Available online at www.sciencedirect.com
ScienceDirect
Physiology & Behavior 93 (2008) 637–650
PHYSIOLOGY & BEHAVIOR
www.elsevier.com/locate/phb

Neuroanatomical specificity of conditioned responses to cocaine *versus* food in mice

Jonathan A. Zombeck, Guan-Ting Chen, Zachary V. Johnson, David M. Rosenberg, Adam B. Craig, Justin S. Rhodes *

Department of Psychology, The Beckman Institute, 405 N Mathews Ave, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

Received 23 July 2007; received in revised form 21 September 2007; accepted 1 November 2007

Abstract

Neural circuits implicated in drug conditioning, craving and relapse overlap extensively with those involved in natural reward and reinforcement. To determine whether specificity could be detected in conditioned brain responses to drugs *versus* food, male outbred HSD:ICR mice were conditioned to a common environment using either 20 mg/kg cocaine (ip) or a familiar food (under food restriction). The mice were then re-exposed to the same environment without the reinforcer and patterns of brain activation were compared using immunohistochemical detection of Fos. Conditioned place preference tests were conducted first to establish relative potency of each reward and facilitate analysis of correlations between Fos and motivation. Place preference was stronger for cocaine than food. Food- but not cocaine-paired cues increased Fos in the paraventricular hypothalamic nucleus whereas the opposite occurred for prefrontal, cingulate and piriform cortices. Individual differences in cocaine place preference were negatively correlated with Fos in the prefrontal cortex. One difference between drugs and natural reinforcers may be lack of feedback from the periphery for drugs which may circumvent control from the hypothalamus in the development of reinforcement circuits. Published by Elsevier Inc.

Keywords: Cocaine; Conditioning; Food; Natural reward; Mice; Fos

1. Introduction

One of the most challenging obstacles for treatment of drug addiction is relapse. Even after years of abstinence, a stressful life event, a small "priming" dose of drug, or exposure to drug-paired cues can trigger an individual to resume compulsive drug seeking behavior [1]. Contact with drug-paired contextual cues is particularly problematic as these cues can be ubiquitous. For example, drug paraphernalia or environments where drugs were taken can produce powerful craving emotions that can overwhelm subjects and lead to relapse [2]. For this and other reasons effort has focused on identifying neural circuits and substrates involved in drug conditioning and reinforcement.

A growing body of literature suggests that the natural reward circuit (*e.g.*, dopamine projections from the ventral midbrain to nucleus accumbens and prefrontal cortex) and connections with amygdala play key roles in drug reward and conditioning [3–5]. The natural reward circuit was originally identified for its role in perception of the appetitive value of natural stimuli. It is thought that the circuit evolved via natural selection to increase fitness (*i.e.*, survival and reproductive success) by generating appropriate behavioral responses to natural stimuli with positive or negative value for the organism [6]. For example, the theory posits that eating or engaging in sexual activities evolved to produce the perception of pleasure as a "solution" or mechanism to motivate and/or reinforce animal behavior essential for survival and reproductive success. Similarly it posits that fear is an emotional response to threat because it motivates and reinforces avoidance behavior [7]. One theory of drug addiction is that drugs of abuse activate the circuit in such a way that the animal interprets the drug stimulus as strongly positive, hence resulting in strong reinforcement of drug seeking behavior [8].

Current opinion is that neural circuits involved in drug addiction overlap extensively with those involved in natural

* Corresponding author. Beckman Institute (room 3315), 405 N. Mathews Avenue, Urbana, IL 61801, USA. Tel.: +1 217 265 0021; fax: +1 217 244 5876.
E-mail address: jrhodes@uiuc.edu (J.S. Rhodes).

0031-9384/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.physbeh.2007.11.004

reward and reinforcement [9]. In fact, whether differences exist or how they might be displayed is not known [10]. If differences could be identified that might provide value for understanding the transition to pathological behavior (*e.g.*, loss of control; not an evolutionary adaptation).

One strategy for identifying potential differences in reinforcement with drugs *versus* natural rewards is to pair a common environment with either a drug or a natural reward and then compare responses elicited when the animals are re-exposed to the same environment in absence of the reinforcer [10]. Any differences in the response must therefore be due to the prior history of reinforcement, and not to the sensory content of the immediate experience as that will be common to all subjects.

This approach has been used in rat studies in which immunohistochemical detection of Fos, the protein product of the gene, *c-fos*, was used to identify brain regions responsive to contextual cues paired with nicotine, morphine or chocolate [10,11]. Similar patterns of activation were found in frontal cortical areas in animals conditioned to drugs or food but interesting differences occurred in the preoptic area of the hypothalamus where cues elicited a greater activation when they were paired with chocolate than drugs. Because Fos is a transcription factor (*i.e.*, it combines with other factors which bind to DNA to affect the expression of hundreds of genes) and because Fos protein levels increase with a discrete time-course following a stimulus, these data identified brain regions likely undergoing a large amount of transcriptional regulation in response to the conditioned stimulus [12–16].

One of the principal aims of this study was to determine whether differences could be found in the Fos responses to contextual cues paired with cocaine *versus* a familiar food. Because we suspected cocaine would be a much stronger stimulus than normal laboratory chow under satiated conditions, the value of food was increased by food restriction. Animals were only given access to their normal lab chow (more than they could eat) once per day for 90 min in a specific context. We reasoned that this would increase the strength of the association between the food reward and context without having to introduce a novel palatable item. Moreover, we thought this would represent a form of conditioning with obvious adaptive evolutionary significance. The ability of an animal to remember environmental cues that predict food availability has clear implications for survival and reproductive success.

As described above, previous studies have explored the neuroanatomy of conditioned brain responses to drugs using immunohistochemical detection of Fos in rodents [10,11,17–22]. These experiments typically proceed in two phases: training and testing. Animals are first trained over a series of days to associate a novel environment with the subjective effects of a drug by repeatedly placing them into that environment after drug administration (drug-paired group). Control animals are typically placed into the environment the same number of times after an injection of saline (unpaired). In some cases, control animals are administered the same amount of drug as the drug-paired animals but in a different environment such as the home cage (*i.e.*, explicitly unpaired) to keep drug administration a controlled variable [17,20]. The animals are then placed into the environment without drug and brain regions are measured for number of Fos positive nuclei. In these experiments, many cues distinguish the drug-paired environment from the unpaired one because the drug-paired environment is either the home cage for control animals or a novel cage for experimental animals. Hence, removal from the home environment and all the cues associated with the new environment such as lighting, texture, smells, and sounds, represent contextual cues associated with the drug experience.

The other major aim of this study was to determine whether we could detect correlations between the Fos signals in the brain and behavioral indices of motivation for cocaine or food [22]. This was accomplished by performing the conditioned place preference (CPP) test before measuring Fos responses in the same animals. The typical method for behavioral assessment of CPP uses two different environments which are similar to each other except for one particular salient feature, such as the texture of the floor (*e.g.*, hole or grid) [23]. The conditioned Fos studies, as described above, typically use more conspicuous contextual cues to distinguish the paired from unpaired contexts (*e.g.*, home *versus* test cage). Thus, to relate behavior to physiology, we modified the typical experimental design for conditioned Fos and used the two floor textures, hole or grid, as the paired or unpaired contexts. The conditioned Fos signals in this study therefore represent more subtle differences attributed to floor-texture cues as compared to previous studies which used a larger composite of cues.

2. Materials and methods

2.1. Subjects and husbandry

A total of 44 genetically variable, male, albino HSD:ICR (or CD1) mice (Harlan Sprague Dawley, Indianapolis, IN) were used. Animals arrived at the Beckman Institute Animal facility at 5 weeks of age and were acclimated for 2 1/2 weeks prior to testing. During the first 1 1/2 weeks, mice were housed 4 per cage. They were then transferred to individual housing for the last week of acclimation and duration of the experiments. Animals were housed in standard polycarbonate shoebox cages with Bed-o-Cob™ bedding. Rooms were controlled for temperature (21±1 °C) and photo-period (12:12 L:D). A reverse light/dark cycle was used in which lights turned on at 2200 h and off at 1000 h Central Standard Time. Red incandescent lamps were kept on continuously so that investigators could handle mice during the dark phase. Food (Harlan Teklad 7012) and water were provided *ad libitum*, except during the food conditioning described below. The Beckman Institute Animal Facility is AAALAC approved. All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee and adhered to NIH guidelines.

2.2. Drug solutions

Cocaine hydrochloride (Sigma Aldrich, St. Louis, MO) was dissolved in 0.9% saline and was administered at a dose of 20 mg/kg via intraperitoneal (ip) injections in a volume of 5 ml/kg. Dose was chosen based on the literature [24–26] and was prepared according to the salt not the base form.

2.3. Video tracking

Distance traveled in the apparatus and the location of the mouse within the CPP boxes were determined by TopScan (Clever Sys Inc, Vienna, VA) video tracking software.

2.4. Place conditioning chambers

The place conditioning chambers were designed following Cunningham et al. [23] with some modifications to enable video tracking from above. They consisted of 20 identical black acrylic boxes (30 cm × 15 cm × 15 cm) with removable clear plastic tops. The floors were interchangeable and consisted of three types of distinct textures: hole, grid and hole/grid. The hole floor consisted of perforated 16-gauge stainless steel with 6.4 mm round holes on 9.5 mm staggered centers. The grid floor consisted of 2.3 mm stainless steel rods mounted 6.4 mm apart. The hole/grid floor consisted of half grid and half hole textures which together had the same dimensions as the solid textures. All three floor types were mounted on a black acrylic frame (33 cm × 18 cm × 5 cm) with 4 holes (1 cm diameter) drilled in the sides for ventilation.

