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Conditioned Fear Inhibits c-fos mRNA Expression in the Central Extended Amygdala

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Abstract

We have shown previously that unconditioned stressors inhibit neurons of the lateral/capsular division of the central nucleus of the amygdala (CEAl/c) and oval division of the bed nucleus of the stria terminalis (BSTov), which form part of the central extended amygdala. The current study investigated whether conditioned fear inhibits c-fos mRNA expression in these regions. Male rats were trained either to associate a visual stimulus (light) with footshock or were exposed to the light alone. After training, animals were replaced in the apparatus, and 2 hours later injected remotely, via a catheter, with amphetamine (2 mg/kg i.p.), to induce c-fos mRNA and allow inhibition of expression to be measured. The rats were then presented with 15 visual stimuli over a 30 minute period. As expected, fear conditioned animals that were not injected with amphetamine, had extremely low levels of c-fos mRNA in the central extended amygdala. In contrast, animals that were trained with the light alone (no fear conditioning) and were injected with amphetamine had high levels of c-fos mRNA in the CEAl/c and BSTov. Animals that underwent fear-conditioning, and were re-exposed to the conditioned stimulus after amphetamine injection had significantly reduced levels of c-fos mRNA in both the BSTov and CEAl/c, compared to the non-conditioned animals. These data suggest that conditioned fear can inhibit neurons of the central extended amygdala. Because these neurons are GABAergic, and project to the medial CEA (an amygdaloid output region), this may be a novel mechanism whereby conditioned fear potentiates amygdaloid output.

Keywords

amygdala; bed nucleus of the stria terminalis; fear; stress; c-fos

Introduction

Models of classical or Pavlovian fear conditioning in which an animal learns to predict danger based on prior experience have been the subject of intense study, and understanding the mechanisms underlying this type of fear conditioning is thought to be very relevant to understanding human anxiety disorders (Bouton *et al.*, 2001). The central nucleus of the amygdala (CEA) is a critical structure for the expression of conditioned fear, with outputs from the medial CEA thought to control many conditioned fear responses, including potentiation of

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the acoustic startle reflex for example (Davis, 1992). The neural circuitry involved in classical or Pavlovian fear conditioning is relatively well understood (Pare *et al.*, 2004; LeDoux, 2007), with activation of thalamic and cortical amygdaloid inputs by both the conditioned stimulus (such as a visual or auditory cue) and somatosensory unconditioned stimulus (such as pain from footshock) leading to convergence of inputs in the lateral amygdala. Current evidence suggests that synaptic plasticity occurs in both the lateral/basolateral amygdaloid complex (Malkani and Rosen, 2000; Blair *et al.*, 2001; Maren and Quirk, 2004; LeDoux, 2007) and CEA (Pascoe and Kapp, 1985; Samson and Pare, 2005). Subsequent exposure to the conditioned stimulus alone results in information flow from the lateral amygdala, via the basal nucleus, accessory basal nucleus and/or intercalated cells of the amygdala, and ultimately to the medial subdivision of the central nucleus of the amygdala (CEA).

In addition to these pathways, the medial CEA receives input from the lateral and capsular divisions of the CEA (CEAl/c) and the oval (or dorsolateral) nucleus of the bed nucleus of the stria terminalis (BSTov) (Petrovich and Swanson, 1997; Jolkkonen and Pitkanen, 1998; Dong *et al.*, 2001). The amygdala and BST are highly interconnected, and distinct subregions have often been associated under an “extended amygdala” concept, primarily based on afferent and efferent projections, morphological features and neurochemical phenotypes (Alheid *et al.*, 1995). Under this system of categorization, the lateral CEA and BSTov are parts of the central extended amygdala due to their highly similar neuroanatomical features (McDonald, 1982; Cassell *et al.*, 1986; Ju and Swanson, 1989; Ju *et al.*, 1989; Moga *et al.*, 1989; McDonald, 2003). The capsular CEA also receives direct input from the lateral amygdala (Pitkanen *et al.*, 1995), and thus the BSTov and CEAl/c are potentially in a position to provide modulatory input to amygdaloid output neurons and possibly modify fear conditioned responses.

