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Conditioned Fear-induced Changes in Behavior and in the Expression of the Immediate Early Gene *c-fos*: With and Without Diazepam Pretreatment

C. H. M. Beck[1] and H. C. Fibiger[2]

[1]Department of Psychology, University of Alberta, Edmonton, Alberta, T6G 2E9, Canada and [2]Division of Neurological Sciences, Department of Psychiatry, University of British Columbia, Vancouver, British Columbia, V6T 1Z3, Canada

The synthesis of Fos, the protein product of the immediate early gene *c-fos*, was used to map metabolically some of the neural substrates of conditioned fear in the rat. Analysis of the behaviors emitted by the rats during the test session provided strong evidence that the conditioning procedure was effective. Exposure to the environment in which they had previously received footshock significantly increased the number of Fos-like immunoreactive neurons in nearly 50 brain regions, both cortical and subcortical. Among the structures showing the most dramatic increases in fear-induced *c-fos* expression were the cingulate, piriform, infralimbic, and retrosplenial cortices, the anterior olfactory nucleus, claustrum, endopiriform nucleus, nucleus accumbens shell, lateral septal nucleus, various amygdalar nuclei, paraventricular thalamic nucleus, ventral lateral geniculate nucleus, the ventromedial, lateral, and dorsal hypothalamic nuclei, the ventral tegmental area, and the supramammillary area. These data demonstrate that a relatively simple classical conditioning procedure activates a large number of widely dispersed cortical and subcortical structures. Some of the structures showing increased *c-fos* expression have important autonomic functions and may therefore have reflected centrally mediated changes in blood pressure and respiration produced by the anxiogenic stimuli.

In a second experiment, the effects of pretreatment with the anxiolytic drug diazepam (2.5, 5.0, or 10 mg/kg) were evaluated. The benzodiazepine produced dose-related decreases in the frequency of crouching (freezing) elicited by the aversively conditioned contextual cues. Diazepam also produced dose-related decreases in conditioned stress-induced *c-fos* expression in all but one structure, the effects being statistically significant in 38 of 60 sampled structures. Diazepam dose dependently increased fear-induced *c-fos* expression in the central nucleus of the amygdala. There was considerable regional variability with respect to sensitivity to diazepam, the retrosplenial cortex and the supramammillary area being the only two structures to show decreases after the lowest dose of diazepam. In contrast, the entorhinal cortex, nucleus accumbens core, ventromedial and posterior hypothalamic nuclei, median raphe, and locus coeruleus were particularly resistant to diazepam, all failing to show statistically significant decreases in conditioned fear-induced *c-fos* expression even at the highest dose. The extent to which diazepam decreased conditioned stress-induced *c-fos* expression was unrelated to previous estimates of benzodiazepine receptor density in the sampled structures.

[Key words: stress, conditioning, fear, behavior, ethological analysis, immediate early gene, C-fos, brain, limbic, amygdala, rat]

The neuronal synthesis of Fos, the protein product of the immediate early gene *c-fos*, increases in some brain regions in response to mild forms of stress such as an injection of isotonic saline, brief restraint, brushing of whiskers, and exposure to a novel environment (Campeau et al., 1991; Chastrette et al., 1991; Sharp et al., 1991; Mack and Mack, 1992; Smith et al., 1992). The structures in which this has been shown to occur include isocortical and allocortical regions as well as a variety of subcortical nuclei. Increases in *c-fos* expression by such relatively temperate stimulation suggest that neutral stimuli previously paired with biologically significant events (i.e., conditioned stimuli) may also produce such effects. Indeed, in two recent studies, exposure to cues that were conditioned to footshock stress were found to elevate *c-fos* mRNA or Fos-like immunoreactivity (FLI) in the amygdala (Campeau et al., 1991; Pezzone et al., 1992). In the present study we sought to provide a more anatomically detailed and quantitative assessment of the effects of conditioned stimuli that had previously been paired with aversive stimuli (footshock) on regional *c-fos* expression in the brain. While previous studies have provided reasonably detailed anatomical analyses of the effects of unconditioned stressors (e.g., immobilization, handling, inescapable swimming) on regional *c-fos* expression (Sharp et al., 1991; Duncan et al., 1993; Senba et al., 1993), to date the effects of conditioned stressors have been analyzed in less detail and nonquantitatively (Campeau et al., 1991; Pezzone et al., 1992; Smith et al., 1992). The analysis of the effects of a conditioned stressor on regional *c-fos* expression has the potential of providing information about the neural substrates of fear at a level of anatomical resolution that cannot be attained with other approaches. The first exper-

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Correspondence should be addressed to Dr. H. C. Fibiger, Division of Neurological Sciences, Department of Psychiatry, 2255 Wesbrook Mall, University of British Columbia, Vancouver, BC, V6T 1Z3, Canada.

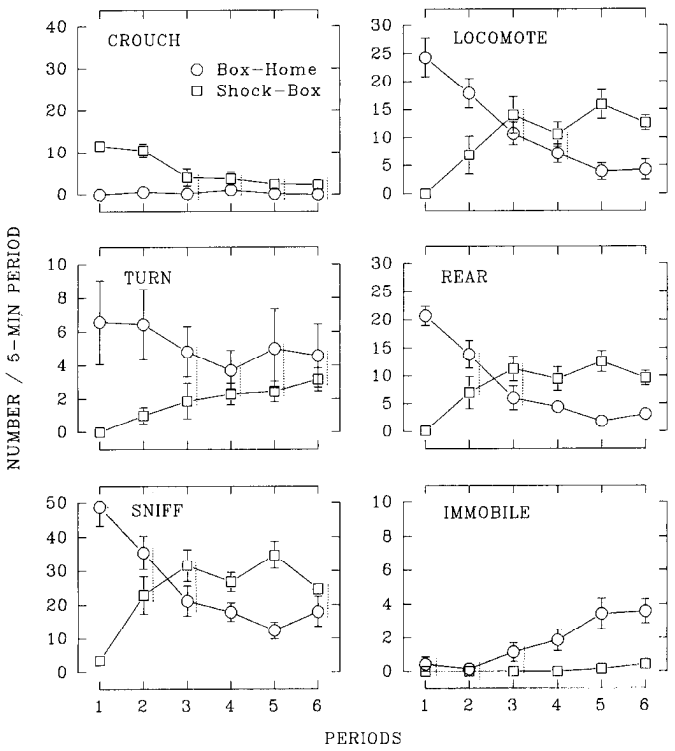

Figure 1. Time course of behavioral events over the six 5 min periods of day 4 for the control group (box-home, ○), and of day 7 for the conditioned fear group (shock-box, □) (both groups $n = 7$). Data represent means and SEs of the frequencies of several behaviors. Fs (df = 5,60) are group by period interactions from two-way ANOVA. Crouch $F = 4.87$, $p < 0.01$; locomote $F = 16.92$, $p < 0.01$; turn $F = 2.53$, $p > 0.05$; rear $F = 18.04$, $p < 0.01$; sniff $F = 19.96$, $p < 0.01$; and immobile $F = 4.38$, $p < 0.05$. *Dashed vertical lines* join means which are not significantly different (Tukey, $p < 0.05$).

iment therefore sought to provide a detailed examination of the effects of a conditioned stressor on regional FLI in the brain and to analyze the results quantitatively. It was found that exposure of rats to an environment in which they had previously received footshock produced increases in *c-fos* expression in widely distributed cortical and subcortical structures.

A second experiment was conducted with two objectives in mind. If increased *c-fos* expression is directly associated with fear produced by the conditioned stimuli, it was hypothesized that an anxiolytic agent should decrease their effects on *c-fos* expression. This conjecture is supported indirectly by observations indicating that increases in the incidence of FLI produced by a convulsant are reduced by anticonvulsant doses of benzodiazepines (Morgan et al., 1987). Another objective of the second experiment was to clarify the effects of benzodiazepines on behaviors induced by stimuli contextually conditioned to footshock.

Experiment 1

Materials and methods

Animals. Twenty-eight male Long–Evans rats (Charles River, Montreal) weighed 306 ± 14 gm (mean ± SE) at the beginning of the experiment. The animals were housed singly in wire mesh cages. The colony room was maintained at 21°C and was on a 12/12 hr light/dark cycle (lights on at 8.00 hr). Food and water were available ad libitum in the home cages.

Apparatus. The behavioral apparatus was a Plexiglas box measuring 25 × 35 × 35 cm high. The brass rods of the grid floor were wired to an electric shock generator. The walls of the box were opaque but the ceiling was clear so that the rat could be viewed via a mirror mounted at 45° to the ceiling. A video camera, video recorder, and a microprocessor were used to record and code each animal's behavior.