2.5. Preconditioning

Each experiment consisted of a preconditioning phase to determine individual biases in preference for the textures before they were paired with reward [23]. Animals were placed on the hole floor in the morning (1100 h, 1 h after lights shut off) and grid floor in the afternoon (1500 h, 5 h after lights shut off) or *vice versa* for 4 consecutive days without any treatment. Trials lasted 30 min in experiment 1 and 90 min in experiment 2. Ninety minute conditioning trials were used in experiment 2 because this time was needed for the animals to eat in the chambers during CPP (see below). On day 5 of preconditioning, in both experiments, animals were placed in the apparatus with half hole and half grid floor types for 30 min in the morning and again for 30 min in the afternoon. In experiment 1, animals were retested with half hole and half grid floors in the morning and afternoon 4 days later (day 9) after remaining undisturbed in home cages (see Fig. 1).

2.6. CPP

CPP proceeded exactly as the preconditioning phase except animals were placed in the apparatus with reward (*i.e.*, cocaine or food) either in the morning or afternoon during the 4 conditioning days. If an animal received a reward in the morning then it was placed in the apparatus without a reward in the afternoon (and *vice versa*). The typical procedure in which the reward is administered on alternate days [24] was not used in order to implement an experimental design for food conditioning in which food is offered only once a day and always in a specific context (hole or grid).

Mice were randomly assigned to two groups, grid-paired or hole-paired (*i.e.*, without regard to baseline preferences). Grid-paired mice were trained to associate grid floor with reward and hole floor without reward. Hole-paired mice were trained to associate hole with reward and grid without. The order (*i.e.*, whether animals received reward in the morning or afternoon on grid or hole) was counterbalanced. Preference tests consisted of placing the mice in the apparatus with half hole and half grid floor types without reward either in the morning or afternoon (counterbalanced) for 30 min.

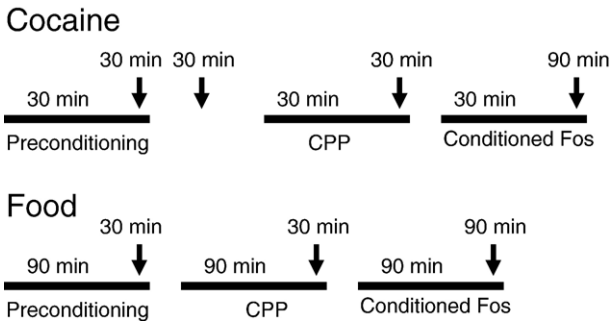

Fig. 1. Schematic diagram of the experimental design. The horizontal bars represent the different phases of the experiments. The arrows show when tests were administered (either to measure CPP or conditioned Fos). In both experiments, animals experienced 1 week of preconditioning to establish baseline texture preferences, 1 week of CPP and 1 week for conditioned Fos. Differences were that in the food experiment 90 min conditioning trials were used instead of 30 min to enable enough time for the animals to eat in the chambers, but note that 30 min preference tests were used in both experiments. In addition, the food experiment proceeded in 3 consecutive weeks whereas in the cocaine experiment, an additional week was inserted after the preconditioning phase for a second preference test. During this week, besides the preference test which occurred in the middle of the week, animals remained undisturbed. Finally, in the food experiment, water was always available in the chambers in a petri dish whereas water was not available in the cocaine experiment.

2.7. Conditioned Fos

The same procedure as outlined for the conditioning phase (above) was repeated except on the test day animals were placed into the apparatus containing a floor with entirely grid or hole textures. Half the animals were placed onto the texture where they had previously experienced the reward, and the other half where they had not experienced the reward. Note that animals were NOT given a choice of floor types during this test to control duration of exposure on the reward-paired context [17]. For both experiments this test lasted 90 min after which animals were euthanized and processed for immunohistochemical detection of Fos (see below).

2.8. Immunohistochemistry

Following Hasen et al. [27], mice were decapitated and their brains were removed and placed into 5% acrolein in phosphate buffered saline (PBS) for fixation overnight. Brains were then transferred to 30% sucrose in PBS for 2 days and sectioned using a cryostat. Sections (40 μm thick) were placed into tissue cryoprotectant (PBS containing 30% sucrose w/v, 30% ethylene glycol v/v, and 10% polyvinylpryrrolidone w/v) in a 24 well plate, then stored at −20 °C. Immunohistochemistry for Fos was performed on free-floating sections that were pretreated with sodium borohydride (100 mg per 20 ml PBS) for 30 min at room temperature. Sections were subsequently washed in PBS

containing 0.2% v/v Triton X-100 (PBS-X) and blocked with 6% v/v Normal Goat Serum (NGS) for 1 h at room temperature. Sections were then incubated with primary antibody against Fos made in rabbit (Calbiochem, San Diego, CA) at a dilution of 1:20,000 in PBS-X containing 2% NGS for 48 h at 5 °C. Next, sections were washed in PBS-X and then incubated in secondary biotinylated antibody against rabbit immunoglobulin made in goat (Vector Labs, Burlingame, CA) at a dilution of 1:500 in PBS-X with 2% NGS for 90 min at room temperature. To visualize the antibody, the peroxidase method was used (ABC system, Vector Labs, Burlingame, CA; 37 µl A, 37 µl B in 15 ml PBS-X) with diaminobenzidine (DAB) as chromogen, enhanced with 0.008% w/v nickel chloride (Sigma, St. Louis, MO). The reaction was stopped by washing the sections in PBS. Sections were mounted from PBS onto subbed slides (pretreated with 1% w/v pig skin gelatin and 0.05% w/v chromium potassium sulfate). The following day, after the sections were dry, they were dehydrated using the following series: 70% ethanol for 5 min, 95% ethanol for 5 min, fresh 95% ethanol for 5 min, 100% ethanol for 10 min, fresh 100% ethanol for 10 min, xylenes for 15 min, and finally fresh xylenes for 15 min. Slides were then removed and coverslipped while still partially wet with xylenes using Permount (Sigma, St. Louis, MO).

2.9. Image analysis

Microscopic (Zeiss) images of the sections were captured via a Zeiss Axiocam digital camera interfaced to a personal computer. All Fos positive cells were automatically counted using NIH ImageJ software within a box (1 × 0.63 mm) placed at the locations shown in Fig. 2 following Paxinos and Franklin [28]. If the brain regions were smaller than this box, as was the case for the paraventricular hypothalamic nucleus and the dentate gyrus, then the nucleus was outlined by hand and particles were counted only within the outlined structures. The counting was done unilaterally, in three alternate sections for each brain region, to obtain an average cell count per brain region for analysis [17,29]. Stereological procedures were not used because we assumed shrinkage of tissue and sizes of nuclei would not differ between treatment groups within an experiment, given that the only difference between groups was whether animals were placed onto a grid *versus* a hole texture. Moreover, stereological procedures would be extremely difficult and time consuming to implement in cortical areas where boundaries are ambiguous. The following steps were taken to ensure immunohistochemical detection of Fos was measured consistently between samples. All sections were exposed to diaminobenzidine for exactly 5 min.

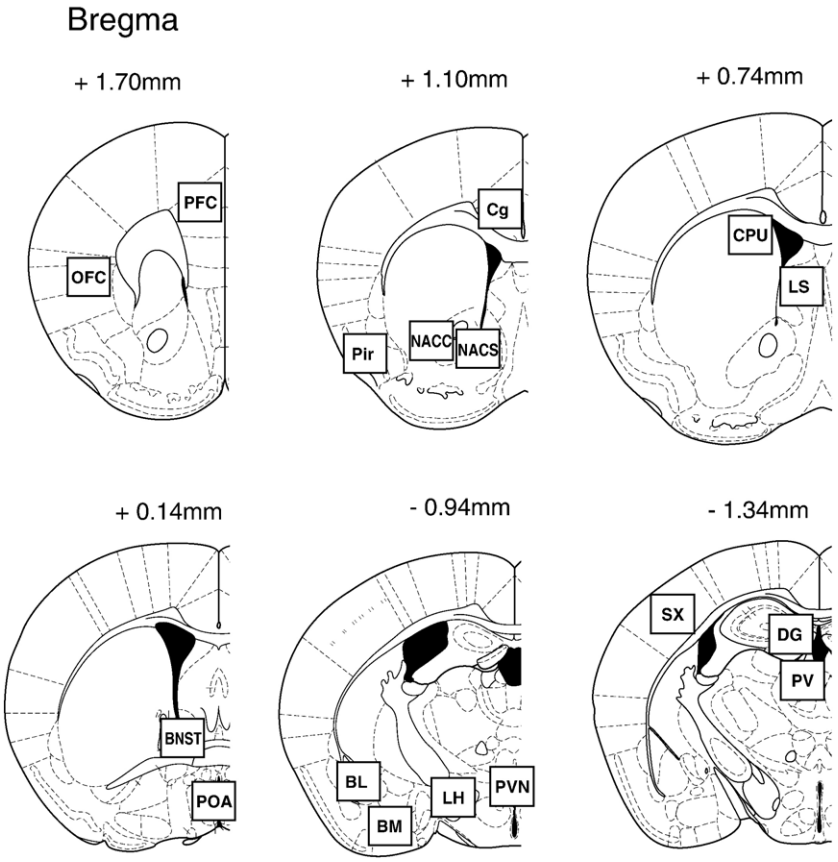

Fig. 2. Locations where Fos positive nuclei were counted (boxes, shown roughly to scale, were 1 X 0.63 mm). Reprinted from The Mouse Brain in Stereotaxic coordinates, 2nd edition, G. Paxinos and K. Franklin, Figures 17, 22, 25, 30, 39, 42, Copyright 2001, with permission from Elsevier. As noted, for the paraventricular hypothalamic nucleus and the dentate gyrus, the nucleus was outlined by hand and particles were counted only within the outlined structures. Legend: PFC = prefrontal cortex, OFC = orbitofrontal cortex, Cg = cingulate cortex, NACC = nucleus accumbens core, NACS = nucleus accumbens shell, PIR = piriform cortex, CPU = caudate, LS = lateral septum, BNST = bed nucleus of the stria terminalis, POA = preoptic area, BL = basolateral amygdala, BM = basomedial amygdala, LH = lateral hypothalamus, PVN = paraventricular hypothalamic nucleus, SX = somatosensory cortex, DG = dentate gyrus, PVA = paraventricular thalamic nucleus.