Surprisingly, given the involvement of the CEA in conditioned fear, the levels of c-fos mRNA or Fos protein expression, used as a tool to assess neuronal activation, are surprisingly meager in this nucleus (and in the BSTov) following conditioned fear (Pezzone *et al.*, 1992; Beck and Fibiger, 1995; Campeau *et al.*, 1997). A similarly low level of c-fos expression has been observed following exposure to unconditioned psychological or processive stressors (Cullinan *et al.*, 1995; Campeau and Watson, 1997; Emmert and Herman, 1999; Day *et al.*, 2001; Dayas *et al.*, 2001). However, we have previously demonstrated that under unconditioned stress conditions such as exposure to a novel environment (Day *et al.*, 2001), loud noise or restraint (Day *et al.*, 2005), levels of c-fos mRNA expression, induced by either amphetamine or interleukin-1 β , are *reduced* in both the BSTov and CEAl/c. Similarly, exposure to a single footshock (1.5 mA, 1 s) reduced diazepam-induced EGR-1 mRNA expression in the CEA (Malkani and Rosen, 2000). This suggests that at least some neurons in these regions may be inhibited by unconditioned stressors. The present study was conducted to determine whether a similar inhibition of c-fos mRNA in the BSTov and CEA occurs during exposure to conditioned fear. The effect of cued fear conditioning (visual stimulus) on amphetamine-induced c-fos mRNA expression in the CEA and BSTov was determined by semi-quantitative *in situ* hybridization. The data support the hypothesis that conditioned fear can inhibit neurons of the CEAl and BSTov, which may reflect a novel and specific mechanism by which modulation of amygdaloid output neurons is achieved.

Results

Experiment 1

As previously shown, amphetamine administration (2 mg/kg i.p.) resulted in high levels of c-fos mRNA expression in the BSTov and CEA. Previous exposure to footshock, in a different environment, and using the same parameters as the fear conditioning procedure in Experiment 2, (15 trials at 0.6 mA for 500 ms, average ITI 2 min., on each of the 2 previous days), did not

alter the amphetamine-induced c-fos mRNA response in these areas, compared to a control group, naïve to footshocks (BST: $p = 0.805$; CEA: $p = 0.945$; Figure 1).

Experiment 2

Animals were divided into experimental groups on the basis of their baseline startle amplitudes, so that the mean startle amplitudes (mean of 30 baseline trials at 95, 100 and 105 dB on the second test day) were not significantly different between groups: Fear Conditioned + Conditioned Stimulus (Light) = 3.39 ± 0.63 N; Fear Conditioned + context = 3.33 ± 0.53 N; Light alone + Light = 3.30 ± 0.56 N. Previous work in our laboratory has shown that the training procedure used is effective at producing fear-potentiated startle (data not shown). A highly abbreviated fear-potentiated startle test indicated that the procedure was effective in this experiment also, with the presence of the visual stimulus resulting in a significantly greater increase in acoustic startle amplitude in conditioned compared with control animals ($F(1,20) = 8.40$; $p = 0.009$; Figure 2).

The levels of c-fos mRNA induced by the conditioned stimulus alone, or following amphetamine injection under control or conditioned stimulus conditions, were determined in the BSTov and CEA by in situ hybridization. As shown in Figure 3, Figure 4A and 4D, levels of c-fos mRNA were extremely low in both the BSTov and CEA when the animals were exposed to the conditioned light stimulus that had previously been paired with footshock. In contrast, all animals that received an amphetamine injection, 2 mg/kg i.p., had significantly higher levels of c-fos mRNA in both the BSTov and CEA, compared to the no amphetamine/conditioned fear group. (Figure 3 and Figure 4; Group 2: train with light alone (no shock) and test with light + amphetamine: BST: $p < 0.001$, Figure 4B; CEA: $p < 0.001$, Figure 4E; Group 3: Train with light paired with shock and test with light + amphetamine: BST: $p = 0.005$, Figure 4C; CEA: $p = 0.016$, Figure 4F; Group 4: train with light and shock and test with context (no light) + amphetamine BST: $p = 0.002$; CEA: $p = 0.004$). Of particular note, group 3 rats (train with light paired with shock and test with light + amphetamine) had significantly lower c-fos mRNA levels in the BSTov ($p = 0.018$) and CEA ($p = 0.021$) compared with rats in group 2 (train with light alone (no shock) and test with light + amphetamine). Levels of c-fos mRNA in the BSTov and CEA of rats in group 4 (train with light and shock and test with context (no light) + amphetamine) were intermediate, and not significantly different from levels expressed by rats in either groups 2 or 3 (Figure 3).