Experimental protocol. Animals were adapted to handling for 3 d, and on the fourth day, experimental day 1, were individually adapted to the test box in a 30 min pretraining session without shock. During this session each rat's exploratory behavior was coded as described below. The animals were then assigned in a quasi-random fashion to one of three groups (each $n = 7$) of individuals matched for latency to begin exploring the box. The groups received a combination of three types of sessions: *home* sessions in which the animal was transported to the test room and then immediately returned to the home cage in the colony room, *box* sessions in which the rat was placed in the test box for a 30 min no-shock session, and *shock* sessions in which the subject experienced a 30 min session of footshock. The shock was delivered as 30 unsignaled unavoidable shock trains presented on a VI 60 sec schedule. Each train consisted of five 1.0 sec duration shocks alternating with 1.0 sec no-shock intervals. Experimental days 2, 3, and 4 were training days and day 7 was the test day. No training or testing was done on days 5 and 6. Groups were designated on the basis of their treatment in the training and test sessions, respectively.

The principal group of interest was the conditioned fear group, the *shock-box* group which was shocked on the three days of training, days 2, 3, and 4, and placed in the box for 30 min without shock on the test day, day 7. This was a conditioned group for which the unconditioned stimuli were the footshocks and the conditioned stimuli were the contextual cues provided by the box and the test room. The animals were undisturbed on days 5 and 6 to permit any direct effects of shock on *c-fos* expression to dissipate. Control groups included a group with exposure to the box but without shock during the three training sessions, the *box-home* group; and a chronic shock group that was not exposed to the cues of the box on the test day, the *shock-home* group. On the test day, rats in both control groups were treated according to the home session protocol described above.

Behavioral coding. Behavior during all sessions in the box was coded continuously by a trained observer. The behavioral events and their times of occurrence were stored by a microprocessor. The behavioral categories used for coding included *flinch,* the rat twitched during a footshock; *jump,* the rat leapt clear of the floor; *crouch,* the rat maintained a flattened posture while not moving; *locomote,* the rat's forequarters entered a quadrant of the box floor; *turn,* the rat turned body through 180°; *rear,* the rat raised its forepaws from the floor while not grooming or jumping; *sniff,* the rat's whiskers moved and its head made scanning movements; *immobile,* the rat was motionless with a normal resting posture; and *groom,* the rat licked, combed, or scratched itself. Test-retest and interobserver reliability measures of behavioral coding produced agreements greater than 79% and all yielded significant coefficients of concordance. One- and two-way ANOVAs with repeated measures as appropriate were followed by Tukey tests for differences between groups for each behavior.

Immunohistochemistry. Two hours after the beginning of the final test session on day 7, the animals received an overdose of sodium pentobarbital and were perfused with saline and 4% paraformaldehyde. The brains were removed, soaked overnight in fixative, and cut into 30 mm sections on a Vibratome. Three to six sections at each of 11 AP levels (see below) were selected for staining, and 60 brain regions were included for analysis. These areas were selected partially by observing increased FLI in pilot studies and in part on reports in the literature (Chastrette et al., 1991; Sharp et al., 1991; Pezzone et al., 1992). Some structures expected to show effects contained such low levels of FLI that they were not counted. These included the ventral pallidum, substantia innominata, most thalamic nuclei (see below), medial preoptic area, anterior, paraventricular and supraoptic nuclei of the hypothalamus, brainstem reticular formation, cerebellum, and autonomic nuclei of the brainstem. The AP coordinates of sections included for detailed analysis (Paxinos and Watson, 1986) and associated structures were *AP +3.2,* anterior olfactory nucleus, anterior cingulate cortex area 3; *AP +2.7,* posterior cingulate cortex area 3, piriform cortex, dorsopeduncular cortex, infralimbic cortex, frontal cortex area 2, lateral orbital cortex, insular cortex, tenia tecta, anterior claustrum; *AP +1.0,* forelimb cortex, frontal cortex area 1, posterior claustrum, endopiriform nucleus, anterior dorsomedial caudate putamen, shell and core of the nucleus accumbens, olfactory tubercle, islands of Calleja, lateral and medial septal nucleus, nucleus of the diagonal band; *AP −0.92,* bed nucleus of stria

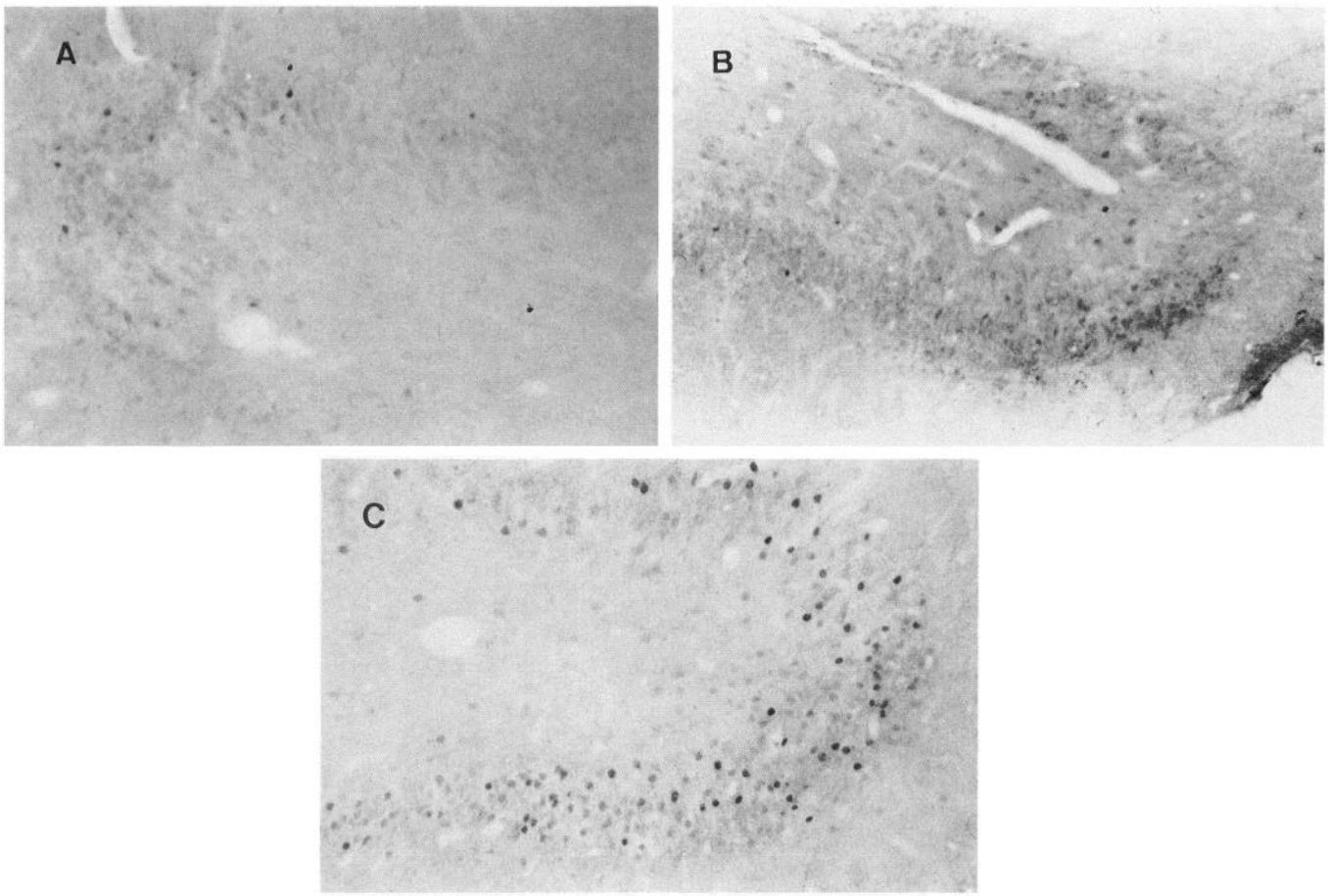

Figure 2. Fos-like immunoreactivity in coronal sections (100× magnification) through the piriform cortex in *A*, the home control group (box-home); *B*, the chronically shocked group (shock-home); and *C*, the conditioned fear group (shock-box).

terminalis; *AP −2.8,* retrosplenial cortex, hindlimb cortex, parietal cortex area 2, perirhinal cortex, posterior dorsomedial caudate putamen, the central, basolateral, basomedial, and cortical nuclei of the amygdala; *AP −3.3,* the dentate gyrus and CA1 field of the dorsal hippocampus, ventromedial, and dorsal hypothalamic nuclei; *AP −3.8,* temporal cortex area 3, amygdalohippocampal area, paraventricular and central medial nuclei of the thalamus, lateral habenula, lateral and posterior nuclei of the hypothalamus; *AP −4.8,* occipital cortex area 2, entorhinal cortex, the dentate gyrus and CA1 field of the ventral hippocampus, ventral lateral geniculate, posterior intralaminar nucleus of the thalamus, anterior pretectal nucleus, ventral tegmental area, substantia nigra, supramammillary nucleus; *AP −7.3,* central gray, dorsal raphe nucleus; *AP −8.0,* pontine nuclei, medial raphe nucleus; *AP −9.3,* locus coeruleus, and lateral parabrachial nucleus.