The background was normalized by automatically adjusting light levels. In the image analysis, a thresholding procedure was applied to distinguish Fos positive nuclei automatically following Rhodes et al. [17]. 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 to be positive for Fos, and were counted. The same threshold was used for all sections within a brain region. The counting was done by individuals who were blind to the experimental conditions. Only particles within a specified size range were counted (between 20 and 300 pixels, under 100 × magnification).

2.10. Statistical analysis

SAS (Release 9.1) Proc Mixed or R (2.3.1) were used for all analyses.

2.10.1. Locomotor activity

Distance (meters) was analyzed by repeated measures analysis of variance with day, time (am or pm), or treatment (*e.g.*, cocaine *versus* saline or food *versus* no food) as within-subject factors.

2.10.2. CPP

Data were analyzed in two ways. First, duration (min) spent on the hole side of the apparatus was compared between hole-paired *versus* grid-paired mice using unpaired *t*-tests. Second, duration on the reward-paired floor (either hole or grid depending on the animal) was compared to duration spent on that floor type under baseline conditions using paired *t*-tests. In experiment 1, baseline scores were calculated by taking the average of the last 3 tests. In experiment 2, they were the second test. The logic was that these values represent the bias in texture preference that is established after acclimation to the test.

2.10.3. Immunohistochemical detection of Fos

Number of Fos positive cells in a brain region were analyzed using a multiple linear regression model with the following parameters. The dependent variable was number of Fos positive nuclei in a brain region. Independent variables were batch (a categorical variable indicating the episode when sections were stained), cumulative distance traveled in the apparatus preceding euthanasia (a continuous variable), place preference (a continuous variable, see below), and treatment (whether the animals were placed into the context where they were expecting reward or not, a categorical variable).

We considered two different ways to quantify individual differences in place preference. One was time spent on the reward-paired texture. Another was the difference between time spent on the reward-paired texture minus time spent on that texture under baseline conditions before it was associated with any reward. The latter was used as the covariate in the multiple linear regression model because it adjusts for individual biases in floor preference.

Batch was included even though treatment groups were counterbalanced within batches to reduce the mean square error, resulting in less inflated type 2 error rates and *p*-values following Rhodes et al. [17]. Distance traveled was included following Rhodes et al. [17] because it was a strong predictor of Fos positive cells in previous studies, and we wished to examine effects of treatment after adjusting for variation explained by locomotor activity. Place preference was included to determine whether individual variation in a measure of motivation for the rewards was correlated with Fos levels. Treatment was included to identify conditioned brain responses.

Because multiple tests were conducted comparing Fos counts between groups for 17 different brain regions, we determined the level of significance per test that would produce a global false discovery rate of 5% for the entire study using qvalue software [17,30]. This value turned out to be 0.02. Hence for the Fos analyses, we considered results statistically significant if the *p*-value was less than or equal to 0.02.

In addition, we carried out a principal component analysis. The first principal component (*i.e.*, the linear combination of Fos values, scaled to a unit variance, across all 17 regions explaining the most variation in the data) was then analyzed using the linear regression model described above and was correlated with Fos levels in each region to analyze loading patterns on the variables.

2.11. Experiment 1: Cocaine

Twenty mice were tested for preconditioning, CPP and conditioned Fos using 20 mg/kg cocaine ip as the unconditioned stimulus.

2.12. Experiment 2: Food

An additional 20 mice were tested for preconditioning, CPP and conditioned Fos using food as the unconditioned stimulus. All trials included continuous access to water in a 3.5 cm diameter clear plastic Petri dish bolted to the middle of the floor. Food was removed 22.5 h before the first conditioning session when food was given (which was the morning of the following day for some animals and the afternoon for others). Four additional age-matched animals, that were not part of the conditioning experiment, were maintained with *ad libitum* food to monitor free feeding weight gain for the duration of the experiment. Animals that lost more than 80% free feeding mass were humanely euthanized.

The following day after food restriction began, animals were placed on grid or hole floors with or without food (but always with water) for 90 min in the morning or afternoon. Food was provided by placing 3 pieces of regular laboratory chow around the water dish. Grid-paired mice were placed on grid with food and hole without. Conversely, hole-paired mice received no food on grid and food on hole. Immediately after the 30 min CPP test on day 5 animals were given *ad libitum* access to food in their home cages through the weekend.

After 2 days of *ad libitum* access to food (see above), food was removed again 22.5 h before the first conditioning session for the conditioned Fos phase of the experiment (see Fig. 1).

3. Results

3.1. Locomotor activity

See Fig. 3. Under baseline conditions, during preconditioning, animals moved between 40 and 60 m in 30 min in the apparatus. Baseline activity decreased as days passed ($F_{5,209}=6.1$, $p<0.0001$) and was consistently higher in am (at the onset of the dark cycle) as compared to pm (middle of the dark cycle) ($F_{1,209}=16.4$, $p<0.0001$; interaction was not significant).

Cocaine (20 mg/kg) elevated locomotor activity above saline by approximately 3 fold during both CPP ($F_{1,125}=597.2$, $p<0.0001$) and conditioned Fos ($F_{1,162}=601.2$, $p<0.0001$). Locomotor activity in response to cocaine remained consistent across days (*i.e.*, sensitization was NOT observed) ($F_{3,54}=0.94$, $p=0.43$). No evidence of a conditioned effect on locomotor activity was observed on the last day of the cocaine experiment when animals were placed on either a cocaine-paired or saline-paired texture after a saline injection ($t_{17}=0.72$, $p=0.48$).

During food CPP (Fig. 3, bottom panel), animals that were not given food in the apparatus moved approximately the same distance each day (approximately 60 m in the first 30 min), but those with food showed reduced locomotor activity as the days passed (approximately 43 m on day 1 *versus* 26 m on day 4), presumably because they were eating rather than moving around. This was reflected in a significant interaction between treatment (food *versus* no food) and day ($F_{3,138}=3.9$, $p=0.01$). During the following conditioned Fos phase, animals again moved approximately half as far when they had food as compared to when they had no food ($F_{1,105}=284.9$, $p<0.0001$) and activity in both groups decreased as the days passed ($F_{3,105}=3.5$, $p=0.02$). The interaction between day and food

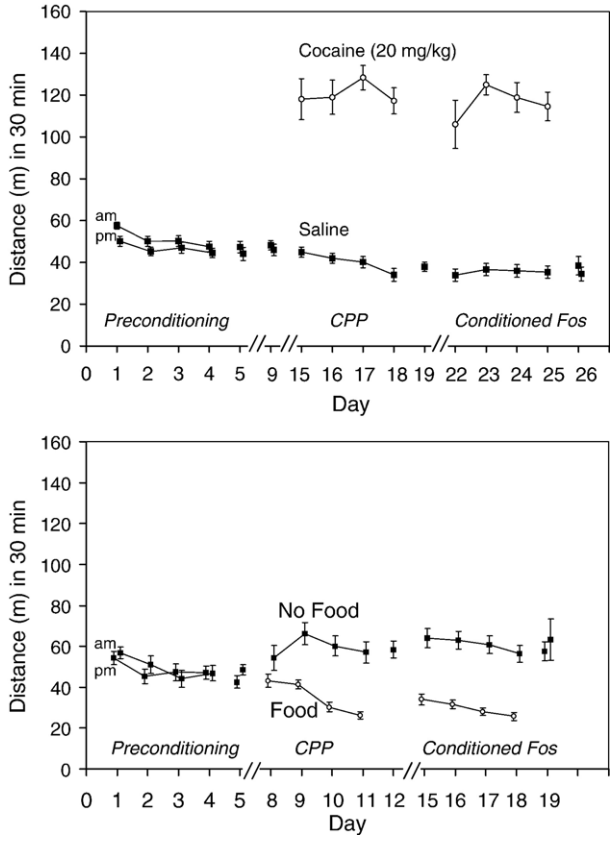

Fig. 3. Locomotor activity. Top panel. Mean (±SEM) distance (meters) traveled in 30 min in the apparatus during each phase of experiment 1. For preconditioning, data are shown separately for am (1 h after lights off) *versus* pm (5 h after lights off). For CPP and conditioned Fos phases, data are shown separately for cocaine *versus* saline injections. On day 26, means for cocaine-paired *versus* saline-paired mice are shown separately (the mean for cocaine-paired mice is above saline-paired). The same animals ($n=20$) are represented in each data point, except day 26 where the animals were split into two groups ($n=10$ per data point). Bottom panel. Same as above for experiment 2. Only the first 30 min of 90 min tests are shown for all days except 5 and 12 which were 30 min tests. The same animals are represented in each data point, $n=20$, for preconditioning, and $n=16$ for CPP and conditioned Fos except day 19 when the animals were split into two groups, food-paired *versus* unpaired ($n=8$ per data point). Open circles represent response to the unconditioned stimulus, cocaine (top panel) or food (bottom panel) treated animals. Closed squares represent either saline-treated (top panel, CPP and conditioned Fos) or untreated (all other data points).

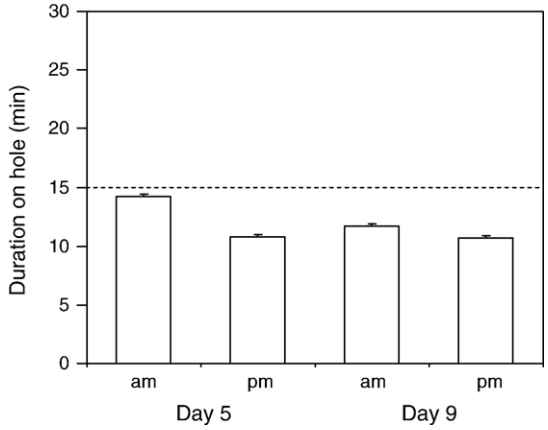

Fig. 4. Baseline bias in preference for grid texture. Mean (±SEM) duration (min) spent on the hole floor for each of the baseline preference tests during the preconditioning phase of experiment 1.