Discussion

The present data show that conditioned fear reduces amphetamine-induced expression of c-fos mRNA in the BSTov and CEA (Experiment 2). In contrast, prior exposure to footshock alone, in a different context, has no effect on amphetamine induced expression of c-fos mRNA in these regions (Experiment 1). In these experiments, rats experienced two daily sessions of 15, 500 ms, 0.6 mA footshocks, with the sessions approximately 48 and 24 hours before amphetamine injection. This contrasts with the inhibition of immediate early gene expression observed for the BSTov and CEAl/c when unconditioned stressors, including footshock, are experienced concurrently with the activating stimulus (Malkani and Rosen, 2000; Day *et al.*, 2001; Day *et al.*, 2005), and suggests that, at least under these conditions, previous stress does not lead to long term changes in responsiveness of the BSTov and CEAl/c. It is generally accepted that the magnitude of c-fos mRNA or protein expression reflects the approximate strength of a stimulus (Hoffman and Lyo, 2002). Hence a decrease in stimulus-evoked c-fos mRNA expression in a given region implies that the strength of the incoming signal to that region is decreased. Although this decrease in activated signal transduction pathways does not necessarily reflect the extent of depolarization of the cells, the current data indicate that exposure to a learned aversive stimulus results in decreased input to cells of the BSTov and

CEAl and are consistent with the hypothesis that conditioned fear inhibits at least some neurons of the BSTov and lateral/capsular CEA. This study extends our previous finding that amphetamine or interleukin-1 β induced expression of c-fos mRNA in the BSTov and CEAl/c is reduced by exposure to unconditioned aversive stimuli, such as novelty (Day *et al.*, 2001), restraint and loud noise (Day *et al.*, 2005).

It should be noted that in the present study, amphetamine administration was performed in the same chambers that the fear conditioning took place. We had expected that the lack of a grid floor and the 2 hour acclimation period prior to amphetamine administration would be sufficient to prevent any effects of contextual fear. However, c-fos mRNA in the BSTov and CEA of rats that had been previously trained to associate the visual stimulus with footshock but were injected with amphetamine in the absence of the visual stimulus (Group 4) was at an intermediate level, between non-conditioned animals (Group 2) and cued conditioned animals (Group 3). This suggests that despite the acclimation period, conditioned animals may have remained somewhat fearful of the context, especially given the fact that previous footshock did not produce a residual effect on inhibition of the BSTov and CEA when amphetamine was injected in a novel environment (Experiment 1). It is therefore possible that the inhibition of c-fos observed in the cued conditioned animals reflects both cued and context conditioning. The amygdala is well known to be important for responses to contextual fear (Phillips and LeDoux, 1992), and post-training lesions of the BST have also been shown to attenuate responses to contextual fear (Sullivan *et al.*, 2004). It would be of interest to more formally test whether cued versus contextual fear results in similar reductions in c-fos mRNA in the BSTov and CEAl/c.

Both amphetamine and interleukin-1 β increase levels of c-fos mRNA expression in the BSTov and CEAl/c almost exclusively in GABAergic neurons that also contain enkephalin (Day *et al.*, 1999; Day *et al.*, 2001). The present data are therefore consistent with the idea that both unconditioned and conditioned aversive stimuli can inhibit GABAergic cells of the BSTov and CEAl/c that also express enkephalin. However, there is at least one other neurochemically distinct neuronal population in the BSTov and CEAl that contains corticotropin releasing hormone (CRH) (Day *et al.*, 1999). It is not known whether or not GABAergic neurons expressing CRH are similarly inhibited by either unconditioned or conditioned aversive stimuli. CRH is a peptide that produces anxiogenic-like behaviors when injected into either the lateral ventricles (Koob and Thatcher-Britton, 1985) or amygdala (Liang and Lee, 1988; Gray and Bingaman, 1996), distinct from its role in regulation of the hypothalamic pituitary adrenal axis (Britton *et al.*, 1986), and thus it would be of great interest to determine if there is a specific interaction between conditioned or unconditioned aversive stimuli and CRH containing neurons of the BSTov and CEA.

At this time, the mechanisms of activation and inhibition of the BSTov and CEAl/c are not clear but, in the specific case of amphetamine activation, may involve dopamine. There are several sources of dopaminergic input to these regions, including the ventral tegmental area (A10), substantia nigra, pars compacta (A9), retrorubral nucleus (A8) and the dorsal raphe nucleus (Hasue and Shammah-Lagnado, 2002), and electron microscopic studies in the CEA have suggested that the distinct dopaminergic pattern of intrinsic versus extrinsic connections may be important for maintaining an integrated and balanced amygdaloid output (Asan, 1997). Interestingly, a 6-hydroxydopamine lesion of the mesostriatal dopamine system results in profound decreases in CRH mRNA expression in both the BSTov and CEA, but does not affect enkephalin mRNA expression in these regions (Day *et al.*, 2002), and does not affect amphetamine-induced c-fos mRNA expression in either the BSTov or CEA (Day *et al.*, 2001), suggesting differential modulation of different cell types by dopamine. The circuitry involved in the inhibition of these regions by conditioned fear is also not clear. Because unconditioned and conditioned aversive stimuli lead to a similar inhibition of the CEAl/c and

BSTov, we hypothesize that similar mechanisms are involved. In the case of unconditioned stress, inhibition of the BSTov and CEA is observed after injection of either amphetamine, interleukin-1 β or diazepam (Malkani and Rosen, 2000; Day *et al.*, 2001; Day *et al.*, 2005). It is likely that these diverse stimuli utilize different neural circuitry to activate the CEAl/c and BSTov. Therefore we hypothesize that unconditioned stress and conditioned fear result in activation of a direct GABAergic input to the BSTov and CEAl/c, causing a competitive inhibition, rather than direct inhibition of the stimulatory pathway.

