HHS Public Access

Author manuscript

Brain Struct Funct. Author manuscript; available in PMC 2017 June 01.

Published in final edited form as:

Brain Struct Funct. 2016 June ; 221(5): 2817–2830. doi:10.1007/s00429-015-1074-z.

Effects of Acute or Chronic Environmental Enrichment on Regional Fos Protein Expression following Sucrose Cue-reactivity testing in Rats

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Abstract

Exposure to environmental enrichment (EE) reduces sucrose seeking by rats with a history of sucrose self-administration. The present experiment examined whether acute or chronic EE also reduces brain Fos levels, a protein marker indicative of neuronal activation. Fos levels were also examined after either 1 or 30 days of forced abstinence to examine whether Fos levels vary with the incubation of sucrose craving. Fos expression was examined in 18 regions and was identified in brain slices using immunohistochemistry. Fos levels were higher in most regions after 30 d of forced abstinence and were decreased in most regions by either acute or chronic EE. Eleven regions had some statistically significant effect and/or interaction of EE or incubation on Fos; the most salient of these are listed here. In the prelimbic cortex there was an incubation of Fos and EE reduced Fos at both forced abstinence time points. In contrast, in the orbitofrontal cortex there was no Fos incubation but EE reduced Fos at both forced abstinence time points. An interaction of EE and incubation was observed in the anterior cingulate cortex and nucleus accumbens core and shell where Fos incubated but EE only decreased Fos at the Day 30 forced abstinence time point. In contrast, in the dorsolateral striatum Fos incubated, but EE robustly decreased Fos expression at both forced abstinence time points. These differential expression patterns provide rationale for more detailed, site-specific molecular functional studies in how they relate to the ability of EE to reduce sucrose seeking.

Keywords

addiction; corticosterone; craving; Fos; incubation; relapse; sucrose

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Conflict of Interest

The authors declare that they have no conflict of interest. The manuscript does not contain clinical studies or patient data.

Introduction

Relapse (Wadden 1993) is a feature of persistent pathological behaviors such as drug addiction (O'Brien et al. 1988;Mendelson and Mello 1996) and is a feature of disordered eating (e.g. weight recidivism; Laddu et al. 2011). Relapse can be triggered by exposure to stimuli previously associated with drug taking (Childress et al. 1999; Carter and Tiffany 1999; Epstein et al. 2009). Likewise, food-predictive stimuli can lead to increased food craving and intake (Jansen et al. 2003; Sobik et al. 2005).

The neurobiology of relapse has been studied extensively using a rodent model where rats or mice respond for cues previously associated with drug or food availability (Shalev et al. 2002). A handful of recent studies have observed that cue-reactivity, a model of "relapse" in animal studies, can be substantially reduced following exposure of rodents to environmental enrichment (EE). Studies have shown this to be the case for rodents with a history of either drug (Chauvet et al. 2009; Thiel et al. 2009) or food self-administration (Grimm et al. 2008; Grimm et al. 2013). Our most recent finding was that overnight (acute) EE was just as, if not more, effective at reducing sucrose seeking compared to one month of EE (Grimm et al. 2013). Furthermore, we found that acute EE also reduced sucrose intake. These findings demonstrate a non-pharmacological means to reduce relapse.

Basic research studies on the neurobiology of relapse have identified several relapse-related structures fitting into functional pathways sub-serving habit, motivation, memory, and emotion (Bossert et al. 2013). Initial activation "mapping" of these, and other, brain regions of rats in a relapse condition following EE have been published for rats with a history of cocaine self-administration (Thiel et al. 2010). These studies utilized Fos, the protein produced following expression of the c-fos oncogene, as a marker of activation as Fos is known to be produced following neuronal activation (Herrera and Robertson 1996). A mapping study was also conducted with mice demonstrating a cocaine conditioned place preference (CPP) (Chauvet et al. 2011). In both studies, EE markedly reduced relapse (cocaine-seeking, rat study; cocaine CPP, mouse study) accompanied by decreased Fos expression in several corticolimbic brain regions.

The aim of the present study was to map regional brain activation during relapse in rats with a history of food (sucrose) self-administration. Sucrose was chosen as the reinforcer to both extend the results of these previous studies with cocaine and gain a better understanding of the neurobiology of food-directed relapse. Given the severity of the obesity epidemic (CDC 2014; WHO 2015), its associated negative health outcomes (Ogden et al. 2007), and also other negative outcomes related to excess food consumption (e.g. sugar; Johnson et al. 2009), further evaluation of factors that maintain feeding behaviors is warranted. In addition we included both acute (overnight) and chronic (one month) EE conditions to examine how the extent of EE and/or length of abstinence from self-administration affects neuronal activity. Mapping was done by quantitating levels of Fos protein in multiple brain regions.

Materials and Methods

Subjects

Sixty-five male Long-Evans rats (approximately 3.5 months old at start of study), bred in the Western Washington University vivarium were used for this experiment. Experimental group sizes (5 experimental conditions described below) were n=13 per group. Prior to any enrichment treatment, rats were housed individually in Micro-Isolator chambers (20 × 32 × 20 cm; Lab Products, Inc., Seaford, DE) under a 12-h reverse day/night cycle with lights off at 0700 h. All Training and Testing occurred between 0900–1100 h. Food (Purina Mills Inc. Mazuri Rodent Pellets, Saint Louis, MO) and water were available *ad libitum* throughout the study except for pre-training water deprivation, noted below. Body weights were recorded every Monday, Wednesday, and Friday for the duration of the study. All procedures followed the guidelines outlined in the "PHS Policy on Humane Care and Use of Laboratory Animals" (PHS 2002) and were approved by the Western Washington University Institutional Animal Care and Use Committee.

Apparatus

Operant procedures took place in operant conditioning chambers (30 × 20 × 24 cm; Med Associates, St. Albans, VT) equipped with one retractable lever to the left side of the tray where sucrose solution was dispensed. A stationary lever was located on the opposite wall. Each chamber was also equipped with four infrared photobeams (Med Associates) that criss-crossed the chamber. Beam-breaks were recorded in Training and Testing sessions. The operant conditioning chambers included a red houselight on the wall opposite the retractable lever. Above the retractable lever was a white stimulus light and a sound generator (2 kHz, 15 dB over ambient noise). The operant conditioning chambers were enclosed in sound-attenuating chambers equipped with fans to provide air flow and white noise.

Behavioral procedures

Training—For each Training session rats were placed into operant conditioning chambers and those doors and the sound-attenuating chamber enclosure doors were closed. The session began with illumination of the house light and insertion of the retractable lever. Rats underwent 10 daily 2-h sessions wherein they learned to press the retractable lever for a 0.2 mL delivery of 10 % sucrose solution. These "active" lever presses were reinforced under a fixed-ratio 1 schedule with a 40 s "time-out". Specifically, an active lever press was accompanied with a 5 s combined presentation of the white stimulus light and the tone. For this 5 s and the following 35 s, active lever responses were not reinforced but were recorded. Presses on the stationary ("inactive") lever elicited no response and were recorded as a control for discriminated responding and motor activity.

Forced abstinence—Following the tenth Training session, rats were randomly assigned to a Treatment condition consisting of a cross between duration of forced abstinence and type of housing condition. The forced abstinence period was either from the end of the tenth Training session to a Testing session the next morning (~22 h; "Day 1") or to a Testing session 30 days later ("Day 30").

Environmental enrichment—EE consisted of a mixture of housing and social enrichment. The EE housing was a large, 4-level wire-mesh environment (91 × 51 × 102 cm; Quality Cage Company, Portland, OR) with novel toys replenished each M,W, F. Three rats were co-housed in EE.

EE was provided as either acute (EE Acute) or chronic (EE Chronic). EE Acute groups were created so that rats experienced EE from the end of the tenth day Training session or the 29th day of forced abstinence until Testing the next morning (~17 h). These were the Day 1 and Day 30 EE Acute conditions. The one EE Chronic condition was exposure to EE from the end of the tenth day Training session until Testing on Day 30 of forced abstinence. All Control (CON) conditions were simply allowing rats to remain single-housed.

Testing—Testing was identical to Training but sucrose was not available and the session duration was 1 h. The Testing session was shorter to better match optimal Fos expression indicative of neuronal activation near the start of the 1-h session (Hope et al. 1994).