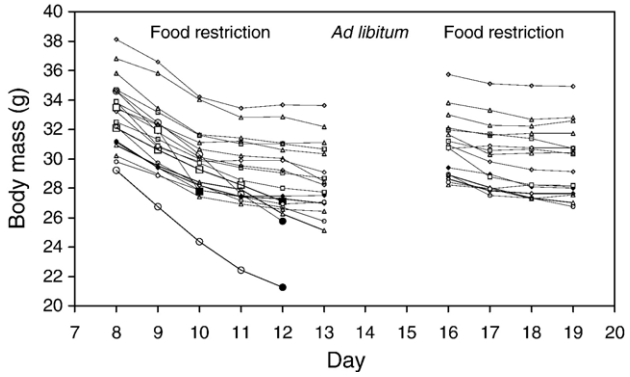

Fig. 5. Food restriction. Body mass (g) is plotted separately for each animal over the course of food restriction (CPP and conditioned Fos phases of experiment 2). Food was removed on day 8 after body mass was measured. On days 9–12 and 16–19 animals were given food only while in the apparatus for 90 min. On day 13 animals were tested for CPP for food (without food in the apparatus) and then they were returned to their cages with *ad libitum* food for the weekend (days 14 and 15). The symbols with connecting lines represent different animals. A filled square indicates that the animal died. A filled circle indicates that the animal was euthanized because it lost greater than 80% free feeding mass.

treatment was not significant. No evidence of a conditioned effect on locomotor activity was observed on the last day of the food experiment when animals were placed on either a food-paired or unpaired texture ($t_{12}=0.60$, $p=0.56$).

3.2. Baseline texture bias

See Fig. 4. During the preconditioning phase of the cocaine experiment, no significant preference for hole *versus* grid was detected on the first test (day 5, am), but a significant bias in preference for grid was established on the second test (pm) (paired $t_{19}=4.8$, $p=0.0001$), and remained consistent on the third and fourth test (day 9 am and pm) (both $p<0.0001$). On average, animals spent approximately 60% of their time on grid and 40% on hole in tests 2–4.

In the food experiment, no significant preference for a floor texture (hole *versus* grid) was detected on the first test (day 5, am). On the second test, on average, animals spent approximately 55% of their time on grid and 45% on hole in pm (the second test), but this difference was not statistically significant (paired $t_{19}=1.2$, $p=0.23$) (data not shown).

3.3. Food restriction

See Fig. 5. By day 5 of the CPP phase of experiment 2, body mass was reduced to an average of 86% (range was 80 to 91%) of initial mass measured the day before food restriction began ($F_{5,89}=126.8$, $p<0.0001$). Two animals died, presumably from the food restriction treatment, and 2 others were euthanized because their body mass was reduced below 80% estimated free feeding weight. After CPP, animals were given 2 days with *ad libitum* food before food restriction resumed for the conditioned Fos phase. During this 2-day *ad libitum* period, the animals recovered from an average of 86% initial weight on day 6 to an average of 93% initial weight on day 9 (paired $t_{15}=11.8$, $p<0.0001$). Over the remaining 4 days of food restriction, weight was reduced but not as much as the first time ($F_{3,45}=29.8$, $p<0.0001$). At the end of the experiment, the animals weighed, on average, 90% their initial weight 11 days earlier before food restriction began (paired $t_{15}=8.1$, $p<0.0001$). The four additional age-matched control animals with *ad libitum* food gained 3% over this period ($F_{9,27}=14.5$, $p<0.0001$).

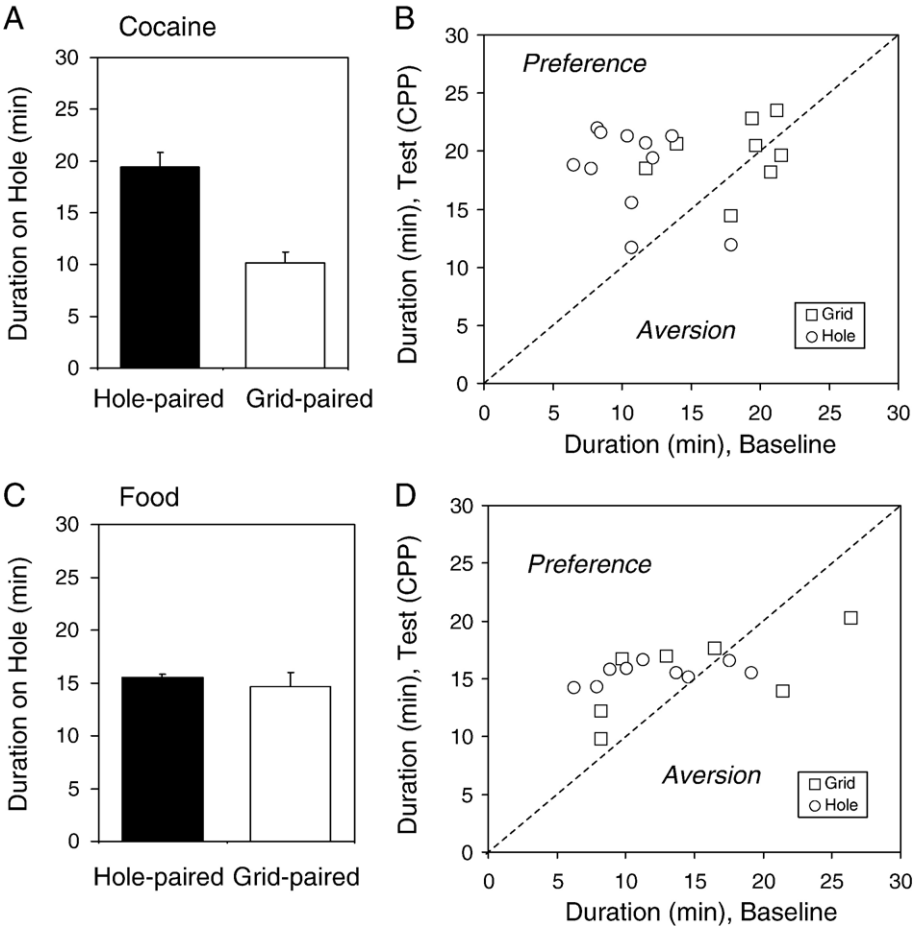

Fig. 6. CPP. A) Mean (±SEM) duration (min) spent on the hole floor for grid-paired *versus* hole-paired mice in the cocaine experiment. (B) Duration (min) spent on hole floor (for hole-paired mice) or grid floor (for grid-paired mice) during cocaine CPP plotted against baseline duration (min) spent on those floor types. The one-to-one line is shown. C) Same as A for the food experiment D) Same as B for the food experiment.

Table 1
Mean (±SE) number of Fos positive nuclei in saline-paired *versus* cocaine-paired mice

Brain region		Least-square means		Coefficient estimates		Multiple linear regression statistics		
		Sal-paired	Coc-paired	Distance	Preference	Treatment	Distance	Preference
Cortex	Prefrontal	332±28.2	441±29.1	3.6±1.89	−0.19±0.069	$F(1,12)=7.2$, $p=0.02$	$F(1,12)=3.6$, $p=0.08$	$F(1,12)=7.3$, $p=0.02$
	Orbitofrontal	144±31.9	232±33.0	2.4±2.14	−0.11±0.078	$F(1,12)=3.7$, $p=0.08$	$F(1,12)=1.3$, $p=0.28$	$F(1,12)=2.0$, $p=0.19$
	Cingulate	346±28.6	499±29.5	6.6±1.91	−0.16±0.070	$F(1,12)=13.8$, $p<0.01$	$F(1,12)=11.8$, $p<0.01$	$F(1,12)=5.2$, $p=0.04$
	Piriform	222±14.0	288±14.4	3.6±0.94	−0.04±0.034	$F(1,12)=11.1$, $p<0.01$	$F(1,12)=14.8$, $p<0.01$	$F(1,12)=1.5$, $p=0.25$
	Somatosensory	264±27.2	266±28.1	1.7±1.82	−0.05±0.066	$F(1,12)=0.0$, $p=0.98$	$F(1,12)=0.9$, $p=0.37$	$F(1,12)=0.5$, $p=0.49$
Basal ganglia and septum	Caudate	29±13.0	64±13.0	0.5±0.84	0.01±0.031	$F(1,12)=3.8$, $p=0.08$	$F(1,12)=0.3$, $p=0.57$	$F(1,12)=0.1$, $p=0.76$
	Lateral septum	249±16.5	300±17.0	4.0±1.11	0.02±0.040	$F(1,12)=4.7$, $p=0.05$	$F(1,12)=13.2$, $p<0.01$	$F(1,12)=0.3$, $p=0.60$
	Accumbens core	93±21.7	153±22.4	1.2±1.45	0.01±0.053	$F(1,12)=3.8$, $p=0.08$	$F(1,12)=0.7$, $p=0.41$	$F(1,12)=0.1$, $p=0.83$
	Accumbens shell	147±15.4	164±15.9	2.0±1.03	−0.07±0.038	$F(1,12)=0.6$, $p=0.46$	$F(1,12)=3.8$, $p=0.08$	$F(1,12)=3.2$, $p=1.0$
	Bed nucleus stria terminalis	60±9.3	83±9.6	1.0±0.62	−0.01±0.023	$F(1,12)=2.9$, $p=0.12$	$F(1,12)=2.7$, $p=0.13$	$F(1,12)=0.2$, $p=0.70$
Hippocampus and thalamus	Dentate gyrus	33±2.5	36±2.6	0.3±0.17	0.00±0.006	$F(1,12)=0.5$, $p=0.51$	$F(1,12)=4.0$, $p=0.07$	$F(1,12)=0.2$, $p=0.69$
	Paraventricular thalamic nucleus	161±16.3	162±16.8	1.2±1.09	0.05±0.040	$F(1,12)=0.0$, $p=0.95$	$F(1,12)=1.1$, $p=0.31$	$F(1,12)=1.7$, $p=0.21$
Hypothalamus	Lateral	127±12.7	164±13.1	2.5±0.85	0.01±0.031	$F(1,12)=4.0$, $p=0.07$	$F(1,12)=8.5$, $p=0.01$	$F(1,12)=0.1$, $p=0.83$
	Paraventricular	187±20.8	195±21.5	1.1±1.39	−0.04±0.051	$F(1,12)=0.1$, $p=0.77$	$F(1,12)=0.7$, $p=0.44$	$F(1,12)=0.7$, $p=0.41$
	Preoptic area	98±10.3	118±10.6	0.0±0.00	−0.00±0.03	$F(1,12)=2.1$, $p=0.18$	$F(1,12)=0.6$, $p=0.45$	$F(1,12)=0.0$, $p=0.87$
Amygdala	Basolateral	60.2±8.3	73±9.2	1.0±0.58	−0.05±0.020	$F(1,11)=1.1$, $p=0.32$	$F(1,11)=2.8$, $p=0.12$	$F(1,11)=6.7$, $p=0.03$
	Basomedial	123±16.4	165±18.2	2.4±1.14	−0.09±0.041	$F(1,11)=3.1$, $p=0.11$	$F(1,11)=4.6$, $p=0.06$	$F(1,11)=5.3$, $p=0.04$