Functionally, the neural circuitry of the BSTov and CEAl/c is consistent with the hypothesis that inhibition of these neurons leads to enhanced amygdala output. This is because both the BSTov and lateral CEA have extensive projections to the medial CEA (Petrovich and Swanson, 1997; Jolkkonen and Pitkänen, 1998; Dong *et al.*, 2001), a major amygdaloid output nucleus (Schwaber *et al.*, 1982; Veening *et al.*, 1984; Rizvi *et al.*, 1991). Because these projections are GABAergic (Day *et al.*, 1999), and presumably inhibitory, their inhibition by conditioned or unconditioned aversive stimuli potentially decreases inhibitory drive to the medial CEA and potentially enhances medial CEA output. Because the medial CEA projects to many of the motor response regions associated with behavioral fear responses (LeDoux *et al.*, 1988; Rosen *et al.*, 1991; Bellgowan and Helmstetter, 1996; De Oca *et al.*, 1998), this may be one way in which fear responses may be modulated. Traditional accounts of conditioned fear circuitry propose association of the unconditioned stimulus (footshock) and conditioned stimulus in the lateral amygdala, which projects via other amygdaloid regions (perhaps the basal, accessory basal and intercalated nuclei) to the medial CEA (Pare *et al.*, 2004; LeDoux, 2007). The medial CEA then projects to brain regions associated with fear responses, such as the startle reflex pathway in the brainstem. Our data are consistent with the idea that when an animal learns to be fearful of a specific stimulus, BSTov and lateral/capsular CEA neurons are inhibited which potentially results in disinhibition of medial CEA output neurons, leading to enhanced amygdaloid output and responses associated with fear, such as potentiated startle. This neural circuitry could potentially provide an additional source of modulatory input to the medial CEA, distinct from the inhibitory inputs from the intercalated nuclei of the amygdala, which are thought to provide an important connection between the lateral amygdala and the medial CEA (Paré *et al.*, 2004). One argument against this model is the fact that neither unconditioned nor conditioned aversive stimuli lead to a significant increase in c-fos mRNA in the CEAm. While this fact remains surprising, a lack of expression of c-fos mRNA should be treated with caution, and cannot be interpreted as a lack of involvement of a region in a particular process. For example, despite the fact that unconditioned footshock stress does not substantially increase c-fos expression in the CEAm (Pezzone *et al.*, 1992; Rosen *et al.*, 1998), increases in CEAm neuronal activity following footshock have been recorded electrophysiologically (Rosenkranz *et al.*, 2006). Similarly, as described above, an abundance of data indicate that the CEAm is likely involved in conditioned fear responses, yet conditioned fear does not result in a substantial elevation in c-fos expression in the CEAm (this manuscript, Campeau *et al.*, 1997).

While a specific role of the CEAl/c in modulation of fear conditioned behaviors would be difficult to establish due to the close neuroanatomical proximity to the medial CEA, which is critical for many conditioned fear responses, the potential role of the BST has been quite extensively studied. However, in contrast to lesions of the CEA, many studies have failed to show an effect of either pre- or post-training lesions of the BST on cued conditioned fear responses (Hitchcock and Davis, 1991; Lee and Davis, 1997; Walker and Davis, 1997; Sullivan *et al.*, 2004; Waddell *et al.*, 2006). In contrast, unconditioned potentiated startle responses to CRH infusion (Lee and Davis, 1997) and bright light (Walker and Davis, 1997) are inhibited by lesions of the BST, but not the CEA. These and other data have led to the current view that the BST and amygdala may have differential functions, with the BST more involved in anxiety-like responses to unconditioned aversive stimuli, and the amygdala more involved in fear