Molecular procedures

Throughout all molecular procedures, assays and analyses were conducted blind to Treatment condition.

Corticosterone ELISA—Immediately following Testing, each subject was deeply anesthetized with pentobarbital (Soccumb; Butler-Schein, Dublin, Ohio) and prepared for trans-cardial perfusion. Prior to perfusion, 1 mL of blood was drawn from the left ventricle of the heart into a heparinized micro-tube (Eppendorf, Hauppauge, NY). This sample was then spun at 2,000 g for 15 min at 4° C. The supernatant was collected into a non-heparinized micro-tube and frozen at −70° C until assayed. Samples were assayed in duplicates using the corticosterone enzyme-linked immunosorbant assay (ELISA) kit from Enzo Life Sciences (Farmingdale, NY) (Catalog No. ADI-900–097). Duplicate values were averaged and are reported as ng/mL.

Fos immunohistochemistry—Following the blood draw (above) 1000 USP units heparin in 1 mL (Baxter, Deerfield, IL) was injected into the left ventricle of heart. A subject was then trans-cardially perfused with 200 mL ice-cold PBS followed with 200 mL ice-cold 4 % paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA). The brain was extracted and placed into 4 % paraformaldehyde for ~24 h, then 20% sucrose solution (MP Biomedicals, Santa Ana, CA) for 24 h, and finally into 30% sucrose for 24 h or until the brain sank, all at 4° C. The brain was then frozen in dry ice powder, wrapped in foil, and stored at −70° C until sectioned. Forty μm sections were taken in three serial sets through 5 brain regions (A/P +3.2, +1.6, −0.26, −2.56, −5.6; Paxinos and Watson, 2007) using a Leica (Buffalo Grove, IL) CM1950 cryostat. Slices were placed into pH 7.4 cryoprotectant consisting of 20 % glycerol and 2 % DMSO (both from Fisher, Pittsburgh, PA) in 6-well plates (Corning, Tewksbury, MA) and stored at −70° C until assayed. Each plate contained a random assortment of subjects representing the 5 treatment conditions.

For the Fos immunohistochemistry assay, a plate was allowed to thaw and then slices were run through the following steps with the aid of Netwells (500 μm mesh; Corning). Briefly,

the series consisted of 13 steps: 1.) PBS wash, 2.) quench endogenous peroxidase with 0.3 % H_2O_2, 3.) PBS wash, 4.) block 1 h with 3 % normal goat serum (NGS) (Vector Laboratories, Burlingame, CA), 5.) incubate 24 h with 3 % NGS and 1:6000 Fos anti-rabbit polyclonal primary antibody (sc-52; lot F1212, Santa Cruz Biotechnology, Dallas, TX), 6.) PBS wash, 7.) incubate 2 h with 1 % NGS and 1:600 biotinylated anti-rabbit H+L secondary antibody (BA-1000, Vector Laboratories), 8.) PBS wash, 9.) incubate 2 h with avidin-biotin complex (PK-6100, Vector Laboratories), 10.) PBS wash, 11.) incubate with diaminobenzidine ~1.5 min (Sigma, St. Louis, MO), 12.) PBS wash, and 13.) mount on Superfrost (Fisher) gelatinized microscope slides. Slices were subsequently dehydrated through a series of alcohols ending in Safeclear (Fisher). Slides were then cover slipped and sealed using Permount (Fisher). Two sets of slices were assayed for each subject.

Regions of interest (ROIs) (Figure 1) were later imaged using an Olympus BX51 microscope running Neurolucida 9 imaging software (MBF Bioscience, Williston, VT). Images were captured at 10 × magnification. Regions of interest in these images were then quantitated for Fos-immunoreactive (Fos IR) cells using Image J (NIH). Fos IR was obvious in tissue as brown-stained ovoids (Figure 2). Briefly, parameters used in Image J included background subtraction (50.0 pixels, light background), maximum entropy threshold, and particle detection of 50–150 based on a preliminary assessment of Fos IR in 14 samples. Each ROI was sampled with a 250 μm-sided box. Counts of Fos IR cells were made bilaterally for each slice and 2 serial slices were examined for each subject. These 4 (2 × 2) counts were then averaged and multiplied by 16 to provide a value representing Fos IR cells/mm^2.

Statistical analyses

Data were organized by day of Testing (Day; 2 levels: Day 1 or Day 30) and housing condition (HOUSING; 3 levels: CON, EE Acute, EE Chronic). Active lever presses, sucrose deliveries during Training (or cue self-presentations during Testing), inactive lever presses, and photobeam breaks were analyzed separately. Training and Testing data were also analyzed separately. Pre-training body weight and Training data were analyzed to determine if groups differed prior to assignment to treatment conditions. Body weights were compared between Day 30 Testing groups to determine if chronic EE affected body weight.

Behavioral and molecular data (corticosterone levels (ng/mL) and Fos counts (Fos IR cells/mm^2)) were analyzed using two-way ANOVA using the Type IV sums of squares model option to account for the “missing” cell due to not having a Day 1 EE Chronic condition (Shaw and Mitchell-Olds 1993). Statistically significant main effects were followed by Tukey’s HSD post-hoc tests. The criterion for statistical significance was $P < 0.05$. Following a significant interaction, five post-hoc comparison t-tests were made between each CON and groups tested at that time point in forced abstinence (3 tests) and between CON and EE Acute groups tested at the two forced abstinence time points (2 tests). Family-wise error for these tests was reduced by using a Šidák correction; this resulted in the criterion for statistical significance to be $P < 0.01$. To evaluate relationships between some of the measures, Pearson’s r correlations were calculated. The criterion for statistical significance for these calculations was $P < 0.05$. For brevity, in most instances only statistics

for significant main effects and interactions of ANOVA are noted in the text. Means ± standard error of the mean (SEM) are indicated in the text and on the Figures. IBM SPSS Statistics 19 was used for all statistical calculations. All treatment groups are indicated on each Figure regardless of whether a significant interaction was identified. This was done to allow the reader to inspect each group mean and SEM. For a Figure with only a main effect (or 2 main effects), post-hoc comparison results are indicated above the Figure. For a Figure with an interaction, post-hoc comparison results are indicated on the Figure with symbols defined in the Figure caption.

Results

Behavioral procedures

Training—Average body weights (465.0 ± 6.1 g) did not differ between groups prior to the start of the study. There were no significant differences between groups in Training behaviors. Averages for all groups across days 7–10 of Training were active lever responses (129.3 ± 6.6), number of sucrose deliveries (77.4 ± 2.9), inactive lever responses (5.3 ± 1.0), and photobeam breaks (2009.8 ± 100.8).

Testing—Active lever. There were statistically significant effects of DAY $F(1,60) = 23.6$, and HOUSING $F(2,60) = 34.9$, P's < 0.05; the interaction was not statistically significant. Post-hoc tests on HOUSING established significant differences between all three conditions (Figure 3). Number of cue self-presentations. As with active lever responding, there were statistically significant effects of DAY $F(1,60) = 29.5$ and HOUSING $F(2,60) = 42.7$, P's < 0.05; the interaction was not statistically significant. Post-hoc tests on HOUSING established significant differences between all three conditions (Figure 3). Inactive lever. There was a significant effect of HOUSING $F(2,60) = 9.4$, $P < 0.05$; the effect of DAY and the interaction term were not statistically significant. Post-hoc tests on HOUSING established significant differences only between the CON and EE Acute condition (Figure 3). Locomotion. As with active lever responding and cue self-presentations there were statistically significant effects of DAY $F(1,60) = 15.8$ and HOUSING $F(2,60) = 40.9$, P's < 0.05; the interaction was not statistically significant. Post-hoc tests on HOUSING established significant differences between all three conditions (Figure 3). Body weights just prior to Day 30 Testing did not differ between Testing groups. The average weight was 496.4 ± 9.7 g

Molecular procedures

Corticosterone ELISA—Plasma corticosterone levels did not differ between conditions. The average plasma level of corticosterone was 119.0 ± 5.9 ng/mL.