Note. The least-square means were estimated for the average value for each of the following covariates, batch of immunohistochemistry (statistics not shown), distance traveled in the apparatus and individual differences in place preference for cocaine (calculated as the difference in duration on the cocaine-paired texture minus duration on that texture under baseline conditions before it was ever paired with cocaine, from preconditioning, the average of tests 2–4).

3.4. CPP

See Fig. 6. On average mice displayed strong CPP for cocaine and weaker CPP for food. This was established in two separate analyses. First, animals trained to associate hole (as opposed to grid) floor with cocaine spent significantly more time on the hole side of the apparatus on the test day (Fig. 6A) ($t_{17}=5.2$, $p<0.0001$) whereas this was not true for food (Fig. 6C). Second, on average, animals spent more time on the cocaine-paired texture (either hole or grid) as compared to how much time they spent on that texture under baseline conditions before it was ever paired with cocaine (paired $t_{18}=3.7$, $p=0.002$). Note substantial individual variation including 4 individuals that displayed place aversion to cocaine by this measure (Fig. 6B). Relative to baseline, animals also spent significantly more time on the food-paired texture but the effect size was smaller than for cocaine (paired $t_{16}=1.7$, 1-tailed $p=0.035$) (Fig. 6D).

3.5. Conditioned Fos

3.5.1. Cocaine

See Table 1. Three out of 17 brain regions sampled showed a statistically significant difference between cocaine-paired *versus* saline-paired animals at $p<=0.02$ (determined by setting the false discovery rate for the study at 5%; see Materials and methods — Statistical analysis). These included cingulate, prefrontal and piriform cortices. Trends of higher average Fos values for cocaine-paired *versus* saline-paired animals occurred for many other regions. Large effect sizes (though not statistically significant) included orbitofrontal, lateral septum, caudate, nucleus accumbens core, bed nucleus of the stria terminalis, lateral hypothalamus and the amygdala (basolateral and basomedial). The first principal component, extracted from the 17 regions, explained 63% of the variation in the data and was significantly correlated ($p<0.01$) with all regions. When analyzed by the multiple linear regression, it showed a statistically significant difference between cocaine-paired *versus* saline-paired animals ($F_{1,11}=9.1$, $p=0.01$).

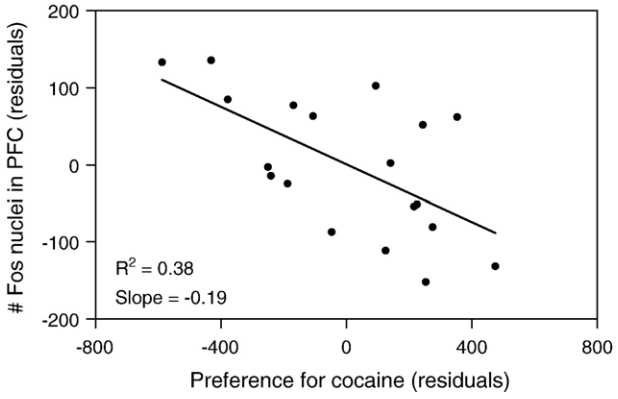

Fig. 7. Basal levels of Fos in the prefrontal cortex negatively correlate with place preference for cocaine. Number of Fos positive nuclei in the prefrontal cortex (residuals) plotted against individual values for place preference for cocaine (residuals). Values for place preference were calculated as the difference in duration on the cocaine-paired texture (hole or grid, depending on the animal) minus duration on that texture under baseline conditions before it was ever paired with cocaine (from phase 1, average of tests 2–4). Residuals were generated from multiple linear regression models that included the following independent variables: batch of immunohistochemistry, distance traveled in the apparatus, and treatment (cocaine-paired *versus* saline-paired groups). The data are shown this way because the slope of the correlation (−0.19) is mathematically equivalent to the Preference coefficient from the multiple linear regression in Table 1, hence illustrating the result for the prefrontal cortex.

Table 2
Mean (±SE) number of Fos positive nuclei in unpaired *versus* food-paired mice

Brain region		Least-square means		Coefficient estimates		Multiple linear regression statistics		
		Unpaired	Food-paired	Distance	Preference	Treatment	Distance	Preference
Cortex	Prefrontal	317±24.5	400±32.5	−2.2±1.38	−0.06±0.089	$F(1,8)=3.38$, $p=0.10$	$F(1,8)=2.51$, $p=0.15$	$F(1,8)=0.39$, $p=0.55$
	Orbitofrontal	14±11.0	58±14.6	−0.5±0.62	−0.02±0.040	$F(1,8)=4.77$, $p=0.06$	$F(1,8)=0.76$, $p=0.41$	$F(1,8)=0.30$, $p=0.60$
	Cingulate	415±25.9	453±34.3	−1.4±1.45	−0.08±0.093	$F(1,8)=0.65$, $p=0.44$	$F(1,8)=0.88$, $p=0.37$	$F(1,8)=0.70$, $p=0.43$
	Piriform	188±34.5	277±45.4	−2.6±1.93	−0.08±0.124	$F(1,8)=2.18$, $p=0.18$	$F(1,8)=1.80$, $p=0.22$	$F(1,8)=0.38$, $p=0.56$
	Somatosensory	273±98.5	542±130.8	−7.6±5.53	−0.19±0.356	$F(1,8)=2.23$, $p=0.17$	$F(1,8)=1.89$, $p=0.21$	$F(1,8)=0.27$, $p=0.62$
Basal ganglia and septum	Caudate	25±4.3	18±5.1	0.4±0.24	−0.03±0.016	$F(1,9)=0.98$, $p=0.35$	$F(1,9)=3.00$, $p=0.12$	$F(1,9)=4.15$, $p=0.07$
	Lateral septum	307±45.9	308±54.2	−1.5±2.58	−0.17±0.169	$F(1,9)=0.00$, $p=0.99$	$F(1,9)=0.32$, $p=0.58$	$F(1,9)=0.98$, $p=0.35$
	Accumbens core	71±12.5	77±16.5	0.9±0.70	0.04±0.045	$F(1,8)=0.07$, $p=0.80$	$F(1,8)=1.61$, $p=0.24$	$F(1,8)=0.85$, $p=0.38$
	Accumbens shell	144±25.6	252±34.0	−4.4±1.44	0.05±0.093	$F(1,8)=5.35$, $p=0.05$	$F(1,8)=9.18$, $p=0.02$	$F(1,8)=0.30$, $p=0.60$
	Bed Nuc. Stria terminalis	117±25.1	194±29.6	−1.5±1.41	−0.13±0.092	$F(1,8)=3.47$, $p=0.10$	$F(1,8)=1.08$, $p=0.33$	$F(1,8)=1.92$, $p=0.20$
Hippocampus and thalamus	Dentate gyrus	62±5.4	58±7.2	0.6±0.31	−0.00±0.020	$F(1,8)=0.22$, $p=0.66$	$F(1,8)=3.40$, $p=0.10$	$F(1,8)=0.02$, $p=0.89$
	Paraventricular thalamic nucl.	211±11.3	241±15.0	0.3±0.63	−0.01±0.041	$F(1,8)=2.13$, $p=0.18$	$F(1,8)=0.28$, $p=0.61$	$F(1,8)=0.13$, $p=0.73$
Hypothalamus	Lateral	170±17.7	170±23.5	0.1±1.0	−0.05±0.064	$F(1,8)=0.00$, $p=1.0$	$F(1,8)=0.02$, $p=0.89$	$F(1,8)=0.63$, $p=0.45$
	Paraventricular	172±26.3	329±34.9	1.2±1.47	0.06±0.095	$F(1,8)=10.74$, $p=0.01$	$F(1,8)=0.62$, $p=0.45$	$F(1,8)=0.35$, $p=0.57$
	Preoptic area	108±16.7	171±22.2	−0.6±0.94	−0.05±0.060	$F(1,8)=4.1$, $p=0.08$	$F(1,8)=0.41$, $p=0.54$	$F(1,8)=0.75$, $p=0.41$
Amygdala	Basolateral	47±9.3	78±12.3	−0.6±0.52	−0.03±0.034	$F(1,8)=3.31$, $p=0.11$	$F(1,8)=1.40$, $p=0.27$	$F(1,8)=0.62$, $p=0.45$
	Basomedial	94±12.6	137±16.8	−1.0±0.71	−0.03±0.046	$F(1,8)=3.48$, $p=0.10$	$F(1,8)=1.98$, $p=0.20$	$F(1,8)=0.35$, $p=0.57$

Note. The least-square means were estimated using the average value for each of the following covariates, batch of immunohistochemistry (statistics not shown), distance traveled in the apparatus and individual differences in place preference for food (calculated as the difference in duration on the food-paired texture minus duration on that texture under baseline conditions before it was ever paired with food (from preconditioning, pm test).