Details of the immunohistochemical methodology for FLI may be found in previous reports from this laboratory (Robertson et al., 1991; Robertson and Fibiger, 1992). Briefly, after washing, sections were incubated with primary antisera, sheep polyclonal antibody (Cambridge Research Biochemicals, CRB 0A-11-823, Wilmington, DE). Further washing was followed by incubation with a biotinylated rabbit anti-sheep secondary antibody (Dimension Laboratories, BA-6000, Mississauga, ON). After another wash, the reaction was made visible with glucose oxidase-3.3′-diaminobenzidene-nickel, and the sections mounted on slides. In this preparation, FLI nuclei appeared darkly stained against a background of lightly stained cells and fibers. To assist in the identification of neural structures, representative sections from selected animals were stained with cresyl violet. Two independent observers counted the number of FLI-positive nuclei in each structure within a 0.5 mm square grid viewed at 100× magnification. Kruskal–Wallis one-way ANOVA was used to test for group effects on counts of FLI-positive nuclei within each structure. Significant group effects, $p < 0.05$, were followed by Mann–Whitney *U* tests to assess group differences. Unless stated otherwise, all statistical effects are $p < 0.05$.

Results

Behavior. The sessions were divided into six 5 min periods to assess the time course of behavioral events within sessions. Two-way ANOVA with one repeated measure was applied to each behavior of the three groups over the six periods on day 1, the no-shock pretraining session. The ANOVAs revealed no significant group main effects or group by period interactions in the frequency of occurrence of any behavior. Therefore, the data provide no evidence that the group differences, in counts of FLI-positive neurons presented below, were the result of group assignment-induced biasing of the groups for reactivity to novelty.

Evidence for habituation to the novelty of the box over three sessions and across the six periods within sessions by the box-home control group was provided by ANOVAs of the session and period effects. The data revealed a trend between and within sessions of decreased frequencies of crouching, locomotion, rearing, and sniffing and increased frequencies of turning, grooming, and immobile resting. As these effects conformed to classical habituation effects (Beck and Chow, 1984), the changes within day 4 for the box-home group are presented as typical (Fig. 1). While formal data were not obtained, casual observation suggested that box-home and shock-home control rats given

Table 1. Number of FLI-positive nuclei (mean ± SEM)

Brain area	Box-home	Shock-home	Shock-box
Allocortex			
Anterior cingulate cortex area 3	1.00 ± 0.38	1.00 ± 0.53	26.71 ± 8.30**
Posterior congulate cortex area 3	1.43 ± 0.61	0.43 ± 0.20	24.71 ± 4.20**
Piriform cortex	1.14 ± 0.51	0.57 ± 0.20	31.00 ± 4.67**
Dosrsopeduncular cortex	0.00 ± 0.00	0.57 ± 0.37	14.86 ± 4.51**
Infralimbic cortex	0.71 ± 0.29	0.86 ± 0.34	21.29 ± 5.41**
Lateral orbital cortex	0.29 ± 0.18	0.57 ± 0.37	17.43 ± 3.46**
Insular cortex	0.29 ± 0.18	0.14 ± 0.14	13.57 ± 3.88**
Retrosplenial cortex	0.71 ± 0.71	0.00 ± 0.00	17.43 ± 2.60**
Perirhinal cortex	0.00 ± 0.00	0.00 ± 0.00	13.14 ± 1.37**
Entorhinal cortex	0.00 ± 0.00	0.00 ± 0.00	4.71 ± 1.58**
Isocortex			
Frontal cortex area 1	0.14 ± 0.14	0.14 ± 0.14	6.71 ± 1.21**
Frontal cortex area 2	0.14 ± 0.14	0.14 ± 0.14	13.57 ± 3.88**
Forelimb cortex	0.00 + 0.00	0.14 ± 0.14	10.71 ± 6.40**
Hindlimb cortex	0.71 ± 0.71	0.00 ± 0.00	7.43 ± 1.53**
Parietal cortex area 2	0.14 ± 0.14	0.00 ± 0.00	6.71 ± 1.21**
Temporal cortex area 3	0.00 ± 0.00	0.14 ± 0.14	16.43 ± 3.11**
Occipital cortex area 2	0.00 ± 0.00	0.29 ± 0.29	12.29 ± 2.54**
Subcortical telencephalon			
Anterior olfactory n.	0.14 ± 0.14	1.14 ± 0.46	21.29 ± 6.46**
Tenia tecta	0.29 ± 0.18	1.00 ± 0.44	19.43 ± 3.88**
Anterior claustrum	1.86 ± 1.39	0.43 ± 0.20	24.71 ± 4.20**
Posterior claustrum	0.57 ± 0.43	1.57 ± 0.78	23.14 ± 5.97**
Dorsal endopiriform n.	0.29 ± 0.18	0.43 ± 0.20	17.86 ± 4.93**
Anterior dorsomedial striatum	0.00 ± 0.00	0.14 ± 0.14	3.86 ± 0.80**
Posterior dorsomedial striatum	0.00 ± 0.00	0.14 ± 0.14	8.29 ± 2.76**
N. accumbens shell	1.57 ± 0.57	2.14 ± 0.26	26.29 ± 5.36**
N. accumbens core	0.29 ± 0.29	0.71 ± 0.29	10.00 ± 3.14*
Olfactory tubercle	0.29 ± 0.29	0.14 ± 0.14	4.43 ± 1.27*
Islands of Calleja	1.00 ± 0.72	0.57 ± 0.30	0.86 ± 0.70
Lateral septal n.	0.71 ± 0.29	0.29 ± 0.18	15.00 ± 5.16**
Medial septal n.	0.29 ± 0.29	0.00 ± 0.00	0.14 ± 0.14
N. diagonal band	0.14 ± 0.14	0.00 ± 0.00	0.14 ± 0.14
Bed n. stria terminalis	0.00 ± 0.00	1.00 ± 0.38	7.43 ± 1.53**
Amygdala central n.	0.00 ± 0.00	0.14 ± 0.14	4.29 ± 1.29**
Amygdala basolateral n.	0.00 ± 0.00	0.00 ± 0.00	10.86 ± 2.69**
Amygdala basomedial n.	0.00 ± 0.00	0.00 ± 0.00	6.71 ± 1.80**
Amygdala cortical n.	0.00 ± 0.00	0.29 ± 0.18	11.57 ± 1.99**
Amygdalohippocampal area	0.00 ± 0.00	1.86 ± 1.86	15.71 ± 3.14**
Hippocampus dorsal dentate gyrus	1.14 ± 1.14	6.57 ± 4.77	7.00 ± 2.27*
Hippocampus ventral dentate gyrus	0.00 ± 0.00	0.29 ± 0.29	6.00 ± 1.73**
Hippocampus dorsal CA1 field	0.14 ± 0.14	0.14 ± 0.14	1.86 ± 0.86
Hippocampus ventral CA1 field	0.00 ± 0.00	0.00 ± 0.00	7.43 ± 0.75**
Thalamus			
Paraventricular n.	2.29 ± 1.36	0.29 ± 0.29	21.00 ± 4.79**
Central median n.	0.00 ± 0.00	0.00 ± 0.00	11.57 ± 1.41**
Lateral habenula	0.71 ± 0.71	3.29 ± 1.87	11.57 ± 5.46
Ventral lateral geniculate n.	6.57 ± 4.87	10.00 ± 4.34	24.86 ± 2.50
Posterior intralaminar n.	0.43 ± 0.43	0.57 ± 0.43	10.14 ± 1.71**
Hypothalamus			
Ventromedial n.	0.00 ± 0.00	0.00 ± 0.00	13.14 ± 2.87**
Lateral n.	0.00 ± 0.00	0.00 ± 0.00	10.71 ± 2.97**
Dorsal n.	0.00 ± 0.00	0.43 ± 0.43	16.71 ± 5.73**
Posterior n.	3.00 ± 2.67	0.14 ± 0.14	0.00 ± 0.00

Table 1. Continued

Brain area	Box-home	Shock-home	Shock-box
Brainstem			
Anterior pretectal n.	0.14 ± 0.14	1.14 ± 0.77	10.57 ± 1.15**
Ventral tegmental area	0.00 ± 0.00	1.14 ± 1.14	25.43 ± 3.11**
Substantia nigra pars compacta	0.00 ± 0.00	0.00 ± 0.00	3.00 ± 0.82**
Supramammillary area	0.00 ± 0.00	0.14 ± 0.14	21.29 ± 5.36**
Central gray	0.14 ± 0.14	0.57 ± 0.57	12.00 ± 3.25**
Dorsal raphe	0.57 ± 0.57	0.14 ± 0.14	1.86 ± 0.86
Median raphe	0.00 ± 0.00	0.00 ± 0.00	5.43 ± 1.11**
Pontine nuclei	6.57 ± 5.29	1.14 ± 1.14	16.43 ± 2.07*
Locus coeruleus	0.00 ± 0.00	0.00 ± 0.00	5.71 ± 2.19**
Lateral parabrachial n.	0.14 ± 0.14	1.14 ± 1.14	3.00 ± 2.67

n., nucleus. Following a significant ($p < 0.01$) within-structure group effect with Kruskal–Wallis 1-way ANOVA, groups ($n = 7$) were compared with Mann–Whitney U tests.