Fos immunohistochemistry—Results of ANOVAs for Fos IR cell counts are indicated in Table 1. Eleven of the 18 regions examined had a statistically significant effect(s) and/or a statistically significant interaction. The means ± SEMs for each of the five experimental groups for each of these regions are indicated in Figure 4–Figure 7. Results of post-hoc tests are also indicated on these Figures. Means ± SEMs for the seven regions without between-group statistically significant differences in Fos IR cell counts are indicated in Table 2.

Group sizes are indicated in the Table notes. Ventral tegmental area group (VTA) sizes are lower as some slices were cut too rostral to provide accurate representations of the VTA.

Correlational analyses

Exploratory analyses were made to examine 1.) the correlation between active lever responding and Fos IR, 2.) the correlation between active lever responding and plasma corticosterone level, and 3.) the correlation between locomotor activity and either active lever responding or corticosterone level. Correlations provide a means to determine if individual differences in a measure predict individual differences in another measure—a question that is not necessarily answered by comparing ANOVA results of two separate measures from the same individuals. That is, correlational analyses provide a means to determine whether overall group differences in a measure (e.g. active lever responses would be assumed to be less in EE vs. CON rats) necessarily account for overall group differences in another measure (e.g. if Fos levels are also lower in EE vs. CON rats). Correlations were first calculated using the data from all of the subjects and then split by the main factors. All r values reported below were significant at least at $P < 0.05$ and are indicated in Table 3. Locomotor activity was found to not be significantly correlated with either active lever responding or corticosterone levels.

Discussion

Testing behavior

Cue-reactivity was highest in the CON conditions with Day 30 responding 178 % greater than Day 1 CON (incubation of craving). Cue-reactivity was below Day 1 CON in all three EE conditions indicating that either acute or chronic EE effectively reduced sucrose cue-reactivity and, arguably, attenuated an incubation of responding. These findings replicate previous results from our laboratory (Grimm et al. 2008; Grimm et al. 2013) and also are similar to previous findings with rats that had a history of cocaine self-administration (Chauvet et al. 2009; Thiel et al. 2009;Thiel et al. 2010;Thiel et al. 2011; Chauvet et al. 2012).

Fos IR

There were three “unique” patterns of Fos IR. First, Fos IR in the orbitofrontal cortex (OFC) was substantially reduced by EE, but there was no incubation of Fos IR (Figure 4). A second pattern, seen in the pre-limbic (PLC) and somatosensory cortex (SSC), was an overall effect of EE reducing Fos IR, but with incubation still apparent (Figure 4 and Figure 6). These first two patterns roughly approximate EE-specific (OFC) or incubation-specific (PLC, SSC) Fos IR patterns. In contrast, the third pattern was an interaction of EE and incubation. Observed in the anterior cingulate cortex (ACC), and nucleus accumbens (NA) core and shell, Fos IR incubated but EE was only effective at reducing Fos IR after 30 days of forced abstinence (Figure 4 and Figure 5). This pattern in the ventral striatum (NA sub-regions) was strikingly different than dorsal striatum (DLS) where Fos IR incubated in the CON rats, but was nearly abolished by EE at both forced-abstinence time points (Figure 5). Other regions with significant effects of EE and/or incubation on Fos IR included the basolateral amygdala (BLA), central nucleus of the amygdala (CeA), CA1 of hippocampus, and infralimbic cortex

(ILC). There was a dissociation between the amygdalar nuclei. Although both had Fos IR reduced by chronic EE, incubation was only apparent in the CeA and, interestingly, there was a trend for Day 1 acute EE to *increase* Fos IR. This same pattern was more robust (and statistically significant) in the ILC.

It is difficult to find direct comparisons of these results in the literature as there are only the two previously-discussed papers assessing Fos IR in rodents that were just tested for seeking behavior following EE (Chauvet et al. 2011; Thiel et al. 2011). As noted, these studies were of animals with a history of cocaine exposure and chronic EE was of primary consideration. Despite these differences (including the fact that Chauvet et al. was a study of CPP rather than operant behavior) there are several consistencies across the studies in terms of regions where EE exposure was associated with less Fos IR. These include cortical regions (ACC, OFC, PLC), striatal (NA core and shell, and DLS), amygdalar (BLA, CeA) regions, and CA1 of hippocampus. We did not find a significant effect of EE in the VTA or BNST as was found in the other studies, but the data (Table 2) indicate a general pattern for both regions where Day 30 CON Fos IR > Day 30 Chronic EE Fos IR. The present results fit with the interpretation of Thiel et al. (2010) that the pattern of Fos IR attenuation by EE generally overlaps with brain regions involved in identifying and then acting to guide behavior to incentive stimuli.

Of particular note regarding EE or incubation affecting neuronal systems involved in incentive-guided seeking behavior were the effects in cortical regions (PLC, ILC, OFC, ACC), striatum (NA and DLS), and amygdala (BLA, CeA). Chronic EE consistently resulted in decreased Fos IR in these regions. As previous studies have indicated functional roles for these regions in primary and conditioned reward-directed responding (Bossert et al. 2013), we hypothesize a widespread diminished response to the sucrose context at several levels including reward valuation (BLA, OFC; McDannald et al. 2014), arousal (CeA; Koob 2009), motivation/habit (striatum; Belin et al. 2013), executive attention (PLC, ILC; Kesner and Churchwell 2011) and cognitive control (ACC; Shenhav et al. 2013) following EE.

The few differences in Fos IR for acute EE (ILC increase) or acute Day 1 EE (NA subregions, ACC decrease) may mean that processing of cues following acute EE is especially exaggerated (ILC) or not susceptible to being attenuated by EE only on Day 1 of forced abstinence (NA subregions, ACC). This latter finding is difficult to interpret, but one hypothesis is that NA and ACC regions participate more in cue-reactivity after 30 days of forced abstinence. This is perhaps relevant to the persistence of motivated cue reactivity (Volkow et al. 2011) vs. responding out of habit (Harkness et al. 2010; Belin et al. 2013). The ILC findings (acute EE increased Fos IR) are intriguing, not only because Fos IR was decreased or unaltered following acute EE in most of the other brain regions, but also because of recent findings suggesting an “anti-relapse” role for the ILC (LaLumiere et al. 2012; Ma et al. 2014). Our results provide a first hint of this in an EE paradigm where one interpretation is that acute EE activates the ILC, setting a brake on relapse behavior.

As noted above, and indicated in Table 2 and Figure 1; Figure 4–Figure 7, Fos IR in most cases followed the magnitude of active lever responding. Previous studies (e.g. Thiel et al. 2010; Zhou et al. 2013; Velez-Hernandez et al. 2014) have reported that ANOVA results do

not always correspond with results when individual differences are examined using correlations. Therefore we calculated correlations between individual Fos IR for the 18 brain regions and individual active lever responses. Statistically significant Pearson *r* correlations are indicated in Table 3 for data grouped either as all subjects, or for the levels of the 2 independent variables. Similar to the previous findings noted above, there were fewer regions with statistically significant correlations between Fos IR and active lever responding than regions with statistically significant ANOVA effects of EE and/or forced abstinence on Fos IR. For all subjects, positive correlations were noted in 7 regions including primarily regions associated with salience tracking and reward valuation (PLC, NA, OFC) but also inputs to these systems (CA1, SSC). Most of these regions have been reported to have correlations between cue-reactivity and Fos IR in previous studies (Thiel et al. 2010; Zhou et al. 2013). We did not find all previously observed correlations, however. Notably, we did not observe correlations in the BLA or VTA. One reason for this discrepancy could be due to the reinforcer (sucrose vs. cocaine), the nature of the testing conditions (discrete cue vs. context + discrete cue), between-study variation in anatomical specificity and/or immunohistochemical technique, and the impact of including acute and chronic EE experienced subjects in the data set. The positive correlation that was identified in the most independent variable levels was the OFC. This was apparent in all but the two EE (acute, chronic) conditions. For both NA core and shell, the positive correlation was also apparent overall and in the CON and Day 30-grouped data and not in EE groupings. But different from OFC, the Day 1 grouping did not have a significant correlation. Finally, the only statistically significant negative correlations were identified for the EE data alone in the PLC and BNST. The negative correlations both were only observed in the Day 30 forced abstinence condition indicating that the most responding by rats after 30 days of forced abstinence was correlated with the least amount of Fos IR.

