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Chronic Desipramine Alters Stress-Induced Behaviors and Regional Expression of the Immediate Early Gene, *c-fos*

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BECK, C. H. M. AND H. C. FIBIGER. *Chronic desipramine alters stress-induced behaviors and regional expression of* c-fos. PHARMACOL BIOCHEM BEHAV **51**(2/3) 331-338, 1995.—This experiment examined the effects of acute or chronic administration of the antidepressant drug desipramine on conditioned stress-induced behaviors and regional *c-fos* expression in the brain. To this end, rats were exposed to three sequential daily sessions of uncontrollable foot-shock and matched, on the basis of crouching, into one of four groups. Two of these groups were exposed to saline injections twice daily and two were exposed to injections of desipramine (5 mg/kg, SC) twice per day, for 9 days. On the 10th day one of the saline groups received saline and the other received desipramine before being exposed to the shock chamber without shock. Likewise, on the 10th day one of the desipramine groups received saline and the other received desipramine before being exposed to the shock chamber without shock. Detailed behavioral analysis showed that compared to the saline-treated controls only the group treated chronically with desipramine, including on the test day, exhibited statistically significant reductions in crouching and increases in exploration during the test session. Similarly, Fos immunohistochemistry revealed that the chronic desipramine group showing positive behavioral effects was the only group in which there were significant reductions in the number of stress-induced Fos-positive neurons in five of 60 structures surveyed. These structures included the anterior cingulate cortex, anterior claustrum, central nucleus of the amygdala, dentate gyrus of the dorsal hippocampus, and paraventricular nucleus of the thalamus. To the extent that repeated exposure to uncontrollable stress is an animal model of depression, these and previous results suggest that these structures are potentially important neural targets for the antidepressant effects of desipramine.

Antidepressant Desipramine Stress Conditioning Fear Behavior Ethologic analysis
Immediate early gene *c-fos* Brain Limbic Amygdala Rat

MOST animal models for testing antidepressant effects do not meet empirical standards of reliability and validity (67). As we wished to conduct a study of the behavioral and immunohistochemical effects of chronic antidepressant treatment, the selection of the most appropriate stressor and measure of stress was a major concern. Electric foot-shock stands out as the only procedure that is involved in two models possessing acceptable standards, learned helplessness (40) and unpredictable stress (51). The ability of foot-shock to cross-sensitize to other stressors and potentiate antidepressant effects also suggests that foot-shock may tap into a final common path to depression (51 64). Psychomotor retardation is one several alternate diagnostic criteria of major depressive episodes (1). Congruently, motor inactivation is a characteristic behavioral consequence of several stress models of depression in animals, including those involving foot-shock (44,51,60,63). Chronic administration of antidepressant drugs reduces the inactivity generated with these models (40,44,51,60). Given these facts,

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we adopted foot-shock as the stressor and inactivity as the principal measure for our study.

Although several studies have examined the neuroanatomical effects of shock stress on the brain (7,17,21,42) and others have assessed the effects of chronic antidepressant treatment on the brains of untreated animals (27,35,46,58), few studies have described the effects of antidepressants on the brains of animals subjected to uncontrollable foot-shock. The latter type of study will be dealt with first because it relates more directly to the issue here.

The medial prefrontal cortex, hippocampus, and lateral geniculate body have been implicated in the alleviation of a shock-induced deficit by intracranial administration of desipramine (41,56). Systemic imipramine treatment reversed shock-induced release of serotonin from frontal cortex and septal slices, and of GABA from hippocampal slices (57). In vivo microdialysis has revealed an imipramine reversal of K^+-stimulated 5-HT release from the medial frontal cortex of shock-stressed rats (39). The serotonin pathway to the frontal cortex and septal area arises in the raphe nuclei (10), whereas the GABA response of the hippocampus may be mediated by local interneurons or by cells projecting from the medial septal nucleus (24). More tangentially, studies of antidepressant treatment in unstressed animals have implicated a variety of neurotransmitter systems including serotonergic (11,22), GABAergic (15,26), noradrenergic (3,36,45,53,62,68), cholinergic (45), enkephalinergic (12,47), dopaminergic (13,35,48), and corticotropin-releasing factor pathways (20), and neuropeptide Y pathways (58).