*Mann–Whitney $p < 0.01$, shock-box group mean greater than the lesser of the other two means.

**Mann–Whitney $p < 0.01$, shock-box group mean greater than both of the other two means.

the "home" treatment in the final session spent most of the time in the home cage motionless in a resting posture.

Comparison of the time course of the behavior of the box-home group and the shock-box groups on their last day in the test apparatus (day 4 and day 7, respectively) with two-way ANOVA with one repeated measure provided behavioral evidence for conditioning. Significant group by period interactions were obtained for the frequencies of crouch, locomote, turn, rear, sniff, and immobile (Fig. 1). Of the behaviors associated with the receipt of shock during shock sessions (flinch, jump, and crouch), only crouch was at a higher frequency in the conditioned (shock-box) group compared to the box-home control group [$F(1,12) = 29.28$, $p < 0.01$]. The incidence of crouching in the conditioned group decreased over the session, whereas crouching did not occur in the control group (Fig. 1). Further evidence of conditioning was found in the suppression of exploratory behaviors, locomote, turn, rear, and sniff, early in the session in the conditioned group relative to the box-home control group. Over the course of the session, the rats in the conditioned group became more active—that is, more locomotion, turning, rearing, and sniffing—and less inactive—that is, less crouching. By contrast, the animals in the control group exhibited progressively less locomotion, turning, rearing, and sniffing and more immobility and grooming [for grooming $F(5,60) = 4.58$, $p < 0.01$, data not shown]. Aside from crouch, the only behavior to yield a significant group effect was immobile [$F(1,12) = 18.96$, $p < 0.01$]. In summary, the effects of footshock conditioning were manifest in the conditioned group's high initial frequencies of crouching which subsequently declined and low initial levels of exploration which gradually increased. The control group decreased its activity level over the course of the session. Thus the groups differed not so much in overall activity levels but rather in the manner in which activity changed during the session.

Immunohistochemistry. Representative photomicrographs of FLI in the piriform cortex for the three groups are presented in Figure 2. FLI-positive nuclei appear as dark spots. Note the greater number of FLI-positive nuclei in the conditioned (shock-box) group.

Kruskal–Wallis one-way ANOVA was used to assess the significance of the group main effect for each anatomical structure. Significant group effects were followed by Mann–Whitney U tests to determine the significance of differences between treatment groups within structures. The mean FLI counts for the box-home and shock-home groups were generally low and did not differ significantly in any structure (Table 1), indicating that shock itself had little effect on *c-fos* expression 72 hr after the last shock. In contrast, compared to the box-home and shock-home control groups, exposure to the shock-associated environment increased *c-fos* expression in almost 50 cortical and subcortical structures (Table 1). With four exceptions (nucleus accumbens core, dentate gyrus of the dorsal hippocampus, olfactory tubercle, and pontine nuclei), the pattern of significant differences between the fear-conditioned group and the two control groups was identical (Table 1). In no instance were there more FLI-positive neurons in a brain structure of one of the control groups than in the fear-conditioned group.

Discussion

Experience prior to the final session had no apparent effect on the regional distribution of FLI-positive neurons if the animal was simply handled and transported to and from the test room on the final session. This was true even if the previous experience involved three sessions of footshock. Thus no differences in *c-fos* expression were found in any of 60 structures studied between the group habituated to the novelty of the box (the box-home group) and the group shocked in the box (the shock-home group).

The principal finding of behavioral significance was that neuronal activation as reflected by increased *c-fos* expression was significantly greater in the fear-conditioned group than in both the shocked and the unshocked controls in 47 of the 60 structures studied. These differences were not simply due to prior exposure to shock or to the stress involved in handling and transport because rats shocked chronically but exposed to the test room only momentarily on the final session exhibited low numbers of FLI-positive neurons. Clearly, therefore, the evocation of *c-fos* expression depended on the elicitation of conditioned fear by the contextual cues of the test apparatus. The presumption that fear conditioning did indeed occur is supported by the differences in behavior between the unshocked and the shock-conditioned rats. The critical difference was not one of overall sessional levels of behavior because the groups were similar in this regard. Rather, the salient distinction was in the intrasessional trends in behavior. Whereas the unshocked

group became less active as the session progressed, the conditioned group initially crouched and then later engaged in increased locomotion, sniffing, and rearing. Therefore, in contrast to the habituation of the control group, the conditioned group was engaging in a behavioral process that has been referred to as warm up (Golani, 1992).

The principal finding of anatomical significance was that 85% of the 60 structures surveyed exhibited increased *c-fos* expression in fear-conditioned rats. The fact that all of these changes involved increases rather than decreases relative to controls supports data from other studies of conditioned fear (Campeau et al., 1991; Pezzone et al., 1992) as well as data from studies involving unconditioned stress (Ceccatelli et al., 1989; Campeau et al., 1991; Chastrette et al., 1991; Sharp et al., 1991; Pezzone et al., 1992). Since previous reports presented only photomicrographic data from selected brain regions, the present study is the first to demonstrate that the neural substrates of conditioned fear are widely distributed anatomically and are quantifiable. The present study confirmed that *c-fos* expression is increased in a number of structures that have been reported in previous studies to be affected by conditioned stressors. These included various subdivisions of the amygdala (Campeau et al., 1991) such as the central and the basomedial nuclei (Pezzone et al., 1992). We also found activation in the cortical and basolateral amygdalar nuclei and the amygdalohippocampal area, regions not identified in previous studies. It is noteworthy that despite the demonstrated importance of the amygdala in fear and stress reactions (Davis, 1992), not all previous studies have reported increased *c-fos* expression in this structure following exposure to conditioned or even unconditioned stressors (cf. Arnold et al., 1992; Smith et al., 1992; Duncan et al., 1993). The circumstances under which stressors increase or fail to increase *c-fos* expression in the amygdala are presently not clear and require further definition.

Smith et al. (1992) observed increased *c-fos* mRNA in the cingulate and piriform cortices, and in the hippocampus, after exposure to a tone CS that had previously been paired with footshock. Conditioned stress-induced *c-fos* expression was confirmed in these structures in the present study (Table 1). With regard to the hippocampus, exposure to the conditioned stressor increased the number of FLI-positive neurons in the dentate gyrus of both the dorsal and ventral hippocampal formation but only in CA1 of the ventral hippocampus. Previous studies have reported increased *c-fos* expression in the cingulate cortex (Sharp et al., 1991; Smith et al., 1992; Duncan et al., 1993; Stone et al., in press), the piriform cortex (Sharp et al., 1991; Bing et al., 1992; Smith et al., 1992), and the hippocampal formation (Sharp et al., 1991; Smith et al., 1992) after exposure to unconditioned stressors.

Three hypothalamic nuclei were found in the present study to be activated by the conditioned stressor. These included the ventromedial nucleus, which has not been reported previously with either conditioned or unconditioned stressors, and the lateral hypothalamic nucleus and dorsal nucleus (both identified previously by Pezzone et al., 1992). Of particular interest is the fact that exposure to the conditioned stressor failed to increase *c-fos* expression in the paraventricular nucleus of the hypothalamus. This was unexpected in view of the fact that many studies have shown that acute exposure to unconditioned stressors increases FLI or *c-fos* mRNA in various subdivisions of this nucleus (Ceccatelli et al., 1989; Chastrette et al., 1991; Sharp et al., 1991; Arnold et al., 1992; Bing et al., 1992; Pezzone et al., 1992; Duncan et al., 1993; Senba et al., 1993). However, in the context of the present negative findings, it is interesting that Smith et al. (1992) have recently reported that neither conditioned nor unconditioned stressors increase *c-fos* mRNA in the paraventricular nucleus of rats that have previously been exposed to footshock, an effect that they attributed to desensitization of the *c-fos* response. The present results are entirely consistent with this finding.