As expected locomotor activity was strongly correlated with Fos levels in many brain regions (see Table 1). Statistically significant correlations were observed for cingulate cortex, piriform cortex, lateral septum, and lateral hypothalamus, with trends in several other regions. The first principal component showed significant correlation with locomotor activity ($F_{1,11}=11.0$, $p<0.01$).

An interesting result occurred for correlations with individual values of CPP for cocaine. A significant negative correlation was observed in the prefrontal cortex (Fig. 7, Table 1). Trends showed negative estimates for all the cortical regions, and large negative estimates in the amygdala.

3.5.2. Food

See Table 2. Only 1 out of the 17 brain regions sampled showed a significant difference in Fos cell number in response to the conditioned context. This was the paraventricular hypothalamic nucleus (Fig. 8). Trends of higher average Fos values for food-paired *versus* unpaired animals occurred for many other regions. Large effect sizes (though not statistically significant) included preoptic area, orbitofrontal cortex, nucleus accumbens shell, bed nucleus of the stria terminalis, and amygdala. The first principal component explained 48% of the variation in the data and was significantly correlated ($p<0.01$) with 13 regions including all of the above with the notable exception of the paraventricular hypothalamic nucleus. When analyzed by multiple linear regression, it showed a statistically significant difference between cocaine-paired *versus* saline-paired animals ($F_{1,8}=6.2$, $p=0.04$).

The estimates of the correlation between Fos numbers and locomotor activity were unexpected. Only 1 region showed a significant correlation, and the coefficient was a large negative value as opposed to positive values in the cocaine experiment. Most correlation estimates were negative under food restriction whereas they were all positive in the cocaine experiment. The first principal component was not statistically correlated with locomotor activity.

4. Discussion

The major finding of this study was identification of neuroanatomical specificity in conditioned responses to cocaine *versus* food in mice. The paraventricular hypothalamic nucleus showed a robust activation to food- but not cocaine-paired cues (Fig. 8). This is consistent with a previous report showing that Fos signals in the paraventricular hypothalamic nucleus are not responsive conditioned stimuli paired with methamphetamine [17]. To the best of our knowledge, only 2 other studies have compared Fos responses to contextual cues paired with natural *versus* drug rewards. In both those studies, the preoptic area of the hypothalamus (adjacent to the paraventricular hypothalamic nucleus) was identified as a key locus for the difference. In the first study, the preoptic area was the only region in which cues elicited a greater activation when paired with chocolate than morphine or nicotine in male rats [10]. In the second study, the preoptic area was also the only region showing greater activation in response to cues paired with access to pups *versus* cocaine in 10-day postpartum female rats [31]. Many studies have established the central role of the hypothalamus in regulating drives for natural reinforcers such as food (for a review see [32]) and sex (for a review see [33]). Moreover, recent studies have identified the lateral hypothalamus as a key

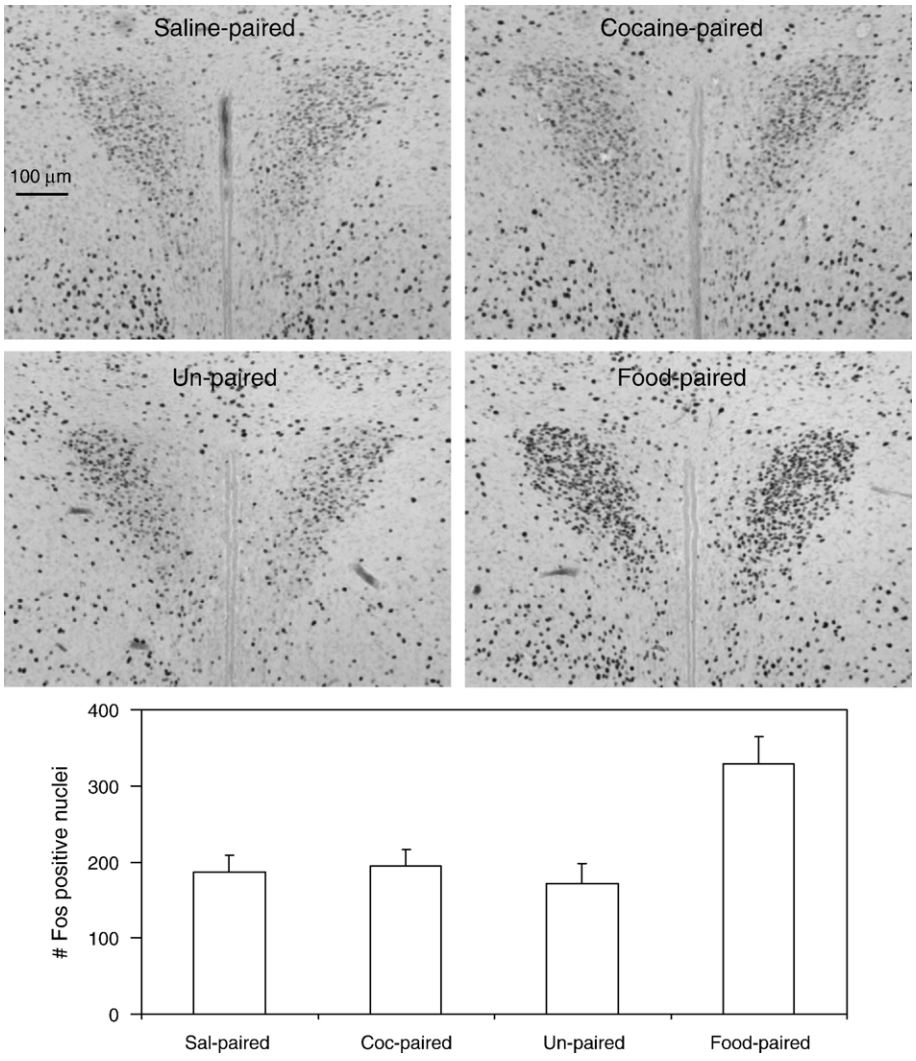

Fig. 8. The paraventricular hypothalamic nucleus shows a robust Fos response to food-paired but NOT cocaine-paired cues. Representative sections showing Fos positive nuclei (black dots) in each group. The graph shows least-square mean (±SEM) number of Fos positive nuclei in the paraventricular hypothalamic nucleus for each group.

region in the regulation of voluntary levels of exercise in mice [29]. It is well established that the hypothalamus receives signals (*e.g.*, leptin, ghrelin) from the peripheral organs with information about "needs" and thereby regulates drives [32]. We speculate that one of the key differences between drugs and natural reinforcers is that drugs activate the natural reward circuit without peripheral feedback mechanisms which circumvents inhibitory control from hypothalamus in the development of reinforcement circuits.

Another interesting discovery was that Fos levels in the prefrontal cortex were negatively correlated with individual differences in CPP for cocaine (see Fig. 7). After adjusting for differences in Fos between the treatment groups, hence effectively removing the contribution of the conditioned stimulus, paired *versus* unpaired, animals with relatively larger preferences for cocaine showed relatively *reduced* levels of Fos activation. One interpretation of this result is that animals with reduced basal activation in the prefrontal cortex are predisposed for increased motivation for cocaine. This is consistent with the idea that neural activity in the prefrontal cortex contributes to inhibitory control over actions and that individuals with less inhibitory control show larger drives for drug rewards [34].

4.1. Conditioned Fos brain responses

Results demonstrate a robust conditioned physiological response in the brain to cues paired with food or cocaine (Tables 1 and 2). The prefrontal and cingulate cortices, which showed significant activation in response to cocaine-paired cues, are well established to play a role in reward and reinforcement in animals and humans [34,35]. These regions have been implicated in the incentive motivational effects of cocaine [19–22,36,37], nicotine [10], methamphetamine [17], amphetamine [38], morphine [11], alcohol [18], chocolate [10] and wheel running [29] in mice and rats. On the other hand, a variety of stimuli, not only cues paired with drugs or natural rewards, appear to activate these regions in humans and rodents. For example, the prefrontal and cingulate cortices are also activated when human [39] or rat [40]

subjects are exposed to cues paired with a shock (*i.e.*, in a model of fear conditioning) or when humans view sexually explicit videos [41]. Moreover, the cingulate cortex is one of the most commonly reported regions 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 prefrontal and cingulate cortices in this study probably reflects a general role in attention or arousal rather than a specific role in motivation or reinforcement for cocaine.

The other region that showed significant activation in response to cocaine-paired cues was the piriform cortex. This region was also identified as responsive to cocaine-paired cues in Le Foll et al. [37] and in response to methamphetamine cues in Rhodes et al. [17] but see [20], where it was not activated by cues paired with cocaine. On the other hand, the piriform cortex was also activated by cues paired with a shock [40] and showed increased activation in mice prevented from their routine exercise on a running wheel [29]. Hence, activation of the piriform cortex in this study probably also reflects a general role in attention or arousal rather than any specific role in drug conditioning.

The paraventricular hypothalamic nucleus showed a strong response to food- but not cocaine-paired cues (Fig. 8). This region is well established to play a key role in the regulation of feeding (for a review see [32]). One pathway involves feedback from arcuate nucleus of the hypothalamus. Leptin released from fat cells throughout the body binds to receptors in the arcuate nucleus which influences the output of projections to other regions of the hypothalamus including the paraventricular hypothalamic nucleus. This allows the brain to adjust appetite depending on metabolic need (*i.e.*, energy stores currently available) [32]. Taken together, this suggests that conditioned responses to food in the brain involve regions that integrate information about metabolic needs from the periphery along with conditioned stimuli predicting food availability. Note that this cannot be true for cocaine because no brain region exists to integrate information from the periphery about "needs" for cocaine.