conditioned responses. In the present study, both the BSTov and CEAl/c showed similar decreases in c-fos mRNA expression in response to amphetamine under cued fear conditions, compared to control animals, in keeping with the parallel responses previously observed (Day *et al.*, 1999; Day *et al.*, 2001; Day *et al.*, 2005). Given the lack of effect of lesions of the BST on cued fear responses, it is possible that this decrease in “activation” of the BSTov has no functional relevance in terms of cued fear conditioned responses, such as fear-potentiated startle. (Walker *et al.*, 2003; Davis, 2006). Alternatively, because inputs from the BSTov represent only a fraction of inputs to the medial CEA, with CEAl/c, with the basal, accessory basal and intercalated amygdala also providing critical or modulatory inputs for conditioned fear responses, it is likely that lesion of the BSTov would not result in the dramatic effects that a lesion of the medial CEA would produce for example. In addition, despite reasonable evidence for differential functions for the BSTov and CEA in learned fear versus anxiety, there are also data that challenge this dissociation. For example, injection of the noradrenergic $\alpha 2$ receptor agonist clonidine into the BST disrupted the acquisition and expression of cued fear-potentiated startle, traditionally viewed as an amygdaloid driven response, as well as light-enhanced startle, which is dependent on the BST (Schweimer *et al.*, 2005). Moreover, in a paradigm very similar to the one used in the present study, where rats were trained over 2 days with 0.6 mA footshock that co-terminated with a 3.7 second light stimulus, muscimol injection into the BST, which would lead to temporary inhibition of this nucleus, led to a significant potentiation of the fear potentiated startle response (Meloni *et al.*, 2006). These data are consistent with the idea that inhibition of the BST can indeed potentiate fear responses, such as an increased acoustic startle reflex response.

Finally, despite evidence supporting a potential role for the BSTov and CEAl/c in modulation of unconditioned stress and conditioned fear responses, activation or inhibition of these neurons should not be construed as an indication of the emotional valence of a stimulus per se. Of particular note, is the fact that a wide range of stimuli increase c-fos mRNA expression in the BSTov and CEA, including both anxiolytic and anxiogenic drugs (Thompson and Rosen, 2006). Furthermore, we have evidence to suggest that anxiolytics (diazepam), and anxiogenics (FG7142 and yohimbine) all increase c-fos mRNA expression selectively in enkephalinergic, but not in CRH containing, neurons (Day, HE, unpublished data). It is possible, and even likely, that this activation occurs in the same enkephalinergic neurons that appear to be inhibited by unconditioned and conditioned aversive stimuli. Therefore, it seems likely that the physiological function of enkephalinergic cells of the BSTov and CEAl/c is independent of the emotional valence of the stimulus, but perhaps reflects regulation of processes that can occur in either the presence or absence of an emotional stimulus. A wealth of evidence has indicated a role for the extended amygdala in cardiovascular processing for example, which can be either related to, or independent of, emotional processing (Kapp *et al.*, 1982; Pascoe and Kapp, 1985; Gelsema *et al.*, 1993). With this in mind, the fact that amphetamine both increases some fear-related responses (Antoniou and Kafetzopoulos, 1991; Markham *et al.*, 2006), and increases c-fos mRNA expression in the BSTov and CEAl/c, is not inconsistent with the proposed modulatory role for these regions on CEAm output, especially given the strong stimulatory response that amphetamine has on the cardiovascular system, independent of any emotional regulation (Simpson, 1975). Clearly much more work needs to be done to elucidate the function of these BSTov and CEAl/c neurons, particularly the role they may play in modulation of CEAm output following conditioned and unconditioned aversive stimuli.

Overall, the present data indicate that c-fos mRNA expression in both the BSTov and CEAl/c can be reduced by learned fear, consistent with the hypothesis that neurons in these regions are inhibited during conditioned fear. The fact that these neurons are GABAergic and project to the medial CEA, a nucleus associated with many fear responses, suggests that inhibition of the BSTov and CEAl/c during conditioned fear may be a novel way to disinhibit neurons of the medial CEA and therefore enhance amygdaloid output. Given the likely importance of learned

fear and associative processes in human anxiety disorders, understanding the interaction of the BSTov and CEAl/c with regions critical for learned fear responses, such as the medial CEA, will be vital in understanding these mental disorders. Thus these nuclei represent an additional and specific target for assessment of dysregulation associated with anxiety disorders.

Experimental Procedure

Animals

Male Sprague Dawley rats (Experiment 1: Harlan, IN; Experiment 2: Charles River, Boston, MA), weighing 200 – 225 g at the time of arrival in the colony, were used for the experiment. Animals were initially group-housed, four per cage, under stable conditions of temperature and humidity and with free access to standard laboratory chow and water. Animals were maintained on a 12h:12h light:dark cycle, lights on at 07:00 a.m. All procedures were approved by The University of Colorado at Boulder Institutional Animal Care and Use Committee, in conformation with NIH guidelines.