Pezzone et al. (1992) reported that conditioned and unconditioned stressors increase FLI in unspecified regions of the "basal ganglia." In the present study, conditioned fear increased *c-fos* expression only in the dorsomedial striatum. The lateral septal nucleus was potently activated by the conditioned stressor (Table 1) and this is in agreement with many previous reports of increased *c-fos* expression in the lateral (particularly ventrolateral) septal nucleus after exposure to either conditioned (Pezzone et al., 1992; Smith et al., 1992) or unconditioned stressors (Sharp et al., 1991; Arnold et al., 1992; Pezzone et al., 1992; Smith et al., 1992; Duncan et al., 1993; Senba et al., 1993). Other structures in which *c-fos* expression has been reported to be increased by exposure to a conditioned stressor include the medial dorsal thalamus and the supraoptic nucleus of the hypothalamus (Pezzone et al., 1992). In the present study, these structures contained such low numbers of FLI-positive neurons that they were not counted. The medial septal nucleus and the nucleus of the diagonal band were included as examples of such low levels of FLI (Table 1). In summary, the present results confirmed previous reports that conditioned fear is accompanied by increased *c-fos* expression in a subset of brain regions including allocortical regions, the amygdala, dorsomedial striatum, lateral septal nucleus, and hypothalamus.

In the present study many structures that have not been identified previously (Pezzone et al., 1992; Smith et al., 1992) exhibited increased *c-fos* expression after exposure to an environment that had previously been paired with footshock. However, exposure to unconditioned stressors has been reported to increase *c-fos* expression in some of these structures. Since unconditioned and conditioned stress often appear to produce similar effects in certain structures (Campeau et al., 1991; Pezzone et al., 1992; Smith et al., 1992), it is interesting to compare the unconditioned effects reported previously with the conditioned effects observed in the present study. Below, these structures are followed by references; structures without references have neither corroboration nor refutation in the literature: *in the allocortex,* the dorsopeduncular cortex, infralimbic, lateral orbital (Duncan et al., 1993), insular, retrosplenial, perirhinal, and entorhinal areas (Bing et al., 1992); *in the isocortex,* the frontal areas 1 and 2 (Sharp et al., 1991, but did not distinguish neocortical areas; Bing et al., 1992; Stone et al., in press), parietal area 2, temporal area 3, and occipital area 2; *in the subcortical telencephalon,* the tenia tecta, anterior and posterior claustrum (Duncan et al., 1993); dorsal endopiriform nucleus (Smith et al., 1992), shell and core of the nucleus accumbens (Arnold et al., 1992), and the bed nucleus of stria terminalis (Sharp et al., 1991; Arnold et al., 1992); *in the thalamus,* the central median (Senba et al., 1993), paraventricular nucleus (Chastrette et al., 1991; Sharp et al., 1991; Senba et al., 1993), and posterior intralaminar nuclei (Sharp et al., 1991; Senba et al., 1993); *in the hypothalamus,* the ventromedial, nucleus; and *in the brainstem,* the anterior pretectal nucleus, ventral tegmental area, substantia nigra pars compacta, supramammillary area, central gray, median raphe, and the locus coeruleus (Ceccatelli et al., 1989;

Bing et al., 1992; Senba et al., 1993). Two of the cited studies examined *c-fos* mRNA by *in situ* hybridization (Sharp et al., 1991; Smith et al., 1992), while the others utilized Fos immunohistochemistry.

Several differences in procedure may have accounted for the greater number of sites exhibiting increased *c-fos* expression in the present study compared to two previous fear-conditioning studies (Pezzone et al., 1992; Smith et al., 1992). These include differences in rat strains (Szechtman et al., 1982), animal housing (Willner et al., 1989), shock intensity (Rescorla, 1974), and the use of explicit versus contextual cues (Selden et al., 1991). Although housing in isolation is stressful for rats (Willner et al., 1989), it was not a methodological difference between the present study and that of Pezzone et al. (1992) because both studies used individual housing. Conversely, housing rats in groups did not preclude the forementioned agreement on a number of structures of Smith et al. (1992) with our study and that of Pezzone et al. (1992). In the present study, the rats received 150 contextually conditioned shocks per day over 3 d compared to 10 explicitly cued shocks per day for 2 d (Pezzone et al., 1992) or 30 explicitly cued shocks per day for 5 d (Smith et al., 1992). It is possible that the greater number of shocks over a more extended period in the present study increased the likelihood of an augmentation in *c-fos* expression. In addition, aversive conditioning of contextual cues and explicit cues may utilize different brain structures (Selden et al., 1991). Any or all of these procedural differences may be important for replication. To sum up, in the present study many previously unrecognized structures were identified as showing increased *c-fos* expression after exposure to a conditioned stressor. The wide distribution of structures with FLI-positive neurons imposes limitations on the external validity of studies which assess *c-fos* effects in only a few structures.

Experiment 2

Materials and methods

Animals, apparatus, and protocol. Thirty-two male Long–Evans rats (Charles River, Montreal) weighed 311 ± 16 gm at the beginning of the experiment. The animal housing arrangement and the apparatus were as described for experiment 1.

Prior to the beginning of the experiment, the animals were adapted to handling and intraperitoneal insertions of a hypodermic needle. On each of the first 3 d of the experiment (days 1, 2, 3) the rats were treated individually to 30 min sessions of inescapable footshocks. The shock parameters within a session were as noted in experiment 1. Subsequently, the rats were matched for the time spent crouching in the third session and assigned in a quasi-random fashion to one of four groups (each $n = 8$): a vehicle group (64% soy bean oil, 21% acetylated monoglyceride, 10% glycerol, 5% egg phospholipid in a volume of 1 ml/kg) and three diazepam groups (Diazemuls, KablVitrum, Newmarket ON, Canada, in same vehicle, at a concentration of 5 mg/ml, and one of three doses: 2.5, 5.0, or 10.0 mg/kg). On day 8, 5 d after the third shock session, each rat was given an intraperitoneal injection 40 min before the start of the test session, the injectate and dose being according to their group assignment. This final session was a 30 min session in the shock box without shock. Thus these groups were treated the same as the conditioned shock group of experiment 1, except for the injections and the 5 d postconditioning interval.

Behavioral coding and immunohistochemistry. The behavior of the rats during the sessions in the box on day 3 (shocked) and day 8 (not shocked) was coded continuously by a trained observer. The behavioral events and their times were scored as in experiment 1 with the addition of one category: *oral,* signifying licking or gnawing the experimental chamber. Test-retest and interobserver reliability measures of coding produced agreements greater than 84% and all yielded significant coefficients of concordance.

Two hours after the beginning of the test session on day 8, the animals were given an overdose of sodium pentobarbital and perfused with saline and 4% paraformaldehyde. The procedures for sectioning, immunohistochemistry, and data analysis were as in experiment 1.

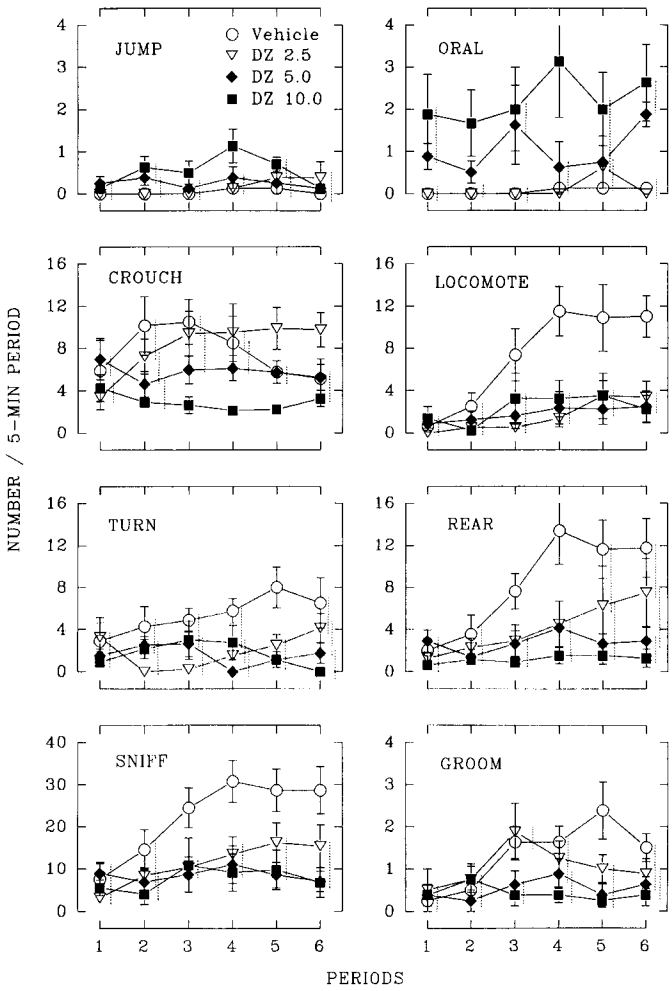

Figure 3. Frequencies of behaviors over six 5 min periods of the final test session, day 8, for groups ($n = 8$) receiving vehicle (○), diazepam 2.5 mg/kg (▽), diazepam 5.0 mg/kg (◆), diazepam 10.0 mg/kg (■). *Vertical dashed lines* join means that are not significantly different (Tukey, $p < 0.05$). ANOVA group effects with df = 3,28; jump $F = 3.28$, $p < 0.05$; oral $F = 3.76$, $p < 0.05$; crouch $F = 4.52$, $p < 0.05$; locomote $F = 7.35$, $p < 0.01$; turn $F = 4.86$, $p < 0.01$; rear $F = 5.95$, $p < 0.01$; sniff $F = 4.86$, $p < 0.01$; groom $F = 5.27$, $p < 0.01$. ANOVA group by period interactions with df = 15,140; jump $F = 0.82$, $p > 0.05$; oral $F = 0.54$, $p > 0.05$; crouch $F = 2.02$, $p < 0.05$; locomote $F = 2.40$, $p < 0.01$; turn $F = 2.32$, $p < 0.01$; rear $F = 2.24$, $p < 0.01$; sniff $F = 2.23$, $p < 0.01$; groom $F = 1.81$, $p < 0.05$.