4.2. Locomotor activity

Several brain regions showed a significant positive correlation between number of Fos positive nuclei and distance traveled in the apparatus in the cocaine experiment (see results for "Distance" in Table 1). These included the cingulate cortex, piriform cortex, lateral septum and lateral hypothalamus. The same brain regions showed positive correlations with locomotor activity in a previous experiment where methamphetamine was used as the reinforcer [17]. It is notable that even when not significant, the estimates were positive for all the brain regions examined (Table 1) which was also true in the methamphetamine experiment [17]. These correlations might reflect increased arousal of the brain associated with movement or a direct role in the control of locomotor activity. In either case, these correlations are not indicative of conditioned responses, since conditioned changes in locomotor activity were not observed in this study. Moreover, the effect of distance occurred over and above effects of conditioning because distance was a covariate in a multiple linear regression model that also included the treatment effect (cocaine-paired *versus* saline-paired).

It is interesting that the food study showed an entirely different result for the distance covariate. Only 1 brain region, the nucleus accumbens shell, showed a significant correlation with locomotor activity, and the estimate was a large negative value! Moreover, a majority of the other brain regions showed negative estimates for the distance covariate, though not significant. The explanation for this is not clear. It might be a reflection of food restriction whereby under food restriction animals that are relatively more active show relatively suppressed brain activation in forebrain areas, but food restriction tends to increase not decrease, Fos expression in the nucleus accumbens shell as well most other regions examined [42]. An alternative is that the positive correlations with locomotor activity are a reflection of chronic cocaine, but that too is unlikely, because even in the absence of cocaine, positive correlations between Fos and locomotor activity were observed in a previous study examining Fos correlations with wheel running behavior [29].

The lack of sensitization or conditioned effects on locomotor activity in this study is most likely attributed to the high doses used for locomotor stimulation (ceiling effect). Typical procedures for sensitization use lower doses and/or intermittent injections [43]. Conditioned effects on locomotor activity are typically subtle and usually also require lower doses (potentially to avoid a ceiling effect) and more context-drug pairing sessions than were implemented here [44].

4.3. CPP

One of the other intriguing findings in this study was the observation of a significant negative correlation between individual differences in place preference for cocaine and Fos levels in the prefrontal cortex (see results for "Preference" in Table 1). Note that the negative correlation occurred after adjusting for variation attributed to the treatment (cocaine-paired *versus* saline-paired). The implication of this result is that within treatment groups animals with relatively greater place preference for cocaine showed relatively reduced brain activation in the prefrontal cortex. The explanation for this is unclear. One possibility is that basal variation in brain activation in the prefrontal cortex contributes to individual differences in motivation for cocaine (*i.e.*, that the relationship is causal). The role of the prefrontal cortex in inhibiting impulsive behavior is well established [45]. Hence, these results are consistent with the idea that individuals with reduced basal prefrontal cortical activation (and hence reduced inhibitory control over behavior) display relatively greater unimpeded drives for rewards [34].

In contrast to the cocaine study, no significant correlations between Fos levels and place preference were observed in the food study. However, the range in individual preferences for food was smaller than for cocaine (see Fig. 6 B vs. D, the data points are closer to the diagonal line for food than cocaine) which reduces statistical power to detect such a correlation. Nonetheless, it is notable that a majority of the preference

coefficient estimates were negative for food as well as for cocaine. Taken together, if the negative correlations are a reflection of a cause and effect relationship as described above, then these results suggest that individuals with relatively reduced prefrontal cortical activation are more inclined to display strong appetitive behavior toward either drugs or food rewards.

One of the reasons for conducting the place preference tests before carrying out the Fos study was to establish the relative potency of the rewards, but results were ambiguous with regard to this aim. Place preference for cocaine was clearly stronger than food (Fig. 6). On the other hand, the magnitude of the conditioned Fos response in the paraventricular hypothalamic nucleus was much stronger for food than cocaine (Fig. 8). Moreover, food must have been perceived as a strong stimulus, because the behavior of the animals in the presence of this stimulus resulted in life or death. Recall that 2 animals died presumably from the food restriction, and 2 other animals had to be euthanized because they lost more than 80% free feeding weight. Our intention was to enhance the positive value of the food stimulus to more closely match potency of cocaine which we assumed was perceived as a very strong positive stimulus. Due to the mortality, the experiment as described this way will not be repeated again.

One possible reason why the effect size for food CPP might have been smaller than cocaine is that the CPP test did not reflect the "true" potency of the food stimulus as compared to cocaine. Recall that the food-paired mice showed reduced locomotor activity and cocaine-paired mice increased locomotor activity during training (Fig. 3). The degree of exploration during training can impact degree of familiarity to the environment, and consequently impact performance during the test phase of the experiment [46]. Moreover, CPP for food has been difficult to establish with the procedure used herein (Christopher Cunningham, personal communication). One reason may have to do with the familiarity of food *versus* cocaine and possible effects of latent inhibition [47]. An alternative reason that food CPP may be difficult to obtain may be related to the idea that the most appropriate behavior for food-seeking would be to forage (explore all places) rather than remain in a place where food had once been. On the other hand, the large conditioned Fos response in the paraventricular hypothalamic nucleus suggests that the animals did in fact make a strong association between the context and food. These issues will be resolved in future studies. For example, it would be interesting to repeat the experiment with a novel highly palatable food for comparison [48] and to include another study where the textures are paired with imminent access to food as opposed to actual eating. This might activate processes mediating expectation or preparation for a natural reward that might show stronger CPP, analogous to cocaine. An alternative would be to pair the texture with post-ingestion or satiety.

4.4. Methodological considerations

Several variables differed between the food and cocaine experiments besides the type of reward used for conditioning that might have influenced conditioned responses. We mentioned already the possibility that the potency of the rewards might have been different. Although the length of sessions on test days was the same for both experiments (always 30 min for CPP and 90 min for conditioned brain activation), the length of conditioning trials was different. Ninety minute sessions were used in the food experiment whereas 30 minute sessions were used in the cocaine study. We needed the longer duration in the food experiment because otherwise the animals would not have enough time to eat a sufficient quantity of food to maintain body mass above 80% free feeding weight. The shorter time (rather than 90 min) was used in the cocaine experiment because cocaine concentrations in the brain wane after approximately 30 min [49,50]. Moreover, 30 min or shorter sessions are typically used for drug conditioning and are known to increase the magnitude of CPP for ethanol in mice [51]. On the other hand, longer trial durations from 15 to 60 min produced greater CPP with cocaine in DBA/2 J mice [24].

Another difference was that in the food experiment animals were food restricted whereas in the cocaine experiment they were not. Cocaine withdrawal *versus* food restriction are part of the differential nature of the rewards and are difficult to separate. This problem was diminished by the experiment design which contrasted conditioned *responses* between reward types. These responses are independent of food restriction (or cocaine withdrawal) to the extent that they represent a difference (change) between two groups that experienced identical food restrictions (or exposure to cocaine). The only difference between groups within an experiment for the brain activation test was whether they were placed in an environment paired with reward or not. The duration of the test was the same (90 min) for all. Nonetheless, it is possible that a different pattern of brain activation would have been elicited from a cocaine-paired environment if those animals were also food restricted (but fed outside the context of the conditioning boxes). The literature would suggest that under food restriction, effects of cocaine conditioning would, if anything, be stronger than what was observed here for frontal cortical regions, and not likely characterized by paraventricular hypothalamic nucleus [52,53]. Likewise, it is possible that the food-paired environment would have elicited a different result if the animals were also withdrawing from cocaine during the test, but probably not likely to deviate from the general pattern of strong activation in the paraventricular hypothalamic nucleus (Fig. 8) as opposed to frontal cortical regions (see Tables 1 and 2) for food as compared to cocaine.

Few studies have attempted to compare conditioned responses to natural *versus* drug rewards [10,31]. The present study provides useful information to be followed up in future studies. For example, we plan to repeat the cocaine experiment using 90 min trials in animals that are also food restricted to 85% free feeding weight. We also will repeat a version of the food experiment in animals withdrawing from cocaine, to empirically test the assumption that the general patterns of conditioned cocaine and food responses reported herein remain qualitatively similar across these different environmental conditions.

4.5. Summary

Here we identify neuroanatomical specificity in conditioned responses to cocaine *versus* familiar food (under food restriction) in mice. The paraventricular hypothalamic nucleus was a key locus for the difference. In this region food- but not cocaine-paired cues increased Fos activation. We speculate that classical conditioning for food involves brain regions that integrate information from the periphery regarding metabolic needs with conditioned stimuli representing food availability whereas for cocaine, no such integration is possible because no signals arrive from the periphery regarding "needs" for cocaine. We also discovered that reduced basal Fos levels in the prefrontal cortex are associated with increased motivation for cocaine as measured by CPP. We interpreted this result as a reflection of the well established theory that reduced activation of prefrontal cortical inhibitory control causes relatively greater motivation for goal directed behavior [34].

Acknowledgements

We thank Nataliya Ryzhenko, Weronika Brzezinska and Cannie Yu Sze-To for their help with data collection. Special thanks to Lisa Foster, Dack Shearer, Reid McClure, Connie Haun, Donnell Parker, and Holly Fairfield for their excellent animal care.