Surgery

Seven to nine days after arrival in the colony, animals underwent surgery to implant an intraperitoneal catheter as previously described (Day and Akil, 1999; Day *et al.*, 2005), in order to reduce the acute stress of handling and injection procedures at the time of the experiment. Briefly, under halothane anesthesia, a piece of silastic tubing (22 cm; 0.020" internal diameter; 0.037" external diameter) was introduced into the abdominal cavity. The silastic catheter was anchored with sutures to the abdominal wall and exteriorized at the back of the skull. The catheter was attached to a cannula connector pedestal (Plastics One, Roanoke, VA) and anchored to the skull with screws and cranioplastic cement. Animals were then housed individually in plastic shoebox cages (22 × 43 × 21 cm) with sawdust bedding on the floor, and allowed to recover for 5–7 days before further experimental manipulation.

Experiment 1: Does previous stress (fear conditioning procedure) result in long term inhibition of the BSTov and CEA, which in itself would cause a decrease in c-fos mRNA expression?

We have previously shown that acute psychological stress can inhibit immediate early gene responses in the BSTov and CEA. However, it was not known whether previous stress experience, such as footshock exposure, would have an effect on subsequent immediate early gene expression in these regions. In the first experiment (N = 16), 2 groups of rats were used, n = 8 per group (control or shock). The experiment was run in 2 equivalent cohorts (n = 4 per group × 2), 1 day apart. Animals in the control group remained in their home cages in the colony room. Seven or eight days after surgery, animals in the stress group were exposed to footshock, with the same parameters used in the fear conditioning procedure (Experiment 2), as follows. On 2 consecutive days, animals were transported in their home cage to a behavioral testing suite, and placed individually into one of eight startle chambers (Kinder Scientific, Poway, CA) that contained a rat holder (SM2001) on a platform load cell that detected displacement. For delivering footshock, a metal grid (SMG-R) connected to a programmable animal shocker (scrambled output; SMSCK) was inserted into each rat holder. After a 5 min. acclimation period, each animal received 15, 0.6 mA, 500 ms duration footshocks, with an average inter-trial interval of 120 s (range 30 to 210 s). In the afternoon after the second shock session, animals (control and shock) were transported to the behavioral testing suite and placed, within their home cages, into one of eight individual noise attenuation chambers, as previously described (Day *et al.*, 2005), which represented a different environment from the shock exposure. The i.p. catheter was connected to an external 1 ml syringe filled with sterile 0.9% saline via a metal spring-covered connector assembly (Plastics One, Roanoke, VA), a fluid swivel mounted on the cage top (Instech Laboratories Inc., Plymouth Meeting, PA) and saline

filled PE tubing that passed outside the box enclosure allowing remote injection. A 1 ml volume of saline was injected to check the patency of the catheter and that there were no leaks. The animals remained in the noise attenuation boxes overnight, with free access to food and water, under light conditions which mimicked those of the colony room. The following morning, approximately 24 hours after the second shock exposure, and under non-stress conditions, 2 mg/ml/kg amphetamine (D-amphetamine sulfate salt; Sigma, St. Louis, MO) was injected remotely via the i.p. catheter, followed by a 0.8 ml saline flush, to stimulate immediate early gene expression in the BSTov and CEA. Animals were killed by rapid decapitation 30 min. after the amphetamine injection. Brains were dissected and stored at -80°C until processing for c-fos mRNA in the BSTov and CEA by in situ hybridization (see below).

Experiment 2: Effect of Conditioned Fear on amphetamine-induced c-fos mRNA Expression in the BSTov and CEA

Rats ($N = 20$) were implanted with an i.p. catheter. Five to seven days after surgery, rats were transported in their home cage to a behavioral testing suite, and tested for baseline startle amplitude as follows. Each animal was placed individually into one of eight startle chambers (Kinder Scientific, Poway, CA) and after a 5 min. acclimation period, the rat's startle amplitude in response to thirty white noise pulses (50 ms; rise-decay: 2 ms) was recorded. Startle amplitude was defined as the maximum load cell output (amplified and digitized) that occurred during the first 200 ms after startle stimulus onset. Ten each of 95, 100 and 105 dB (SPL; A scale) noise bursts were randomly presented with an inter-trial interval of 30 seconds. Background noise level was set at 60 dB. After the startle test the animals were returned to their home cage and the colony room. This startle test was repeated the following day, and the animals' baseline startle amplitudes (from the second day of testing) matched between three experimental groups. The three groups were randomized between 3 experimental cohorts.

One to three days after the baseline startle test, each cohort began the first of 2 consecutive days of training. Animals within each cohort were transported to the behavioral testing suite within the home cage, and then placed individually within a startle chamber. After a 5 min. acclimation period, rats in two of the three experimental groups (randomized between cohorts) received fifteen 0.6 mA footshocks, 500 ms duration, with an average inter-trial interval of 2 min. Each footshock co-terminated with a 3.7 second, 80 lux visual light stimulus, delivered via a pair of custom-made 60 LED panels. The rats in the third experimental group were placed individually in the startle chambers and experienced the visual stimulus under the same schedule as the trained groups, but received no footshocks. After the training session, animals were returned to their home cages and the colony room. The following day the training session was repeated. This paradigm has been used previously in our laboratory to produce a reliable potentiation of the startle response to different intensity noise bursts, in the presence of this conditioned visual stimulus (data not shown).