Given the diversity of putative pathways and structures, the present study, sought to provide a detailed anatomical assessment of structures involved in the effects of antidepressant treatment of stressed rats. Rats subjected to uncontrollable foot-shock were treated chronically with saline or the antidepressant desipramine and tested for recovery from the inactivating effects of shock in a contextual conditioning paradigm. The behavioral effects of the drug treatment on conditioning were assessed by a detailed ethologic description of the animal's activity. At a cellular level, conditioning increases the number of neurons containing Fos, the protein product of the immediate early gene *c-fos*, in active brain structures (7, 42,59). After the conditioning session, the animals were killed and their brains reacted histochemically for Foslike immunoreactivity at 60 different neuroanatomical sites. The sites were chosen partly based on preliminary studies conducted in this laboratory and partly on the literature (7,9,17,21,42,54,59).

Behavioral therapeutic effects of chronic antidepressant treatment have been reported more frequently when the final injection is on the day of the test session (5,28,40,41,43,55,61) than when it is 24 h before the test session (25). The latter was the preferred procedure in the present study because of the recognition that stressful events can influence *c-fos* reactivity acutely (54). To provide comparative data for the more prevalent methodology, another experimental group of stressed animals was given desipramine chronically, including the last day. To control for potential same-day effects of desipramine on *c-fos* reactivity, a group of stressed animals treated chronically with saline and acutely with desipramine on the last day was included. A chronic desipramine group of unstressed animals was not included because we knew from unpublished work that chronic desipramine had the same effect on Fos reactivity as chronic saline: essentially none. This supports the finding that chronic desipramine treatment has no effect on a test of learned helplessness in unstressed animals (25).

METHOD

Animals

We used 28 male Long-Evans rats (Charles River, Montreal) weighing 285 ± 15 g (mean ± SE) at the beginning of the experiment. The animals were housed singly in wire mesh cages. The colony room was maintained at 21 ± 1°C and was on a 12 L : 12 D cycle (lights on at 0800 h). Food and water were freely available in the home cages.

Apparatus

The behavioral test 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 videocamera, videorecorder, and microprocessor were used to videorecord and code the animal's behavior.

Experimental Protocol

The animals were adapted to handling for 3 days. Subsequently, on the first 3 days of the experiment (days 1-3) the rats, in squads of four, were treated individually to 30-min sessions of inescapable foot-shocks. The shocks were delivered as 30 unsignalled shock trains on a FT 60-s schedule. Each train consisted of five 1.0-s duration, 0.5-mA shocks alternating with 1.0-s no-shock intervals. Subsequently, the rats were matched for the time spent crouching in the third session to equate for the effect of individual differences on groups and assigned in a quasirandom fashion to one of four groups (n = 7): Group SAL was injected with 0.9% saline at a volume of 1 ml/kg, subcutaneously (SC), twice daily at approximately 0900 and 1500 h, on days 4-13 and once on day 14; group DEA, the desipramine (desipramine hydrochloride; Sigma, St. Louis, MO) acute group, also received saline, except on the last day, day 14, when they received a single injection of desipramine; group DECN, treated with desipramine chronically but not on the last day, was treated with desipramine twice daily on days 4-13; and group DECL, receiving desipramine chronically including the last day, was injected with desipramine twice daily on days 4-8 and 10-13, and once on days 9 and 14. On day 14 between 0900 and 1200 h, all animals received a final 30-min session in the test apparatus without any shock. For groups DEA and DECL, the last injection was 40 min before the beginning of the last test session, day 14. Desipramine was dissolved in saline (concentration 5 mg/ml, dose 5 mg/kg, SC).