Results

Behavior. Two-way ANOVA of the four groups' behavioral frequencies over the six periods of the shock session on day 3 revealed no significant group, period, or group × period effects, thus confirming the effectiveness of the matching procedure. The absence of behavioral change over the course of the session indicates that the chronic footshock regimen had produced a stable within-session pattern of behavior, all groups exhibiting a constant high frequency of crouching.

Two-way ANOVA of group and period behavioral effects in the no-shock session of day 8 revealed significant interaction effects for the frequency of all of the behaviors except flinch and immobile (Fig. 3). Tukey tests indicated that compared to ve-

Table 2. Number of FLI-positive nuclei (mean ± SEM)

Brain area	Vehicle	Diazepam 2.5 mg/kg	Diazepam 5.0 mg/kg	Diazepam 10.00 mg/kg
Allocortex				
Anterior cingulate cortex area 3	19.38 ± 2.93	13.13 ± 1.97	8.38 ± 2.19*	7.88 ± 1.78*
Posterior cingulate cortex area 3	11.13 ± 3.22	9.13 ± 1.38	5.88 ± 1.55	4.00 ± 1.02*
Piriform cortex	23.13 ± 4.08	16.88 ± 3.11	11.25 ± 2.76*	7.13 ± 1.38**
Dorsopeduncular cortex	13.25 ± 2.37	11.88 ± 2.01	7.63 ± 2.03	5.63 ± 1.13*
Infralimbic cortex	22.00 ± 3.16	15.75 ± 1.50	12.00 ± 2.04*	10.88 ± 1.53**
Lateral orbital cortex	6.63 ± 1.51	7.75 ± 2.41	4.25 ± 1.49	3.63 ± 1.16*
Insular cortex	0.25 ± .016	0.75 ± 0.31	0.75 ± 0.25	4.75 ± 2.72
Retrosplenial cortex	10.25 ± 2.93	3.38 ± 0.84*	2.00 ± 0.82*	2.75 ± 1.11*
Perirhinal cortex	14.75 ± 2.45	15.63 ± 3.35	10.88 ± 2.38	7.75 ± 1.16*
Entorhinal cortex	6.50 ± 1.75	7.13 ± 1.78	5.50 ± 2.36	3.38 ± 1.13
Isocortex				
Frontal cortex area 1	1.63 ± 0.78	1.38 ± 0.65	1.25 ± 0.65	0.75 ± 0.31
Frontal cortex area 2	2.25 ± 0.59	0.88 ± 0.40	2.13 ± 0.83	0.88 ± 0.40
Forelimb cortex	2.38 ± 0.98	0.25 ± 0.16	1.00 ± 0.60	075 ± .049
Hindlimb cortex	2.50 ± 0.60	1.75 ± 0.62	1.50 ± 0.85	1.75 ± 1.06
Parietal cortex area 2	9.63 ± 1.45	0.38 ± 0.18	0.13 ± 0.13	0.88 ± 0.18
Temporal cortex area 3	0.50 ± 0.19	7.13 ± 1.82	6.63 ± 0.92	3.63 ± 0.78**
Occipital cortex area 2	19.38 ± 6.30	11.38 ± 2.23	10.38 ± 2.88	5.75 ± 1.06**
Subcortical telencephalon				
Anterior olfactory n.	14.38 ± 2.35	9.75 ± 2.81	7.75 ± 1.94	5.50 ± 0.87**
Tenia tecta	13.38 ± 2.04	10.88 ± 2.65	5.75 ± 1.32**	5.50 ± 1.18**
Anterior claustrum	17.50 ± 5.55	7.50 ± 1.20	6.88 ± 3.23	4.13 ± 1.34**
Posterior claustrum	14.38 ± 3.71	10.50 ± 2.23	4.75 ± 1.79*	5.13 ± 1.34*
Dorsal endopiriform n.	11.88 ± 1.57	11.63 ± 1.57	5.88 ± 0.93*	6.38 ± 1.08*
Anterior dorsomedial caudate	3.88 ± 1.01	4.50 ± 0.76	2.13 ± 0.90	1.13 ± 0.52*
Posterior dorsomedial caudate	7.13 ± 0.64	5.75 ± 1.62	2.88 ± 0.85**	3.25 ± 0.56**
N. accumbens shell	15.50 ± 3.08	13.50 ± 2.91	7.25 ± 3.64*	6.75 ± 2.33*
N. accumbens core	5.75 ± 1.45	6.88 ± 1.72	3.50 ± 2.03	4.13 ± 1.36
Olfactory tubercle	2.75 ± 0.45	2.38 ± 0.63	0.50 ± 0.19**	0.63 ± 0.32**
Islands of Calleja	8.00 ± 5.18	4.13 ± 2.19	3.88 ± 1.83	2.00 ± 1.25
Lateral septal n.	17.13 ± 4.43	9.38 ± 2.40	9.63 ± 2.80	6.13 ± 2.26*
Medial septal n.	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
N. diagonal band	0.13 ± 0.13	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Bed n. stria terminalis	6.75 ± 2.79	5.00 ± 1.51	3.13 ± 0.61	3.38 ± 1.05
Amygdala central n.	5.88 ± 2.73	15.00 ± 2.67*	18.75 ± 3.37*	20.00 ± 3.04**
Amygdala basolateral n.	6.25 ± 2.24	5.25 ± 2.07	3.00 ± 1.22	2.63 ± 1.36
Amygdala basomedial n.	4.75 ± 1.79	5.25 ± 1.76	2.38 ± 0.89	2.13 ± 0.64
Amygdala cortical n.	9.38 ± 1.53	8.88 ± 1.95	7.38 ± 2.29	5.38 ± 0.92*
Amygdalohippocampal area	16.13 ± 5.49	8.38 ± 1.45	2.38 ± 0.64**	2.13 ± 0.64**
Hippocampus dorsal dentate gyrus	4.13 ± 0.48	3.75 ± 1.33	2.50 ± 0.63	1.25 ± 0.49**
Hippocampus ventral dentate gyrus	5.13 ± 1.71	3.75 ± 1.05	1.13 ± 0.64	1.00 ± 0.73*
Hippocampus dorsal CA1 field	0.75 ± 0.16	0.38 ± 0.26	0.38 ± 0.26	0.50 ± 0.38
Hippocampus ventral CA1 field	4.50 ± 1.12	3.63 ± 1.10	3.13 ± 1.11	2.50 ± 0.82**
Thalamus				
Paraventricular n.	32.50 ± 4.44	32.88 ± 5.55	25.75 ± 7.18	18.13 ± 4.62*
Central median n.	8.50 ± 2.77	6.25 ± 1.40	8.00 ± 3.60	3.75 ± 1.79
Lateral habenula	25.25 ± 6.58	11.38 ± 2.97	9.38 ± 3.15*	9.13 ± 3.15*
Ventral lateral geniculate n.	22.38 ± 3.55	14.25 ± 2.53	10.50 ± 3.16*	7.88 ± 2.82**
Posterior intralaminar n.	5.13 ± 0.83	4.25 ± 1.45	1.75 ± 0.86*	1.50 ± 0.63**
Hypothalamus				
Ventromedial n.	4.00 ± 1.18	4.25 ± 1.41	3.75 ± 1.73	2.88 ± 0.67
Lateral n.	8.25 ± 1.88	7.38 ± 1.78	3.63 ± 1.05	2.88 ± 1.54*
Dorsal n.	1063 ± 2.34	9.00 ± 1.64	4.75 ± 1.58	3.25 ± 1.40**
Posterior n.	5.38 ± 0.91	5.00 ± 1.36	3.50 ± 0.89	3.25 ± 0.84

Table 2. Continued

Brain area	Vehicle	Diazepam 2.5 mg/kg	Diazepam 5.0 mg/kg	Diazepam 10.00 mg/kg
Brainstem				
Anterior pretectal n.	4.63 ± 1.41	2.13 ± 0.52	2.75 ± 2.09*	1.13 ± 0.79*
Ventral tegmental area	12.75 ± 2.19	12.38 ± 3.16	10.00 ± 2.61	4.50 ± 1.02**
Substantia nigra pars compacta	1.88 ± 0.67	1.38 ± 0.71	0.75 ± 0.62	0.75 ± 0.62
Supramammillary area	27.38 ± 6.91	13.13 ± 3.29*	7.63 ± 2.40*	3.75 ± 1.29**
Central gray	18.50 ± 3.38	12.25 ± 2.95	8.50 ± 1.97*	7.00 ± 1.90*
Dorsal raphe	1.50 ± 0.27	0.63 ± 0.42	1.75 ± 0.41	0.75 ± 0.41
Median raphe	7.25 ± 1.41	8.38 ± 2.92	2.88 ± 1.01*	4.25 ± 2.37
Pontine nuclei	20.38 ± 3.49	16.38 ± 4.23	7.75 ± 2.99*	7.25 ± 3.58*
Locus coeruleus	5.13 ± 3.23	4.38 ± 0.68	4.63 ± 0.68	3.25 ± 1.08
Lateral parabrachial n.	21.00 ± 5.66	16.63 ± 4.05	18.75 ± 5.52	7.75 ± 2.39

n., nucleus. Following a significant ($p < 0.05$) within-structure group effect with Kruskal–Wallis 1-way ANOVA, groups ($n = 8$) were compared with Mann–Whitney U tests.