For each cohort, the day after the second training session, a very brief fear potentiated startle test was performed to validate the conditioning procedure in these animals. Animals of each cohort were again transported within the home cage to the behavioral suite and placed within the startle chamber. After a 5 minute acclimation period, 30 noise bursts (10 each of 95, 100 or 105 dB) were presented in quasi-random order (ITI = 30 seconds). This was followed by two presentations of the visual stimulus that co-terminated with either a 95 dB or 100 dB noise burst. These 2 light/noise burst presentations were embedded within a further 6 (2 each of 95, 100 or 105 dB) unpaired noise bursts. The maximum startle amplitude in response to each noise burst was recorded. Only 2 conditioned stimulus presentations were used to minimize extinction of the fear memory. After the fear-potentiated startle test, animals were returned to their home cages and the colony room.

The morning after the fear potentiated startle test, animals were transported in their home cage to the behavioral suite. Animals were placed in the startle apparatus and the catheter was connected to an external 1 ml syringe filled with sterile 0.9% saline via a metal spring-covered connector assembly (Plastics One, Roanoke, VA), a fluid swivel mounted on the Plexiglas ceiling of the startle chamber (Instech Laboratories Inc., Plymouth Meeting, PA) and saline filled PE tubing that passed outside the box enclosure allowing remote injection. A 1 ml volume of saline was injected to check the patency of the catheter and that there were no leaks. Three animals from the two groups that had been fear conditioned had blocked or leaking catheters on the test day. Instead of being excluded, they were tested without amphetamine injection to determine the extent of c-fos mRNA expression to the conditioned stimulus alone. After a 2 hour acclimation period, animals were injected with 2 mg/ml/kg amphetamine via the i.p. catheter, followed by a 0.8 ml saline flush. Immediately after the injection a subset of the animals were exposed to 15, 3.7 second, 80 lux visual stimuli, with an average inter-trial interval of 120 s, as in the training sessions. At the end of the visual stimulus exposures, and 30 min. after the amphetamine injection, animals were killed by rapid decapitation and brains removed and stored at -80°C until processing for c-fos mRNA in the BSTov and CEA by in situ hybridization (see below).

In order to test each cohort at a similar time of day, the 3 cohorts were tested on 3 consecutive days. However, it should be noted that for each cohort, the 2 training sessions, the fear potentiated startle test and the amphetamine injection/test day occurred on each of 4 consecutive days. Groups were randomized between cohorts. Group 1 (n = 3): Train: Shock + Light; Test: No Amphetamine + Light; Group 2 (n = 5): Train: No Shock + Light; Test: Amphetamine + Light; Group 3 (n = 7): Train: Shock + Light; Test: Amphetamine + Light; Group 4 (n = 5): Train: Shock + Light; Test Amphetamine + no light (context).

In situ hybridization

The method for in situ hybridization has been described previously (Day and Akil, 1996). Briefly, 10 μm sections were cut on a cryostat (Leica model 1850) through the BSTov and CEA, thaw-mounted onto polylysine-coated slides and stored at -80°C . A [^{35}S]-UTP-labeled riboprobe against c-fos mRNA (680 mer; courtesy of Dr. T. Curran St Jude Children's Hospital, Memphis, TN) was generated using standard transcription methods. Sections at 60 μm (BSTov) or 120 μm (CEA) intervals, were fixed in 4% paraformaldehyde (1 hour), acetylated in 0.1 M triethanolamine with 0.25% acetic anhydride (10 min.) and dehydrated through graded alcohols. Sections were hybridized overnight at 55°C with a [^{35}S]-UTP-labeled riboprobe diluted in hybridization buffer containing 50% formamide, 10% dextran sulfate, 2 $\times$ saline sodium citrate, 50 mM PBS, pH 7.4, 1 $\times$ Denhardt's solution, and 0.1 mg/ml yeast tRNA. The following day, sections were treated with RNase A, 200 $\mu\text{g}/\text{ml}$ at 37°C (1 hour), and washed to a final stringency of 0.1 $\times$ saline sodium citrate at 65°C (1 hour). Dehydrated sections were exposed to X-ray film (BioMax MR; Eastman Kodak, Rochester, NY) for up to 1 week and the films analyzed as described below.