Determining functional roles in sucrose seeking for the regions found to have Fos IR differ according to experimental condition will require functional studies. Caution is also required in interpreting a lack of correlation between active lever responding and Fos IR in the several regions where group effects were observed with ANOVA. Active lever responding likely only reflects one aspect of “craving” (seeking). That is, it could be that for an individual subject a unique pattern of activation results in their level of seeking behavior, perhaps reflecting relative contributions of contextual cues, discrete cues, and state of arousal, to that particular subject’s behavior. In addition, our design contains a confound in that the number of Testing cue presentations is not constant across subjects due to the fact that our model depends on contingent cue self-presentations (Grimm et al. 2000). Therefore, our preferred interpretation of our Fos IR data is that Fos levels reflect response of the brain to the sucrose-predictive environment including responding for cues. In a sense, the act of responding creates a complete CS + (Bindra 1972). An alternate hypothesis is that absolute number of cue presentations explains Fos levels. As the correlational results show, active lever responding predicted Fos IR in 7 of the 11 regions where significant ANOVA effects were found. Correlations were similar between the number of cue self-presentations and Fos IR (data not shown). Therefore a conservative interpretation of the data is that for some regions, absolute number of cue self-presentations is the primary predictor, or even driver, of

Fos IR. This still leaves open the question of what underlying state drives cue-reactivity behavior. Our results are equivocal on this point.

Finally, of note in the present study was that while group locomotion levels followed along with active lever responding (Figure 1), locomotor responding did not correlate with active lever responding or Fos IR. This lack of effect is of note as locomotor behavior is typically an indication of appetitive, approach behavior (Wise 2004). Clearly there are subtleties in how these behaviors (active lever responding, locomotor activity) relate to each other and to neuronal activation measured as Fos IR. This will require further study.

EE mechanisms

As noted by Solinas et al. (2010), stress increases unconditioned, conditioned, and reinforcing effects of drugs so it is reasonable to hypothesize that EE reduces these as a result of a decrease in stress. Specifically, Solinas et al. suggested that EE may decrease the level of stress of an individual and that this decrease in arousal diminishes cue-reactivity, perhaps due to less negative-reinforcement-driven responding. This hypothesis was derived from studying animals with a history of psychostimulant self-administration, and perhaps is appropriate due to the distinct effects of a drug such as cocaine versus sucrose on stress neurophysiology. In the present study we did not find a difference in plasma corticosterone levels between the five treatment groups. Average plasma levels were 119.0 ± 5.9 ng/mL, a value above what has been reported for non-stressed Long-Evans male rats in some (Konkle et al. 2010) but not other (Kupferschmidt et al. 2012) studies. For example, corticosterone levels in Long-Evans rats prior to a stress challenge but just following a behavioral test were approximately 350 ng/mL (Kupferschmidt et al.). As with the present study, corticosterone was sampled from rats after being in a testing condition for 1h. It is possible that if rats had not been tested, group differences in corticosterone would have been identifiable. Finally, it could also be that corticosterone in all rats was elevated when injected with pentobarbital just prior to perfusion, masking group differences. That being said, we actually observed a negative correlation between corticosterone levels and active lever responding for all of the rats ($r = -0.3$, $P < 0.01$; Table 3). Furthermore, Thiel et al. (2011) found no consistent link between corticosterone and EE. Given the inconsistencies in reported relationships between corticosterone and EE across studies, more detailed examination of a possible stress-specific effect of EE is needed.

An EE-mediated change in sensitivity to external cues predictive of reward has been suggested previously (Thiel et al. 2010,Thiel et al. 2011,; Grimm et al. 2008, Grimm et al. 2013). As with an anti-stress hypothesis, this explanation fits with the behavioral findings across studies where either acute or chronic EE reduced cue seeking and/or primary reinforcement (studies already noted and (Bardo et al. 2001; Green et al. 2002; Brenes and Fornaguera 2008). However, this hypothesis does not require a relationship between seeking and corticosterone level. An “incentive-based” (Thiel et al 2011; also see Gill and Cain 2011) framework for the EE effects would mean that the EE experience either results in some profound recalibration in reward circuitry where reinforcement value of cues and primary reinforcement is reduced in general, or more of a *transient* change where the reward circuitry tracks and adapts to the current perceived value of the environment. This latter

hypothesis would predict a decrease in responding for cues and/or primary reinforcement following EE due to the subject experiencing a negative contrast (Reynolds 1961) between the highly reinforcing EE experience and now the perceived less reinforcing self-administration experience. The transient aspect of this hypothesis would also mean that EE effects would not be enduring. Specific examination of this is yet limited, but so far while acute EE did result in reduced sucrose intake two days after EE, a subsequent test approximately one month later revealed no lingering effect of EE on sucrose seeking (Grimm et al. 2013). Chronic EE reduced cocaine seeking (CPP) was also found to be transient and, furthermore, there was a striking *increase* in cocaine seeking when mice were tested two weeks post EE (Nader et al. 2012). This contrast hypothesis is in line with EE providing "alternate reinforcement" to subjects. This interpretation of EE effects on drug reinforcement was first introduced by Alexander et al. (1978) where rats chronically housed in an EE environment consumed less morphine than rats in isolated housing.

What is unique about the approach of the present study and the majority of more recent studies of the effects of EE on drug seeking or taking is that the EE context is dissociated from the SA context. Therefore the EE effect is not simply a subject reducing responding directed at reward-paired cues due to the distraction of exploring novel stimuli. This might be a confound if EE were simply applied to the self-administration chamber. The more recent findings could indicate that the negative contrast functions across contexts. In addition, it may be alternative reinforcement that produces the negative contrast. If so, it provides a unique form of alternate reinforcement that may be translatable. Alternate reinforcement has already been identified as a clinical approach to reduce drug taking (Bennet et al. 1998; Johnson and Bickel 2003;Tuten et al. 2012; Cutter et al. 2014). Providing opportunities for individuals to engage in more adaptive behaviors (education, exercise, healthier food consumption) could be akin to providing alternative reinforcement in animal models (Carroll 1993).

Concluding remarks

Either acute (17h) or chronic (30d) EE greatly reduced sucrose seeking by rats. This decrease in responding was accompanied by a reduction in Fos IR in a majority of the 18 brain regions examined, several of these regions known to modulate responding guided by incentives. Fos IR was also typically higher in rats after 30 d of forced abstinence from sucrose self-administration (incubation of Fos expression). There were regions where acute EE prior to Day 1 testing either did not alter Fos IR (NA, ACC) or increased it (ILC). Future studies will detail the possible contribution of receptor-mediated transduction pathways (e.g. dopamine and glutamate receptor) to these regional Fos expression effects in conjunction with site-specific functional (site-specific behavioral pharmacology) studies. The present, and potential future, studies will provide a better understanding of the neurobiology of EE, a potential non-pharmacological intervention/treatment strategy for relapse behavior.

Acknowledgements

The authors wish to thank Kylan Dorsey and Dr. Blair Duncan for help with data collection and Dr. Bruce Hope for technical advice on immunohistochemistry. This study was supported by national institutes of health grant DA016285-03 and Western Washington University.