References

[1] Self DW. Neural substrates of drug craving and relapse in drug addiction. Ann Med 1998;30:379–89.
[2] Childress AR, Mozley PD, McElgin W, Fitzgerald J, Reivich M, O'Brien CP. Limbic activation during cue-induced cocaine craving. Am J Psychiatry 1999;156:11–8.
[3] Wise RA. Brain reward circuitry: insights from unsensed incentives. Neuron 2002;36:229–40.
[4] See RE, Fuchs RA, Ledford CC, McLaughlin J. Drug addiction, relapse, and the amygdala. Ann N Y Acad Sci 2003;985:294–307.
[5] Koob G, Kreek MJ. Stress, dysregulation of drug reward pathways, and the transition to drug dependence. Am J Psychiatry 2007;164:1149–59.
[6] Glickman SE, Schiff BB. A biological theory of reinforcement. Psychol Rev 1967;74:81–109.
[7] Salamone JD. The involvement of nucleus accumbens dopamine in appetitive and aversive motivation. Behav Brain Res 1994;61:117–33.
[8] Kelley AE, Berridge KC. The neuroscience of natural rewards: relevance to addictive drugs. J Neurosci 2002;22:3306–11.
[9] Kalivas PW, O'Brien C. Drug addiction as a pathology of staged neuroplasticity. Neuropsychopharmacology 2008;33:166–80.
[10] Schroeder BE, Binzak JM, Kelley AE. A common profile of prefrontal cortical activation following exposure to nicotine- or chocolate-associated contextual cues. Neuroscience 2001;105:535–45.
[11] Schroeder BE, Holahan MR, Landry CF, Kelley AE. Morphine-associated environmental cues elicit conditioned gene expression. Synapse 2000;37:146–58.
[12] Chiu R, Boyle WJ, Meek J, Smeal T, Hunter T, Karin M. The c-Fos protein interacts with c-Jun/AP-1 to stimulate transcription of AP-1 responsive genes. Cell 1988;54:541–52.
[13] Clayton DF. The genomic action potential. Neurobiol Learn Mem 2000;74:185–216.
[14] Zangenehpour S, Chaudhuri A. Differential induction and decay curves of c-fos and zif268 revealed through dual activity maps. Brain Res Mol Brain Res 2002;109:221–5.
[15] Herdegen T, Leah JD. Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. Brain Res Brain Res Rev 1998;28:370–490.
[16] Nestler EJ, Barrot M, Self DW. Delta FosB: a sustained molecular switch for addiction. Proc Natl Acad Sci U S A 2001;98:11042–6.
[17] Rhodes JS, Ryabinin AE, Crabbe JC. Patterns of brain activation associated with contextual conditioning to methamphetamine in mice. Behav Neurosci 2005;119:759–71.
[18] Topple AN, Hunt GE, McGregor IS. Possible neural substrates of beer-craving in rats. Neurosci Lett 1998;252:99–102.
[19] Ciccocioppo R, Sanna PP, Weiss F. Cocaine-predictive stimulus induces drug-seeking behavior and neural activation in limbic brain regions after multiple months of abstinence: reversal by D(1) antagonists. Proc Natl Acad Sci U S A 2001;98:1976–81.
[20] Brown EE, Robertson GS, Fibiger HC. Evidence for conditional neuronal activation following exposure to a cocaine-paired environment: role of forebrain limbic structures. J Neurosci 1992;12:4112–21.
[21] Franklin TR, Druhan JP. Expression of Fos-related antigens in the nucleus accumbens and associated regions following exposure to a cocaine-paired environment. Eur J Neurosci 2000;12:2097–106.
[22] Neisewander JL, Baker DA, Fuchs RA, Tran-Nguyen LT, Palmer A, Marshall JF. Fos protein expression and cocaine-seeking behavior in rats after exposure to a cocaine self-administration environment. J Neurosci 2000;20:798–805.
[23] Cunningham CL, Ferree NK, Howard MA. Apparatus bias and place conditioning with ethanol in mice. Psychopharmacology (Berl) 2003;170:409–22.
[24] Cunningham CL, Dickinson SD, Grahame NJ, Okorn DM, McMullin CS. Genetic differences in cocaine-induced conditioned place preference in mice depend on conditioning trial duration. Psychopharmacology (Berl) 1999;146:73–80.
[25] Brabant C, Quertemont E, Tirelli E. Influence of the dose and the number of drug-context pairings on the magnitude and the long-lasting retention of cocaine-induced conditioned place preference in C57BL/6 J mice. Psychopharmacology (Berl) 2005;180:33–40.
[26] Orsini C, Bonito-Oliva A, Conversi D, Cabib S. Susceptibility to conditioned place preference induced by addictive drugs in mice of the C57BL/6 and DBA/2 inbred strains. Psychopharmacology (Berl) 2005;181: 327–36.
[27] Hasen NS, Gammie SC. Maternal aggression: new insights from Egr-1. Brain Res 2006;1108:147–56.
[28] Paxinos G, Franklin K. The Mouse Brain Atlas in Sterotaxic Coordinates. second edn. San Diego: Academic Press; 2001.
[29] Rhodes JS, Garland Jr T, Gammie SC. Patterns of brain activity associated with variation in voluntary wheel-running behavior. Behav Neurosci 2003;117:1243–56.
[30] Storey J. A direct approach to false discovery rates. J R Stat Soc 2002;64:479–98.
[31] Mattson BJ, Morrell JI. Preference for cocaine- versus pup-associated cues differentially activates neurons expressing either Fos or cocaine- and amphetamine-regulated transcript in lactating, maternal rodents. Neuroscience 2005;135:315–28.
[32] Williams G, Bing C, Cai XJ, Harrold JA, King PJ, Liu XH. The hypothalamus and the control of energy homeostasis: different circuits, different purposes. Physiol Behav 2001;74:683–701.
[33] Paredes RG. Medial preoptic area/anterior hypothalamus and sexual motivation. Scand J Psychol 2003;44:203–12.
[34] Goldstein RZ, Volkow ND. Drug addiction and its underlying neurobiological basis: neuroimaging evidence for the involvement of the frontal cortex. Am J Psychiatry 2002;159:1642–52.
[35] Robbins TW, Everitt BJ. Limbic-striatal memory systems and drug addiction. Neurobiol Learn Mem 2002;78:625–36.
[36] Miller CA, Marshall JF. Altered Fos expression in neural pathways underlying cue-elicited drug seeking in the rat. Eur J Neurosci 2005;21:1385–93.
[37] Le Foll B, Frances H, Diaz J, Schwartz JC, Sokoloff P. Role of the dopamine D3 receptor in reactivity to cocaine-associated cues in mice. Eur J Neurosci 2002;15:2016–26.

[38] Mead AN, Vasilaki A, Spyraki C, Duka T, Stephens DN. AMPA-receptor involvement in c-fos expression in the medial prefrontal cortex and amygdala dissociates neural substrates of conditioned activity and conditioned reward. Eur J Neurosci 1999;11:4089–98.
[39] Fischer H, Andersson JL, Furmark T, Fredrikson M. Fear conditioning and brain activity: a positron emission tomography study in humans. Behav Neurosci 2000;114:671–80.
[40] Beck CH, Fibiger HC. Conditioned fear-induced changes in behavior and in the expression of the immediate early gene c-fos: with and without diazepam pretreatment. J Neurosci 1995;15:709–20.
[41] Garavan H, Pankiewicz J, Bloom A, Cho JK, Sperry L, Ross TJ, Salmeron BJ, Risinger R, Kelley D, Stein EA. Cue-induced cocaine craving: neuroanatomical specificity for drug users and drug stimuli. Am J Psychiatry 2000;157:1789–98.
[42] Carr KD, Park TH, Zhang Y, Stone EA. Neuroanatomical patterns of Fos-like immunoreactivity induced by naltrexone in food-restricted and ad libitum fed rats. Brain Res 1998;779:26–32.
[43] Partridge B, Schenk S. Context-independent sensitization to the locomotor-activating effects of cocaine. Pharmacol Biochem Behav 1999;63: 543–8.
[44] Michel A, Tambour S, Tirelli E. The magnitude and the extinction duration of the cocaine-induced conditioned locomotion-activated response are related to the number of cocaine injections paired with the testing context in C57BL/6 J mice. Behav Brain Res 2003;145:113–23.
[45] Brown SM, Manuck SB, Flory JD, Hariri AR. Neural basis of individual differences in impulsivity: contributions of corticolimbic circuits for behavioral arousal and control. Emotion 2006;6:239–45.
[46] Bardo MT, Bevins RA. Conditioned place preference: what does it add to our preclinical understanding of drug reward? Psychopharmacology (Berl) 2000;153:31–43.
[47] Young AM, Moran PM, Joseph MH. The role of dopamine in conditioning and latent inhibition: what, when, where and how? Neurosci Biobehav Rev 2005;29:963–76.
[48] Schiltz CA, Bremer QZ, Landry CF, Kelley AE. Food-associated cues alter forebrain functional connectivity as assessed with immediate early gene and proenkephalin expression. BMC Biol 2007;5:16.
[49] Azar MR, Acar N, Erwin VG, Barbato GF, Morse AC, Heist CL, Jones BC. Distribution and clearance of cocaine in brain is influenced by genetics. Pharmacol Biochem Behav 1998;59:637–40.
[50] Benuck M, Lajtha A, Reith ME. Pharmacokinetics of systemically administered cocaine and locomotor stimulation in mice. J Pharmacol Exp Ther 1987;243:144–9.
[51] Cunningham CL, Niehus DR, Malott DH, Prather LK. Genetic differences in the rewarding and activating effects of morphine and ethanol. Psychopharmacology (Berl) 1992;107:385–93.
[52] Stuber GD, Evans SB, Higgins MS, Pu Y, Figlewicz DP. Food restriction modulates amphetamine-conditioned place preference and nucleus accumbens dopamine release in the rat. Synapse 2002;46:83–90.
[53] Bell SM, Stewart RB, Thompson SC, Meisch RA. Food-deprivation increases cocaine-induced conditioned place preference and locomotor activity in rats. Psychopharmacology (Berl) 1997;131:1–8.