Semi-quantitative x-ray film analysis

Levels of c-fos mRNA were analyzed by computer-assisted optical densitometry. Analysis was performed blind to the treatment conditions. Anatomical landmarks were based on the white matter distribution of the unstained tissue section, according to a standard rat brain atlas (Swanson, 1992). The anterior commissure was the primary landmark used for the BSTov and the optic tract and boundary of the basolateral amygdaloid complex were used for the CEA. Overall, the relatively discrete expression of amphetamine-induced c-fos mRNA in these brain areas facilitated analysis from x-ray film. Brain section images were captured digitally (CCD camera, model XC-77; Sony, Tokyo, Japan), and the relative optical density of the x-ray film was determined using Scion Image version 4.0 for PC. A macro was written (Dr. S. Campeau)

that enabled signal above background to be determined automatically. For each section, a background sample was taken over an area of white matter, and the signal threshold was calculated as mean gray value of background + $3.5 \times$ standard deviation. The section was automatically density sliced at this value, so that only pixels with gray values above these criteria were included in the analysis. It should be noted that although background criteria were relatively stringent, this thresholding method still results in a few pixels above background in areas off the brain section, indicating that even weak-intensity mRNA signal on the tissue section is detected. A template was used for each brain region, so that the same area was included in the analysis for each region. Left and right sides were analyzed through the rostral-caudal extent of each nucleus. The number of pixels above background was multiplied by the signal above background, to give an integrated density value. This method has been shown to reflect both the number of cells expressing mRNA and the expression level per cell, as determined by cell and grain counts of emulsion dipped slides (Day *et al.*, 2005). The mean of the highest 3 integrated density values for each hemisphere (6 values total) for each animal was calculated, to give a single value for each animal representing the peak of c-fos mRNA expression.

Statistical Analysis

Startle data were analyzed by repeated measures analysis of variance (ANOVA). Data for c-fos mRNA expression levels were analyzed by one-way ANOVA followed by Fisher's Protected Least Significant difference (PLSD) post-hoc multiple comparisons test. Significance was set at $p < 0.05$.

Abbreviations

BST, bed nucleus of the stria terminalis; CEAl/c, central nucleus of the amygdala, lateral/capsular divisions; CRH, corticotropin releasing hormone.

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Figure 1.

Experiment 1: Relative levels of c-fos mRNA in the BSTov and CEA. Animals either remained in their home cages within the colony room (Control) or were exposed to footshock, under the same paradigm used for the fear conditioning procedure (Experiment 2), on 2 consecutive days (Shock), before injection with amphetamine, 2 mg/kg i.p., in a different environment the following day. Amphetamine administration resulted in strong c-fos mRNA expression in both the BSTov and CEA, but previous shock experience did not diminish this expression in either brain region.

Figure 2.

Experiment 2: Startle response amplitudes (% baseline) to a 95dB or 100 dB noise burst, co-terminating with a 3.7 s light stimulus in (i) animals with no previous light-shock association (control group) and (ii) animals which had been trained to associate the light with a 0.6 mA, 0.5 s footshock (fear conditioned group). Data are expressed as a percentage of baseline startle, which was taken as the mean startle response to the previous 3 noise burst stimuli at either 95 dB or 100 dB as appropriate. * $p < 0.01$ with respect to the control group.

Figure 3.

Experiment 2: Relative levels of c-fos mRNA in the BSTov and CEA. Animals were trained to associate a 3.7 s light (L) with a 0.6 mA, 0.5 s footshock (S), or were exposed to the light (L) but did not receive shock (-). On the test day animals were either re-exposed to the light (L) or the startle apparatus alone (-), and either received amphetamine (A; 2 mg/kg i.p.) via the i.p. catheter, or no injection (-). The no injection animals served as a control for c-fos mRNA expression elicited by a conditioned fear stimulus alone. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ with respect to the fear conditioned group that did not receive an amphetamine injection (Train L/S; Test L/-). † $p < 0.05$ with respect to control animals that did not undergo fear conditioning, and received amphetamine on the test day (Train L/-; Test L/A).

Figure 4.

Experiment 2: Photographs of x-ray films showing the expression of c-fos mRNA in the BSTov (A, B, C) and CEA (D, E, F) 30 minutes after (A,D) the presentation of 15 conditioned light stimuli, but with no amphetamine injection (Group 1: animals had been trained to associate the light stimuli with foot shock), (B,E) amphetamine injection, 2 mg/kg i.p. plus the presentation of 15 unconditioned light stimuli (Group 2: animals had been exposed to the visual stimuli alone, without footshock), (C,F) amphetamine injection, 2 mg/kg i.p. plus the presentation of 15 conditioned light stimuli (Group 3: animals had been trained to associate the light stimuli with foot shock).