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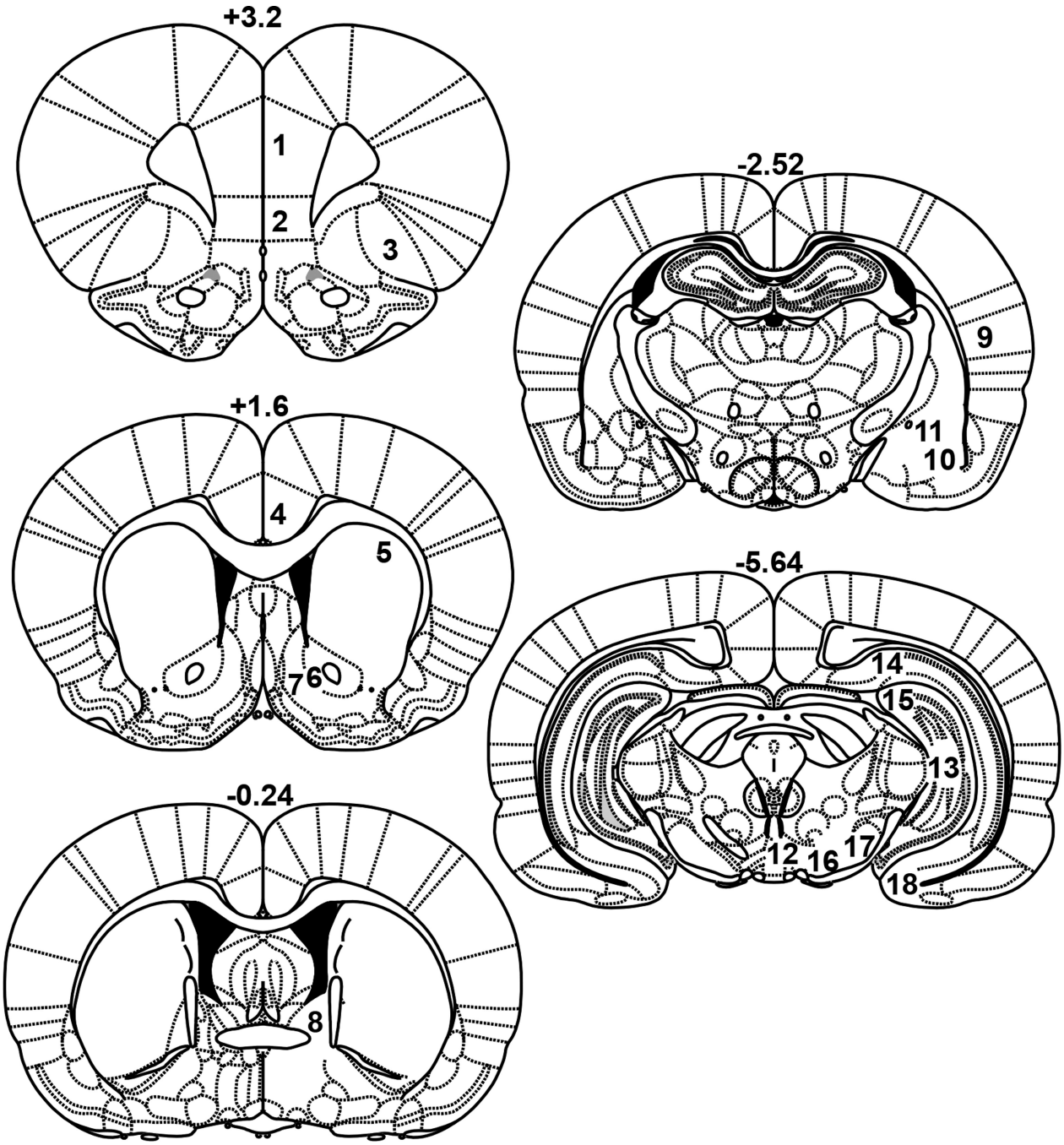

Fig. 1.

Regions selected for quantitation of Fos IR. Selection of regions was based on Thiel et al. (2010) and are identified as: 1 prelimbic cortex, 2 infralimbic cortex, 3 orbitofrontal cortex, 4 anterior cingulate cortex, 5 dorsolateral striatum, 6 nucleus accumbens core, 7 nucleus accumbens shell, 8 bed nucleus of the stria terminalis, 9 somatosensory cortex, 10 basolateral amygdala, 11 central nucleus of the amygdala, 12 ventral tegmental area, 13 CA3 hippocampus, 14 CA1 hippocampus, 15 dentate gyrus of hippocampus, 16 substantia nigra

pars compacta, 17 substantia nigra pars reticulata, and 18 ventral subiculum. Distance from Bregma is indicated in mm. Figure adapted from Paxinos and Watson (2007).

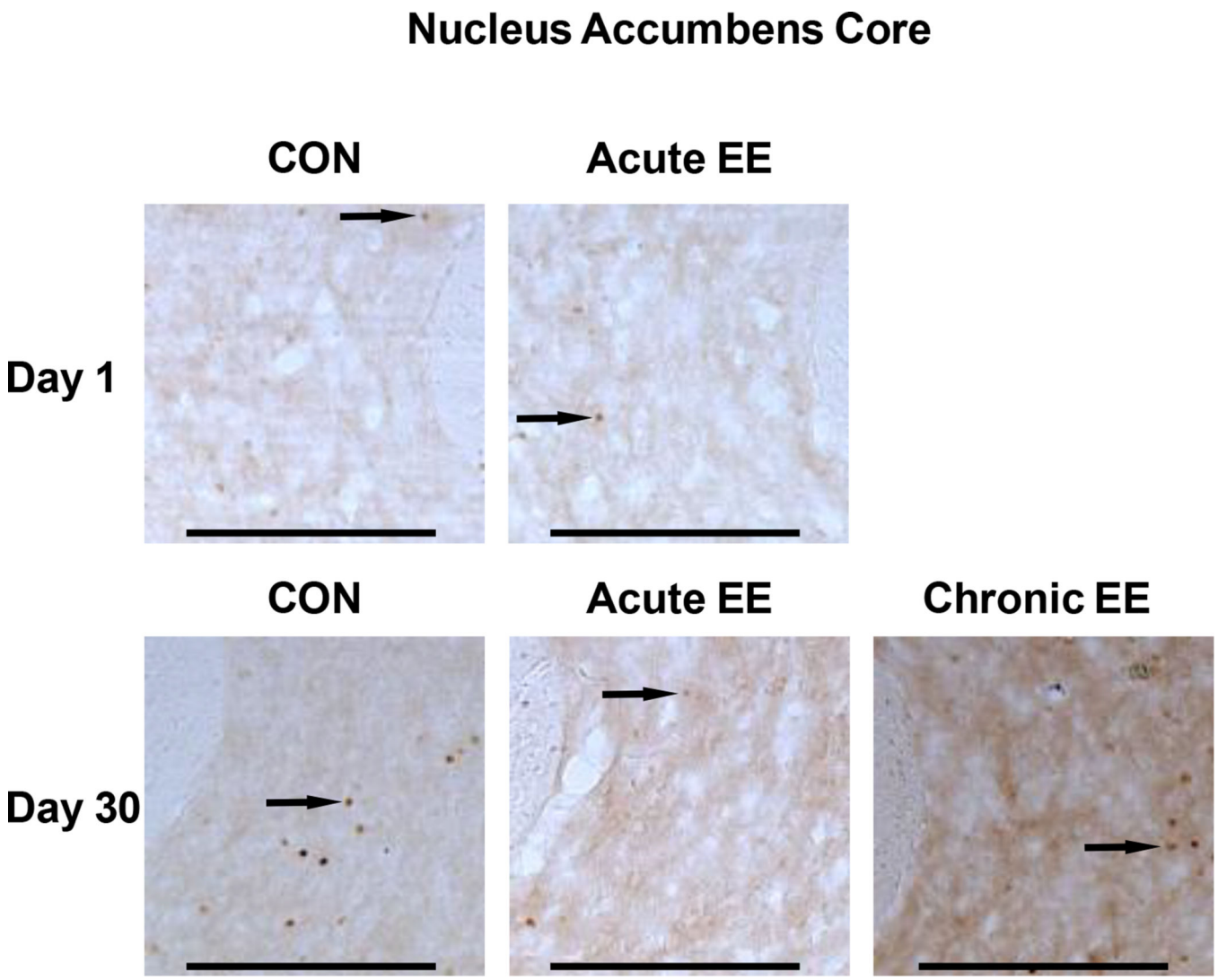

Fig. 2.

Representative Fos IR images from the NA core at 10× magnification. Conditions are indicated on the figure. These are raw images (not color, contrast, or brightness adjusted). The subject with the median Fos IR count from its condition is presented. The dark line is 250 micrometers; the anterior commissure is to the right (Day 1 subjects) or left (Day 30 subjects) in each image Arrows point to representative Fos IR.

