Meyer, P. J., & Phillips, T. J. (2003). Bivalent effects of MK-801 on ethanol-induced sensitization do not parallel its effects on ethanol-induced tolerance. *Behavioral Neuroscience, 117,* 641–649.

Morland, J., Jones, B. L., Palomares, M. L., & Alkana, R. L. (1994). Morphine-6-glucuronide: A potent stimulator of locomotor activity in mice. *Life Sciences, 55,* PL163–PL168.

Morris, R., & Hagan, J. J. (1983). Hippocampal electrical activity and ballistic movement. In W. Seifert (Ed.), *Neurobiology of the hippocampus* (pp. 321–331). London: Academic Press.

Myhrer, T. (1975). Normal jump avoidance performance in rats with the hippocampal theta rhythm selectively disrupted. *Behavioural Biology, 14,* 489–498.

Myrick, H., Anton, R. F., Li, X., Henderson, S., Drobes, D., Voronin, K., et al. (2004). Differential brain activity in alcoholics and social drinkers to alcohol cues: Relationship to craving. *Neuropsychopharmacology, 29,* 393–402.

Neisewander, J. L., Baker, D. A., Fuchs, R. A., Tran-Nguyen, L. T., Palmer, A., & Marshall, J. F. (2000). Fos protein expression and cocaine-seeking behavior in rats after exposure to a cocaine self-administration environment. *Journal of Neuroscience, 20,* 798–805.

Nestler, E. J., Barrot, M., & Self, D. W. (2001). Delta FosB: A sustained molecular switch for addiction. *Proceedings of the National Academy of Sciences, USA, 98,* 11042–11046.

Oddie, S. D., & Bland, B. H. (1998). Hippocampal formation theta activity and movement selection. *Neuroscience & Biobehavioral Reviews, 22,* 221–231.

Panlilio, L. V., & Schindler, C. W. (1997). Conditioned locomotor-activating and reinforcing effects of discrete stimuli paired with intraperitoneal cocaine. *Behavioural Pharmacology, 8,* 691–698.

Paxinos, G., & Franklin, K. (2001). *The mouse brain atlas in stereotaxic coordinates* (2nd ed.). San Diego, CA: Academic Press.

Phillips, T. J. (1999). [Mice injected with methamphetamine and placed into activity monitors]. Unpublished raw data.

Phillips, T. J., Dickinson, S., & Burkhart-Kasch, S. (1994). Behavioral sensitization to drug stimulant effects in C57BL/6J and DBA/2J inbred mice. *Behavioral Neuroscience, 108,* 789–803.

Phillips, T. J., Huson, M., Gwiazdon, C., Burkhart-Kasch, S., & Shen, E. H. (1995). Effects of acute and repeated ethanol exposures on the locomotor activity of BXD recombinant inbred mice. *Alcoholism, Clinical and Experimental Research, 19,* 269–278.

Pinto, A., Jankowski, M., & Sesack, S. R. (2003). Projections from the paraventricular nucleus of the thalamus to the rat prefrontal cortex and nucleus accumbens shell: Ultrastructural characteristics and spatial re-

lationships with dopamine afferents. *Journal of Comparative Neurology, 459,* 142–155.

Rasband, W. (2005). Image J (Version 1.34) [Computer software]. Retrieved from http://rsb.info.nih.gov/ij/download.html

Rhodes, J., Best, K., Belknap, J., Finn, D., & Crabbe, J. (2005). Evaluation of a simple model of ethanol drinking to intoxication in C57BL/6J mice. *Physiology & Behavior, 84,* 53–63.

Rhodes, J. S., & Crabbe, J. C. (2003). Progress towards finding genes for alcoholism in mice. *Clinical Neuroscience Research, 3,* 315–323.

Rhodes, J. S., Garland, T., Jr., & Gammie, S. C. (2003). Patterns of brain activity associated with variation in voluntary wheel-running behavior. *Behavioral Neuroscience, 117,* 1243–1256.

Rivest, S., & Rivier, C. (1994). Stress and interleukin-1 beta-induced activation of c-fos, NGFI-B and CRF gene expression in the hypothalamic PVN: Comparison between Sprague-Dawley, Fisher-344 and Lewis rats. *Journal of Neuroendocrinology, 6,* 101–117.

Ryabinin, A. E., Wang, Y. M., Freeman, P., & Risinger, F. O. (1999). Selective effects of alcohol drinking on restraint-induced expression of immediate early genes in mouse brain. *Alcoholism, Clinical and Experimental Research, 23,* 1272–1280.

Schroeder, B. E., Binzak, J. M., & Kelley, A. E. (2001). A common profile of prefrontal cortical activation following exposure to nicotine- or chocolate-associated contextual cues. *Neuroscience, 105,* 535–545.

Schroeder, B. E., Holahan, M. R., Landry, C. F., & Kelley, A. E. (2000). Morphine-associated environmental cues elicit conditioned gene expression. *Synapse, 37,* 146–158.