Behavioral Coding

The behavior of the rats during the sessions in the box on days 3 (shocked) and 14 (not shocked) was coded continuously by a trained observer who was blinded to the group designation of the individual animals. The behavioral events and their times of occurrence were stored by a microprocessor. The behavioral categories used for coding included: jump (leaping clear of the floor); flinch (twitching during a foot-shock); crouch (maintaining a flattened posture while not moving); escape (biting or rapid pawing at the floor bars); locomote (forequarters entering a quadrant of the box floor); turn (turning the trunk 180°); rear (raising the forepaws from the floor without jumping or grooming); sniff (whisker movements and head scanning); groom (licking, combing, or scratching itself); and immobile (motionless with a normal resting posture). The

reliability of the observer's coding was assessed by comparison with his own coding and with that of another trained observer. Test–retest and interobserver reliability measures of coding produced only agreements > 82%, and all yielded significant coefficients of concordance. To permit analysis of the changes in behavior over the course of a session, sessions were divided into six 5-min periods. Two-way analysis of variance (ANOVA) with one repeated measure was used to assess group × period effects within sessions for each behavior coded. Significant effects were followed by Tukey tests for differences between control and experimental groups.

Immunohistochemistry

Two hours after the beginning of the test session on day 14, the animals were given an overdose of sodium pentobarbital and perfused with saline and 4% paraformaldehyde. The brains were removed, soaked overnight in fixative, and cut in 30-μm frontal sections on a vibratome. Three to six sections were stained from each of 60 areas, distributed over 11 anterior-posterior (AP) levels of the brain. Approximations to the chosen AP levels were AP +3.2, +2.7, +1.0, −0.9, −2.8, −3.3, −3.8, −4.8, −7.3, −8.0, and −9.3 (38).

The immunohistochemical methodology has been used previously in this laboratory (49,50). After washing, sections were incubated with primary antisera, sheep polyclonal antibody (CRB 0A-11-823; Cambridge Research Biochemicals, Wilmington, DE) directed against residues 2–16 of the *N*-terminal region of the Fos molecule. A second sheep polyclonal antibody (PEPA 53; Serotec, Toronto, Ontario, Canada), which also recognizes amino acids 2–16 of the terminal region of Fos, was used to verify results obtained with the CRB antibody. The antibodies produced similar results. Washing three times with 0.02 M phosphate buffered saline (PBS) was followed by incubation in PBS containing 0.3% hydrogen peroxide for 10 min to block endogenous peroxide activity. Sections were then washed three times in PBS and incubated in PBS containing 0.3% Triton-X, 0.02% azide, and Fos primary antisera (diluted 1 : 2000) for 48 h. The sections were then washed three times with PBS and incubated for 1 h with a biotinylated rabbit antisheep secondary antibody (BA-6000, diluted 1 : 500; Dimension Laboratories, Mississauga, Ontario, Canada). Following another wash, three times in PBS, the sections were incubated for 1 h with PBS containing 0.3% Triton-X and 0.5% avidin-biotinylated horseradish peroxidase complex (Dimension). After three washes in PBS, the sections were rinsed with 0.1 M acetate buffer, pH 6.0. The reaction was made visible with glucose oxidase-3.3′-diaminobenzidene-nickel. The reaction was stopped by washing in PBS and the sections were mounted on chrom-alum slides, dehydrated, and coverslipped. To control for specificity of immunoreactivity, some sections were incubated with Fos-antisera that had been preabsorbed with Fos peptide (CRB OP-11-3210). Preabsorption of the CRB and Serotec antibodies with *N*-terminal antigenic sequence eliminated Fos immunoreactivity. In addition, omission of the primary antibody from the immunohistochemical procedure blocked Fos immunoreactivity.