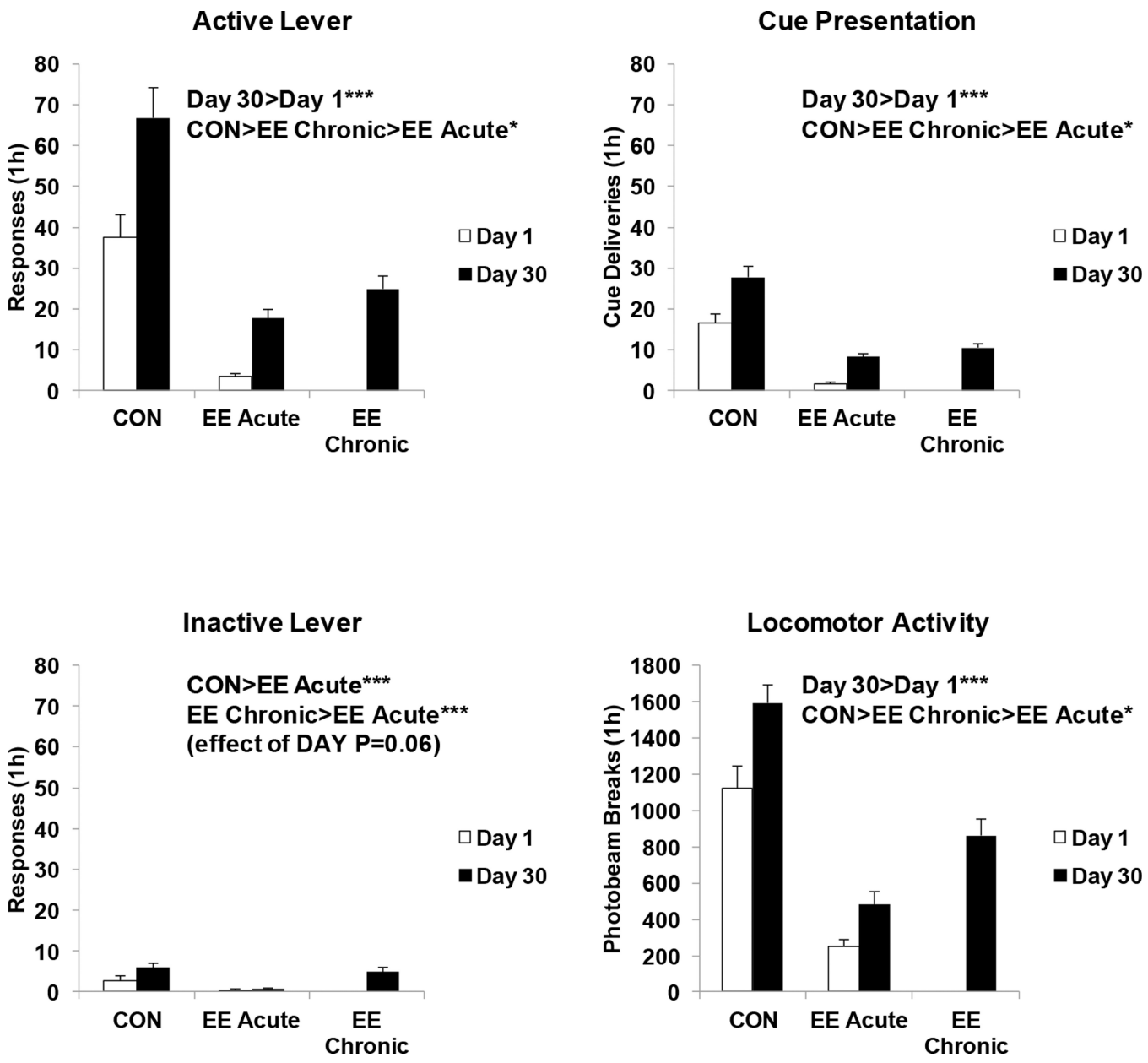

Fig. 3.

Testing behavior. Statistically significant main effects are indicated with statistically significant post-hoc test results, $*P < 0.05$, $***P < 0.001$. There were no statistically significant interaction terms.

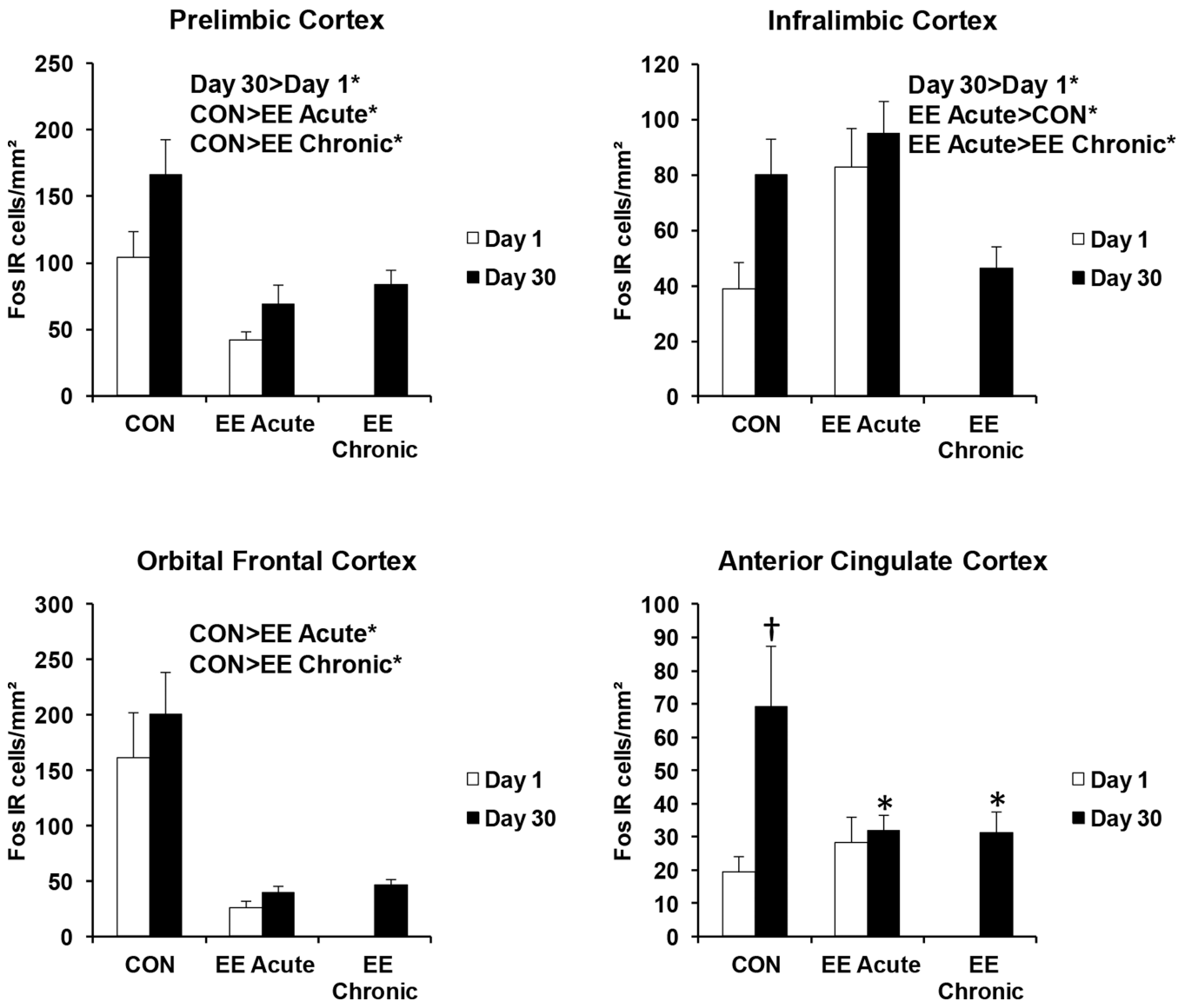

Fig. 4.

Frontal cortex regions with significant effects of EE and/or incubation on Fos IR. Statistically significant main effects are indicated with statistically significant post-hoc test results,*P< 0.05. For anterior cingulate cortex there was a statistically significant interaction term; for the post-hoc test results *indicates significant difference from CON group at that forced-abstinence time point, P < 0.05; † (dagger) indicates significant difference from Day 1 group in that housing treatment condition, P < 0.05.