*Mann–Whitney $p < 0.05$, compared to vehicle group.

*Mann–Whitney $p < 0.01$, compared to vehicle group.

hicle controls, diazepam-treated rats exhibited increased jump and oral, and decreased crouch, locomote, turn, rear, sniff, and groom (Fig. 3). Thus, the diazepam-treated rats were less active than vehicle-pretreated rats, except for their increased frequency of jump and oral. These appeared to be escape behaviors since the jumping was toward the entry way (box lid) and the oral activity took the form of vigorous attempts to bite through the floor bars. These behaviors do not support the suggestion that the decreased locomotion of the rats given the highest dose of diazepam was due to sedation.

Immunohistochemistry. The vehicle group in this experiment was treated the same as the conditioned shock group (shock-box) in the first experiment except that animals in the latter did not receive saline injections and waited 3 d rather than 5 d between the last shock session and the test session. To assess the degree of replication, we compared the ANOVA, Mann–Whitney pattern of group differences between the two control groups of experiment 1 (box-home and shock-home) with the conditioned shock groups of the two experiments. Only five of the 60 structures produced disagreements. These comparative statistics are not presented but may be approximated by scanning the respective columns in Tables 1 and 2. Of the five discrepant structures, the parabrachial nucleus had a significant increase in FLI-positive neurons in the conditioned group in experiment 2 but not experiment 1. The opposite was true of the insular, frontal (areas 1 and 2), and parietal cortical areas and the substantia nigra. The failure to replicate increases in *c-fos* expression in these latter regions may have been due to the longer training–test interval in experiment 2. However, it also raises doubts about the reliability of the effects of the conditioning procedure on *c-fos* expression in these regions. In sum, the effects of conditioned shock on *c-fos* expression were replicated in 55 out of 60 structures.

With few exceptions, in all regions in which *c-fos* expression was increased by exposure to the shock-paired environment, diazepam produced a dose-related inhibition of this response (Figs. 4, 5; Table 2). However, this inhibition was statistically significant in only 38 out of the 60 sampled sites, the remainder showing varying degrees of drug-induced nonsignificant decreases in the number of stress-induced FLI-positive neurons. Six structures in which diazepam did not produce statistically significant effects appeared to be particularly resistant to the effects of the drug. These were the entorhinal cortex, nucleus accumbens core, ventromedial and posterior nuclei of the hypothalamus, median raphe nucleus, and the locus coeruleus (Table 2). Among the areas in which diazepam produced statistically significant decreases in *c-fos* expression, the retrosplenial cortex and the supramammillary area were particularly sensitive, both showing significant effects at the lowest dose (2.5 mg/kg) of diazepam. Twenty structures showed statistically significant inhibition of stress-induced *c-fos* expression at the middle dose (5 mg/kg), and in 18 regions diazepam produced significant effects only at the highest (10 mg/kg) dose. There was considerable variability, therefore, in the regional potency of diazepam with respect to inhibition of the conditioned fear-induced increases in *c-fos* expression. In only one structure, the central nucleus of the amygdala, did diazepam produce statistically significant increases in the stress-induced *c-fos* expression. It is noteworthy that the other amygdalar nuclei were quite resistant to the effects of diazepam (Table 2).

Discussion

Behavioral effects. The behavioral data confirm that the foot-shock conditioning was effective in inducing a stable high frequency of crouching. In the rat, this behavior is considered to be an indicator of fear in situations in which aversive stimulation is inescapable (Blanchard et al., 1968; Blanchard and Blanchard, 1969). It is therefore appropriate to interpret the decrease in crouching produced by diazepam as an anxiolytic effect (Sachs et al., 1966). Benzodiazepines have been observed to decrease freezing in other situations as well (Hard et al., 1985; Boix et al., 1986).

In addition to decreasing the incidence of freezing, diazepam increased escape behavior [chewing on the bars (oral) and jumping] while preventing the gradually emerging increases in exploration (locomote, rear, sniff) and grooming seen in the vehicle-treated rats. This is in agreement with the hypothesis that a common manifestation of benzodiazepine-induced anxiolysis is to reduce behavioral variability by enhancing initial reactions (Loh and Beck, 1989). Escape behavior has been identified as

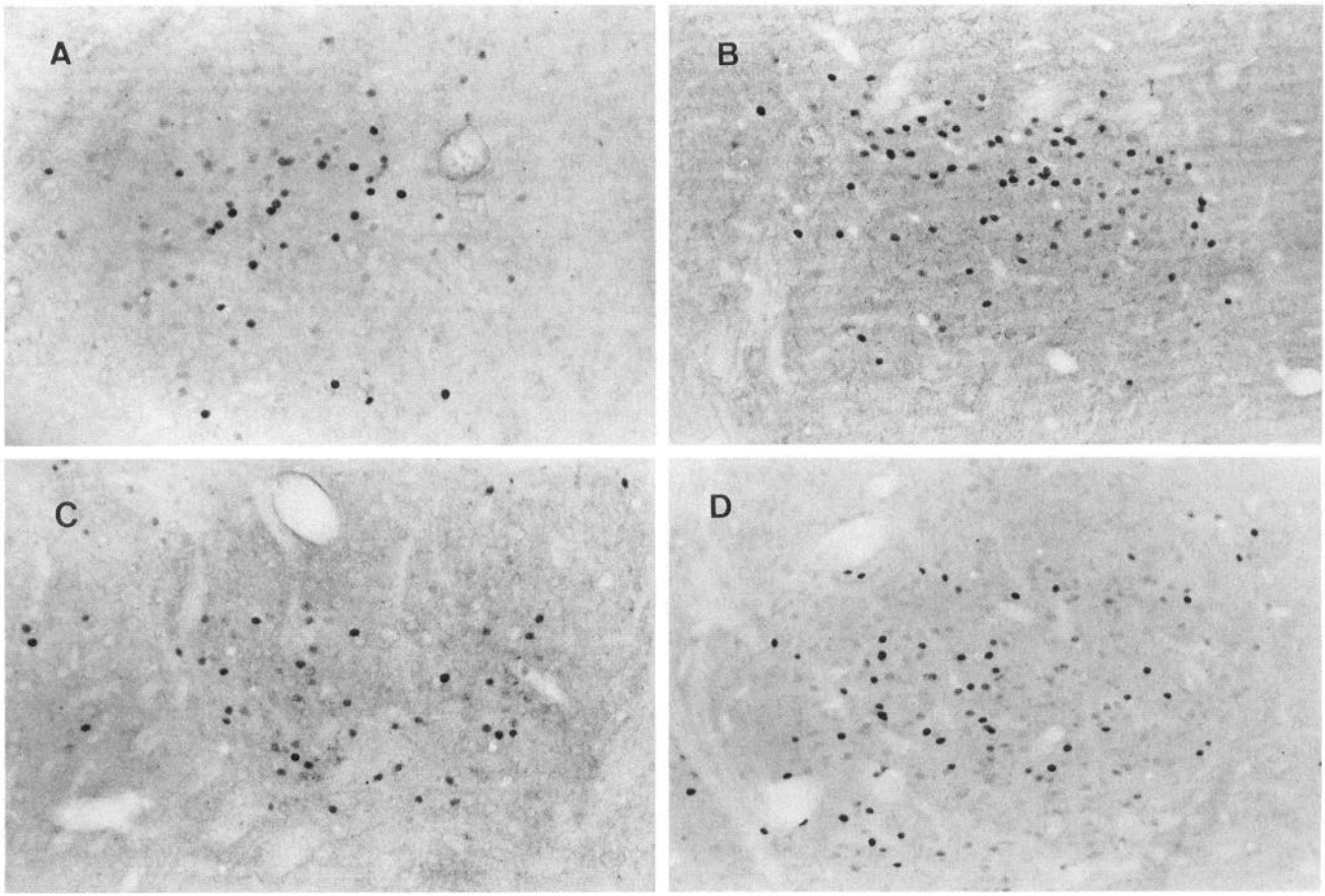

Figure 4. Fos-like immunoreactivity in the central nucleus of the amygdala after pretreatment with vehicle (*A*), diazepam 2.5 mg/kg (*B*), diazepam 5.0 mg/kg (*C*), and diazepam 10.0 mg/kg (*D*). This was the only structure in which diazepam significantly increased fear-induced *c-fos* expression.

a stronger initial response bias than freezing in rats exposed to stressful situations (Bolles, 1970). In summary, diazepam reduced the frequency of conditioned freezing and of exploratory behaviors in favor of a prepotent tendency to escape.