In this preparation, Fos-positive neurons appeared darkly stained against a background of lightly but differentially stained cells and fibres. To help identify neural structures, representative sections from selected animals were stained with cresyl violet. Two independent observers counted the number of Fos-positive neurons in each structure within a 0.5-mm-square grid viewed at ×100 magnification. Kruskal–Wallis one-way ANOVA was used to test for a group effect on counts of Fos-positive neurons within each anatomical site. 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

Two-way ANOVA of group changes in the frequencies and the percent times of each behavior over the six periods of the third shock session revealed no significant group, period, or group by period effects. Each of the 25 shocks in a 5-min period was usually followed by flinching and then a prolonged period of crouching. Most of the session time was spent crouching: mean ± SE percent time for the SAL group was 89.23 ± 2.41; for the DEA group 87.66 ± 3.03; for the DECN group 86.95 ± 4.28; and for the DECL group 89.60 ± 3.58. As expected, the groups did not differ in their behavior during the last shock session; all groups exhibited high levels of freezing (i.e., crouch, behavior, and little investigative activity, such as locomote, turn, rear, and sniff).

Two-way ANOVA of groups by period for behavioral frequency effects in the no-shock session of day 14 revealed no significant group or interaction effects for jump, flinch, or immobile. Significant group and group × period interaction effects, respectively, were found for crouch [$F(3, 24) = 4.24$, $p < 0.05$, $F(15, 120) = 2.14$, $p < 0.05$], escape [$F(3, 24) = 31.87$, $p < 0.001$, $F(15, 120) = 6.36$, $p < 0.001$], locomote [$F(3, 24) = 3.84$, $p < 0.05$, $F(15, 120) = 2.51$, $p < 0.01$], turn [$F(3, 24) = 7.05$, $p < 0.01$, $F(15, 120) = 2.61$, $p < 0.01$], rear [$F(3, 24) = 3.72$, $p < 0.05$, $F(15, 120) = 2.08$, $p < 0.05$], sniff [$F(3, 24) = 3.86$, $p < 0.05$, $F(15, 120) = 2.01$, $p < 0.05$], groom [$F(3, 24) = 6.03$, $p < 0.01$, $F(15, 120) = 1.99$, $p < 0.05$] (Fig. 1). Tukey tests showed that the group main effects were due to the DECL group's exhibiting less crouch and more of the other behaviors than the SAL, DEA, or DECN groups. For the most part, the group × period interactions were related to the steeper slope of the changes in the DECL group's behavior over the course of the session (Fig. 1). In summary, in the conditioned shock session, the behavior of the SAL animals did not differ from the behavior of the animals given desipramine acutely or desipramine chronically except on the last session. The chronically treated animals that received desipramine on the last session exhibited more exploratory behavior and less freezing than did the other two groups.

Immunohistochemistry

Some sites expected to show Fos effects presented such low levels of Fos reactivity to casual inspection that they were not counted. These included the central portion of the caudate/putamen, ventral pallidum, substantia innominata, most of the thalamic nuclei, medial preoptic area, anterior, paraventricular (PVN), and supraoptic nuclei of the hypothalamus, brain stem reticular formation, cerebellum, and autonomic nuclei of the brain stem.

Of the 60 structures counted that failed to show significant group differences, 45 had low (< 20) counts of Foslike positive neurons. These included several cortical areas, specifically the posterior cingulate cortex area 3, frontal cortex areas 1 and 2, lateral orbital cortex, insular cortex, forelimb and hind-