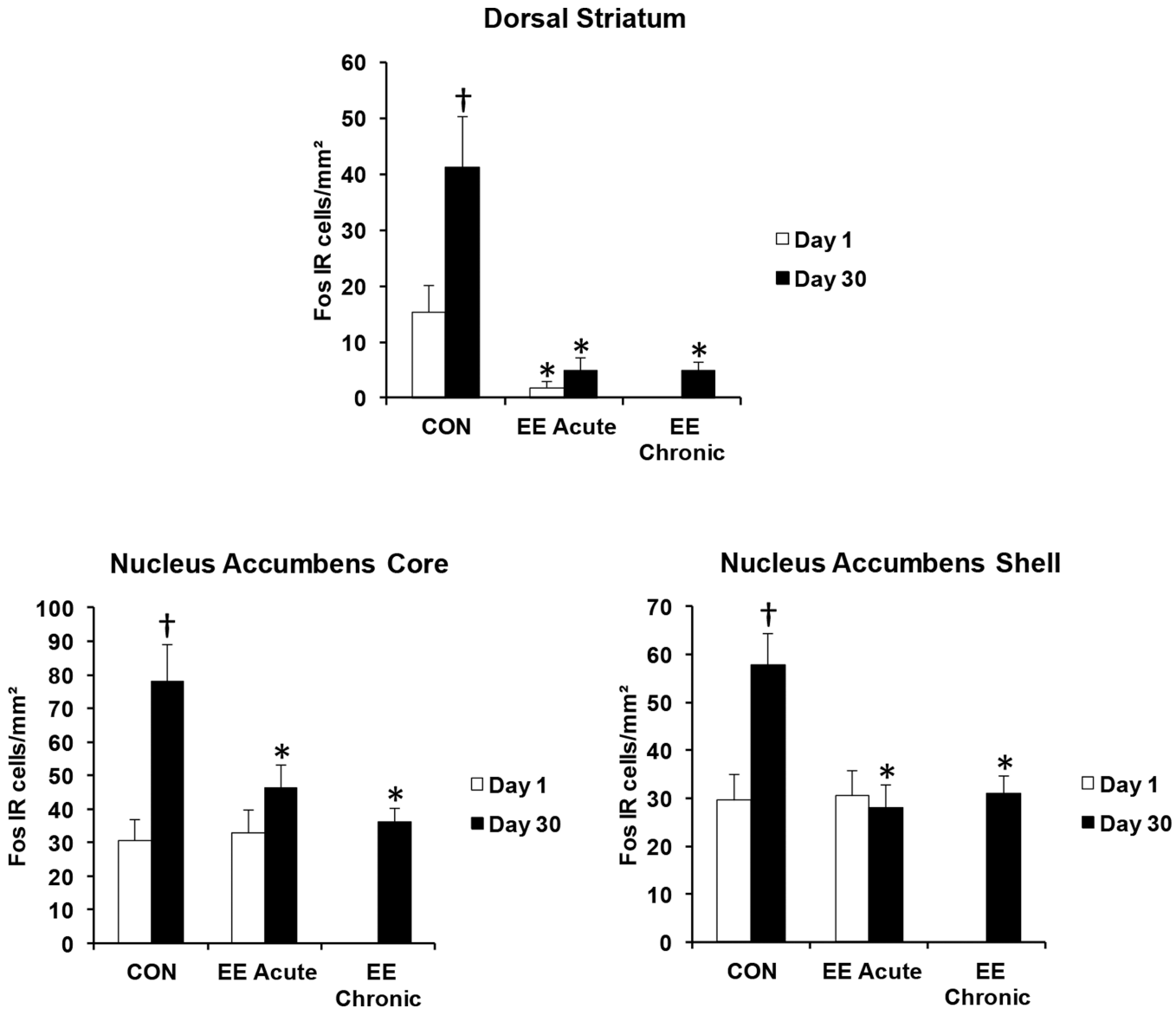

Fig. 5.

Striatal regions with significant effects of EE and/or incubation on Fos IR. All regions had a statistically significant interaction term. For the post-hoc test results *indicates significant difference from CON group at that forced-abstinence time point, $P < 0.05$; † (dagger) indicates significant difference from Day 1 group in that housing treatment condition, $P < 0.05$.

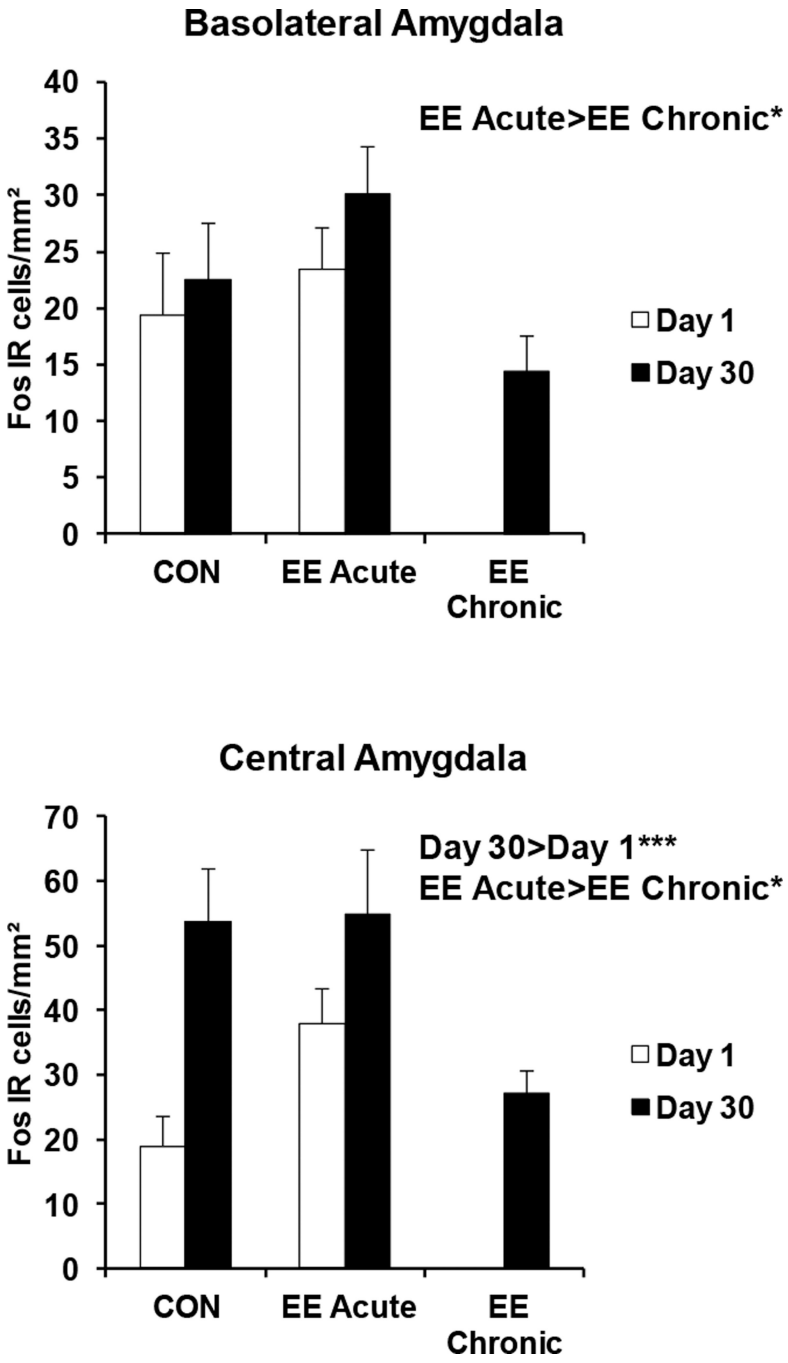

Fig. 6.

Amygdala sub-regions with significant effects of EE and/or incubation on Fos IR. Statistically significant main effects are indicated with statistically significant post-hoc test results,* $P < 0.05$, *** $P < 0.001$.