See, R. E., Fuchs, R. A., Ledford, C. C., & McLaughlin, J. (2003). Drug addiction, relapse, and the amygdala. In P. Shinnick-Gallagher, A. Pitkänen, A. Shekhar, & L. Cahill (Eds.), *Annals of the New York Academy of Sciences: Vol. 985. The amygdala in brain function* (pp. 294–307). New York: New York Academy of Sciences.

Steciuk, M., Kram, M., Kramer, G. L., & Petty, F. (1999). Decrease in stress-induced c-Fos-like immunoreactivity in the lateral septal nucleus of learned helpless rats. *Brain Research, 822,* 256–259.

Storey, J. (2002). A direct approach to false discovery rates. *Journal of the Royal Statistical Society, 64,* 479–498.

Swank, M. W., Ellis, A. E., & Cochran, B. N. (1996). c-Fos antisense blocks acquisition and extinction of conditioned taste aversion in mice. *NeuroReport, 7,* 1866–1870.

Tan, Z., & Nagata, S. (2002). PVN c-fos expression, HPA axis response and immune cell distribution during restraint stress. *Journal of UOEH, 24,* 131–149.

Tapert, S. F., Brown, G. G., Baratta, M. V., & Brown, S. A. (2004). fMRI BOLD response to alcohol stimuli in alcohol dependent young women. *Addictive Behaviors, 29,* 33–50.

Tapert, S. F., Cheung, E. H., Brown, G. G., Frank, L. R., Paulus, M. P., Schweinsburg, A. D., et al. (2003). Neural response to alcohol stimuli in adolescents with alcohol use disorder. *Archives of General Psychiatry, 60,* 727–735.

Tischmeyer, W., Kaczmarek, L., Strauss, M., Jork, R., & Matthies, H. (1990). Accumulation of c-fos mRNA in rat hippocampus during acquisition of a brightness discrimination. *Behavioral and Neural Biology, 54,* 165–171.

Tolliver, B. K., & Carney, J. M. (1995). Locomotor stimulant effects of cocaine and novel cocaine analogs in DBA/2J and C57BL/6J inbred mice. *Pharmacology, Biochemistry and Behavior, 50,* 163–169.

Topple, A. N., Hunt, G. E., & McGregor, I. S. (1998). Possible neural substrates of beer-craving in rats. *Neuroscience Letters, 252,* 99–102.

Uz, T., Akhisaroglu, M., Ahmed, R., & Manev, H. (2003). The pineal gland is critical for circadian period1 expression in the striatum and for circadian cocaine sensitization in mice. *Neuropsychopharmacology, 28,* 2117–2123.

Volkow, N. D., Fowler, J. S., & Wang, G. J. (2004). The addicted human brain viewed in the light of imaging studies: Brain circuits and treatment strategies. *Neuropharmacology, 47*(Suppl. 1), 3–13.

Volkow, N. D., Wang, G. J., Fowler, J. S., Hitzemann, R., Angrist, B., Gatley, S. J., et al. (1999). Association of methylphenidate-induced craving with changes in right striato-orbitofrontal metabolism in cocaine abusers: Implications in addiction. *American Journal of Psychiatry, 156,* 19–26.

Volkow, N. D., Wang, G. J., Ma, Y., Fowler, J. S., Zhu, W., Maynard, L., et al. (2003). Expectation enhances the regional brain metabolic and the reinforcing effects of stimulants in cocaine abusers. *Journal of Neuroscience, 23,* 11461–11468.

Walker, D. L., Toufexis, D. J., & Davis, M. (2003). Role of the bed nucleus of the stria terminalis versus the amygdala in fear, stress, and anxiety. *European Journal of Pharmacology, 463,* 199–216.

Wilson, S. J., Sayette, M. A., & Fiez, J. A. (2004). Prefrontal responses to drug cues: A neurocognitive analysis. *Nature Neuroscience, 7,* 211–214.

Windle, R. J., Kershaw, Y. M., Shanks, N., Wood, S. A., Lightman, S. L., & Ingram, C. D. (2004). Oxytocin attenuates stress-induced c-fos mRNA expression in specific forebrain regions associated with modulation of hypothalamo-pituitary-adrenal activity. *Journal of Neuroscience, 24,* 2974–2982.

Wu, Y. L., Yoshida, M., Emoto, H., & Tanaka, M. (1999). Psychological stress selectively increases extracellular dopamine in the 'shell', but not in the 'core' of the rat nucleus accumbens: A novel dual-needle probe simultaneous microdialysis study. *Neuroscience Letters, 275,* 69–72.

Zangenehpour, S., & Chaudhuri, A. (2002). Differential induction and decay curves of c-fos and zif268 revealed through dual activity maps. *Brain Research. Molecular Brain Research, 109,* 221–225.

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