Immunohistochemistry. Diazepam attenuated or blocked fear-induced *c-fos* expression in all but one of the structures examined. The only previous evidence that benzodiazepines can reduce increases in neuronal *c-fos* expression comes from a report on the anticonvulsant effects of diazepam (Morgan et al., 1987). The present results extend this finding to include conditioned responses produced by contextual stimuli. A particularly interesting effect of diazepam was observed in the central amygdalar nucleus where the drug actually increased the number of FLI-positive neurons induced by fear conditioning. There is ample evidence that this nucleus is involved in conditioned fear (Davis, 1992). However, it is possible that the diazepam-induced increase in *c-fos* expression in the central nucleus of the amygdala is not related to the effects of the drug on conditioned fear. This is supported by the observation that benzodiazepines continue to produce anxiolytic effects in animals with lesions of the amygdala (Yadin et al., 1991).

The relative densities of benzodiazepine (clonazepam and flunitrazepam) binding in the rat brain have been reported for many of the structures examined in the present experiments (Young and Kuhar, 1980; Richards and Mohler, 1984). A review of these data indicates that 30 of the 35 structures showing both conditioned fear and diazepam pretreatment effects on *c-fos* expression have moderate to high densities of benzodiazepine receptors. Nevertheless, the relationship between benzodiazepine receptor density and the extent to which diazepam decreased fear-induced *c-fos* expression does not appear to be strong inasmuch as the two regions which showed the greatest sensitivity to the drug (retrosplenial cortex and supramammillary area) have only moderate numbers of these receptors. In addition, some of the structures showing increased *c-fos* expression were not significantly affected by diazepam pretreatment despite having high densities of benzodiazepine receptors (e.g., insular cortex, locus coeruleus). Thus, it is apparent that an absence of statistically significant changes in *c-fos* expression in response to diazepam is not due to a paucity of benzodiazepine receptors in that structure. In addition, the clear behavioral effects of the highest dose of diazepam argue against the possibility that this dose was subthreshold.

It is noteworthy that in the lateral habenula and the ventral lateral geniculate nucleus diazepam decreased *c-fos* expression, despite the fact that the experiment 1 fear conditioning did not produce statistically significant effects, due perhaps to the rather high variability in FLI. In the posterior intralaminar and ventrolateral geniculate nuclei, FLI-positive neurons formed scattered lines laterally through the intralaminar nuclei and then moved to a dorsal course through the magnocellular portion of the ventrolateral geniculate giving the impression of cell bodies intermittently distributed along a pathway. Candidates for such a pathway include thalamostriatal and thalamocortical projec-

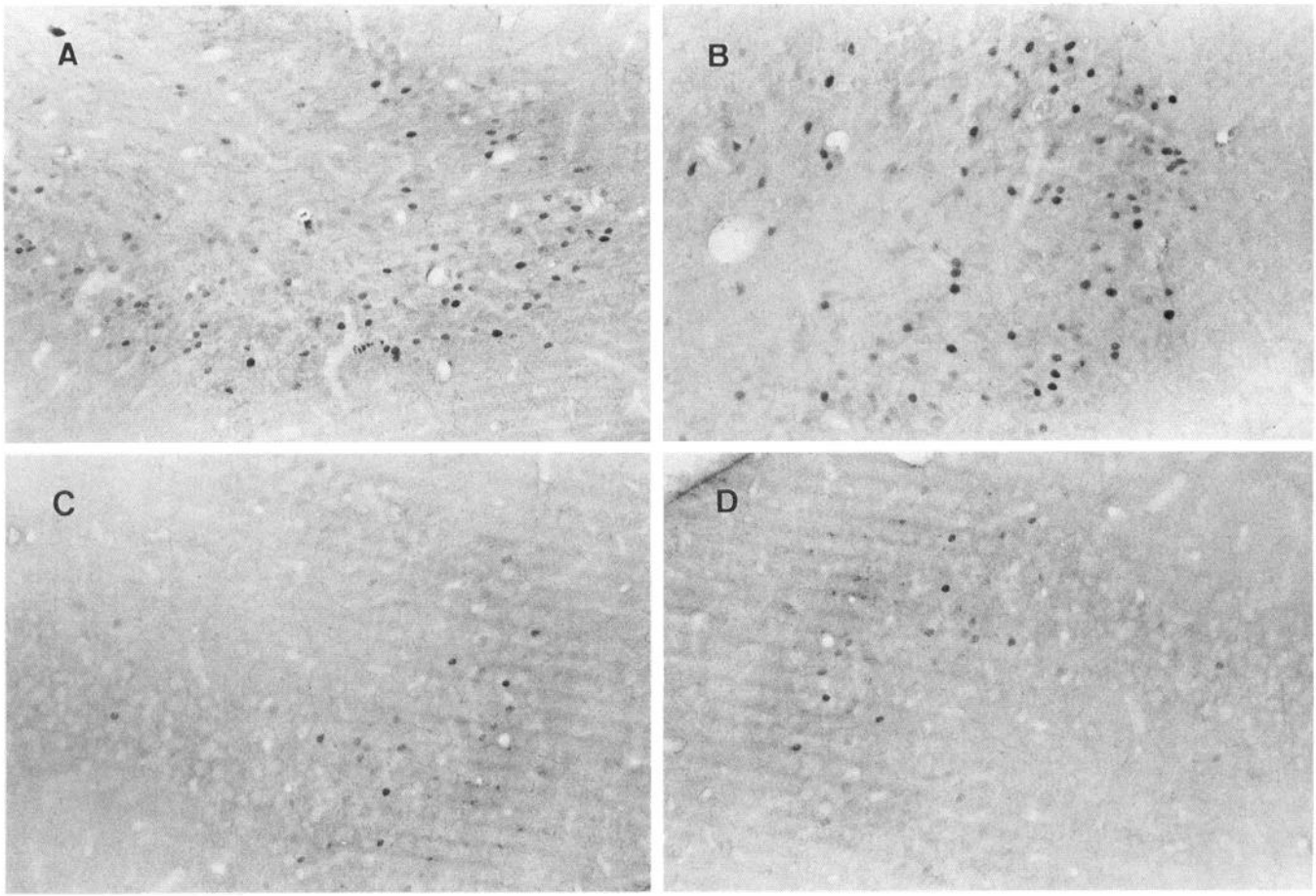

Figure 5. Fos-like immunoreactivity in the piriform cortex after pretreatment with vehicle (*A*), diazepam 2.5 mg/kg (*B*), diazepam 5.0 mg/kg (*C*), and diazepam 10.0 mg/kg (*D*). This was one of 38 structures in which diazepam produced significant decreases in fear-induced *c-fos* expression.

tions from the intralaminar nucleus (Nauta et al., 1974; Herkenham, 1980) and noradrenergic afferents from locus coeruleus to the ventrolateral geniculate (Pasquier and Villar, 1982).

Among the structures surveyed that showed no change in *c-fos* expression in either experiment were the islands of Calleja, medial septal nucleus, nucleus of the diagonal band, CA1 field of the dorsal hippocampus, posterior hypothalamus, dorsal raphe, and lateral parabrachial nucleus. These structures exhibited either high variability in *c-fos* expression (islands of Calleja and the lateral parabrachial nucleus), or low FLI (the remaining areas). The variability in the lateral parabrachial nucleus, a major relay center for the transfer of autonomic information, is interesting because of its involvement in the mediation of respiratory and pressor effects of stress (Frysinger et al., 1988). It would be worth determining whether variability in the *c-fos* expression in this structure was related to individual differences in physiological responses to the conditioned stressor. It is also noteworthy in this regard that Krukoff et al. (1992) have recently demonstrated that electrical stimulation of the parabrachial nucleus, at current intensities that produced significant increases in mean arterial pressure in anesthetized rats, increased the number of FLI-positive neurons in a number of structures that were shown in the present study to be responsive to aversively conditioned contextual cues. These included the central nucleus of the amygdala, the endopiriform nucleus, the insular cortex, and the piriform cortex. This raises the possibility that the increases in *c-fos* expression observed in these structures were related to centrally mediated changes in cardiovascular function produced by exposure to the conditioned stressor.

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