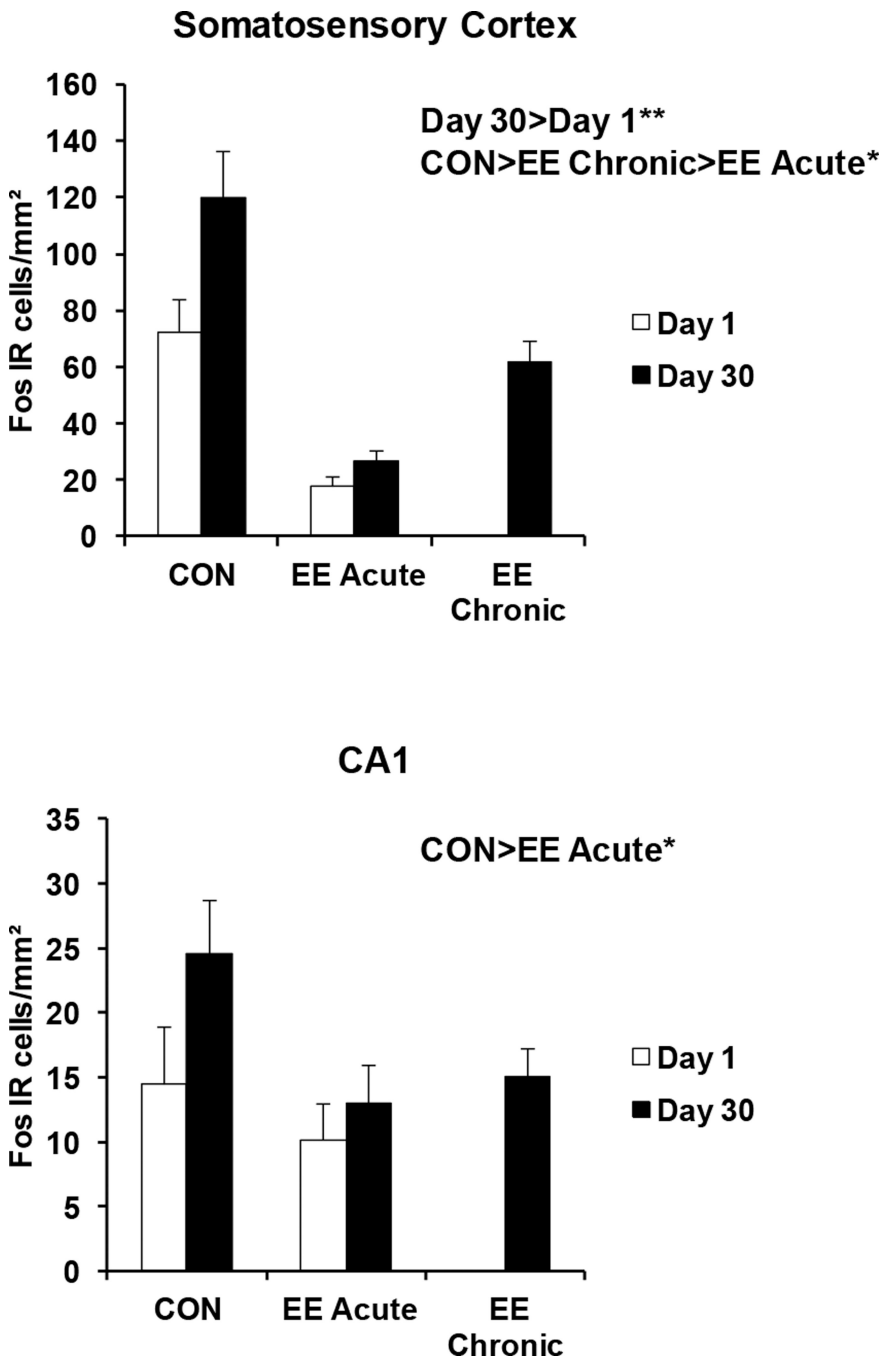

Fig. 7.

Other regions with significant effects of EE and/or incubation on Fos IR. Statistically significant main effects are indicated with statistically significant post-hoc test results,* $P<$ 0.05, ** $P<$ 0.01

Table 1

ANOVA results for Fos IR.

Region	DAY F(1,60)[1]	HOUSING F(2,60)[1]	DAY × HOUSING F(1,60)[1]
1 prelimbic cortex	7.0*	9.7***	1.1 n.s.
2 infralimbic cortex	5.5*	4.9*	0.2 n.s.
3 orbitofrontal cortex	0.3 n.s.	13.0***	0.6 n.s.
4 ant. cingulate cortex	7.7**	5.1**	5.8*
5 dorsolateral striatum	9.3**	19.5***	5.8*
6 nucleus accumbens core	17.7***	9.2***	5.5*
7 nucleus accumbens shell	6.2*	10.0***	8.8**
8 bed nucleus of the stria terminalis	1.3 n.s.	2.3 n.s.	3.5 n.s.
9 somatosensory cortex	8.3**	22.9***	3.8 n.s. ($P = 0.056$)
10 basolateral amygdala	1.3 n.s.	3.2*	0.2 n.s.
11 central nucleus of the amygdala	14.4***	5.3**	1.7 n.s.
12 ventral tegmental area	2.0 n.s. (1,46)	1.8 n.s. (2,46)	2.3 n.s. (1,46)
13 CA3 hippocampus	2.8 n.s. (1,59)	0.1 n.s. (2,59)	0.7 n.s. (1,59)
14 CA1 hippocampus	3.6 n.s. (1,59)	3.3*	1.1 n.s. (1,59)
15 dentate gyrus of hippocampus	2.0 n.s. (1,59)	1.0 n.s. (2,59)	1.1 n.s. (1,59)
16 substantia nigra pars compacta	0.1 n.s. (1,59)	0.4 n.s. (2,59)	0.3 n.s. (1,59)
17 substantia nigra pars reticulata	2.1 n.s. (1,59)	0.2 n.s. (2,59)	0.0 n.s. (1,59)
18 ventral subiculum	1.8 n.s. (1,59)	0.0 n.s. (2,59)	0.0 n.s. (1,59)

Notes:

[1] degrees of freedom unless otherwise noted.

* $P < 0.05$,

** $P < 0.01$,

*** $P < 0.001$.

Regions indicated in Figure 1.

Table 2

Fos IR for regions with non-significant ANOVA results

Region	CON DAY1	EE ACUTE DAY 1	CON DAY30	EE ACUTE DAY 30	EE CHRONIC (DAY 30)
8 bed nucleus of the stria terminalis	10.3 ± 3.2	15.1 ± 4.9	28.0 ± 10.1	10.8 ± 3.5	15.7 ± 4.9
12 ventral tegmental area	53.4 ± 9.8	48.5 ± 13.9	84.3 ± 16.6	59.9 ± 9.3	51.2 ± 8.1
13 CA3 hippocampus	21.3 ± 4.4	16.0 ± 2.9	12.6 ± 3.8	13.0 ± 3.2	10.6 ± 2.7
15 dentate gyrus of hippocampus	37.0 ± 6.8	29.5 ± 5.4	40.6 ± 7.4	54.7 ± 18.9	34.8 ± 5.9
16 substantia nigra pars compacta	14.0 ± 3.8	14.6 ± 4.3	18.2 ± 4.1	13.7 ± 6.8	12.6 ± 3.4
17 substantia nigra pars reticulata	4.3 ± 2.7	4.3 ± 1.8	1.8 ± 0.6	2.0 ± 0.9	3.1 ± 1.3
18 ventral subiculum	131.0 ± 23.1	130.1 ± 20.3	180.9 ± 23.5	167.0 ± 20.1	176.0 ± 56.8

Notes: n = 12 – 13 per group (VTA 8 – 13 per group), means (Fos IR cells/mm^2) ± SEMs indicated. Regions indicated in Figure 1.

Table 3

Significant correlations between active lever responding and regional Fos IR and plasma corticosterone level on the Test day.

	prelimbic cortex	infralimbic cortex	orbitofrontal cortex	dorsolateral striatum	nucleus accumbens core	nucleus accumbens shell	bed nucleus of the stria terminalis	somato-sensory cortex	CA1 hippocampus	plasma corticosterone
All Subjects	0.5		0.7	0.4	0.4	0.6		0.6	0.3	−0.3
CON Acute EE		0.5	0.5		0.4	0.7				−0.6
Chron ic EE	−0.6						−0.6			
Day 1	0.5		0.8					0.6		
Day 30	0.4		0.7	0.4	0.5	0.7		0.6		−0.4

Note. Correlations (Pearson's r) are presented either as all subjects or split into treatment conditions. All correlations are statistically significant, $P < 0.05$.