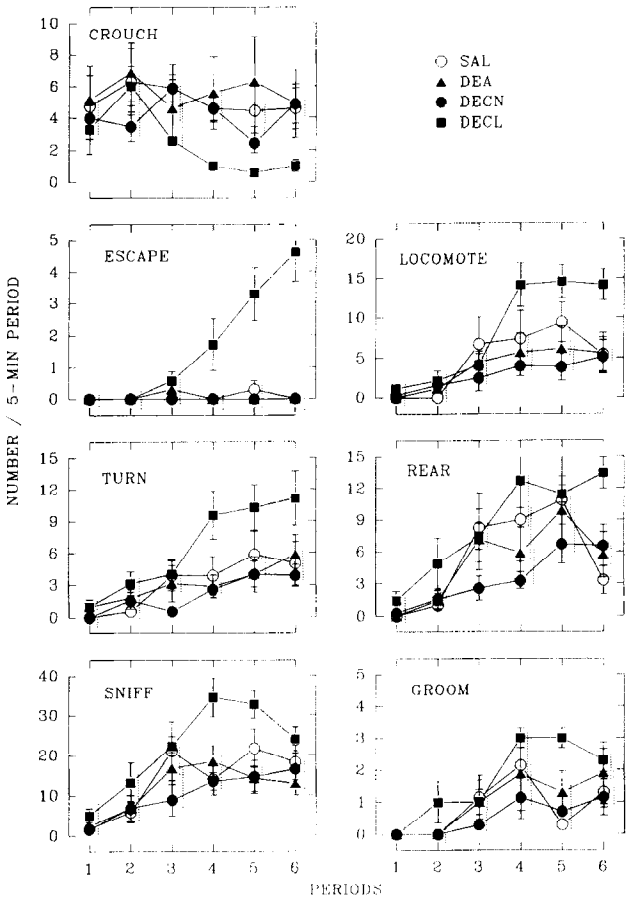

FIG. 1. Means and SEs of the frequency of crouch, escape, locomote, turn, rear, sniff, and groom over six 5-min periods of the no-shock session, day 14, for the SAL group (○), the DEA group given saline except on the last session in which they received desipramine (5 mg/kg, X1) (▲), the DECN group given desipramine chronically (5 mg/kg, X20) but not on the last day (●), and the DECL group given desipramine chronically (5 mg/kg, X20) including on the last day (■). Vertical lines join means that are not significantly different (Tukey, $p < 0.05$).

limb cortex, retrosplenial cortex, parietal cortex area 2, perirhinal cortex, temporal cortex area 3, and entorhinal cortex; several limbic and striatal structures including the olfactory tubercle, tenia tecta, islands of Calleja, endopiriform nucleus, shell and core of the nucleus accumbens, medial septal nucleus, nucleus of the diagonal band, bed nucleus of stria terminalis, and the basolateral, basomedial, and cortical nuclei of the amygdala; amygdalohippocampal area, the CA1 field of the dorsal hippocampus, the dentate gyrus and CA1 field of the ventral hippocampus, anterior, and posterior dorsomedial caudate putamen; several diencephalic structures lateral habenula, central median, ventral lateral geniculate, and posterior intralaminar nucleus of the thalamus, ventromedial, lateral, and posterior nuclei of the hypothalamus; and several brain-stem areas, specifically anterior pretectal nucleus, ventral tegmental area, substantia nigra, central gray, dorsal raphe nucleus, median raphe nucleus, locus ceruleus (LC), and lateral parabrachial nucleus.

Several structures had high numbers (>20) of Foslike positive neurons, but the groups did not differ in these counts (Kruskal–Wallis one-way ANOVA). These structures included the anterior olfactory nucleus, piriform cortex, dorsopeduncular cortex, infralimbic cortex, posterior claustrum, lateral septal nucleus, occipital cortex area 2M, dorsal hypothalamus, supramammillary area, and pontine nuclei.

Only five structures showed significant changes in *c-fos* expression. The number of Foslike positive neurons differed across the groups in the anterior cingulate cortex, [Kruskal–Wallis $H(3) = 12.56$, $p < 0.01$] (Fig. 2), anterior claustrum [$H(3) = 11.69$, $p < 0.01$], central nucleus of the amygdala [$H(3) = 9.34$, $p < 0.05$], dentate gyrus of the dorsal hippocampus [$H(3) = 10.77$, $p < 0.05$], and paraventricular nucleus of the thalamus [$H(3) = 7.99$, $p < 0.05$] (Fig. 3). Mann–Whitney *U*-tests showed that the DEA and DECN groups did not differ from the SAL group in any of these structures. By contrast, the number of Foslike positive neurons were smaller for the DECL group than for the saline controls in each of these four structures.

In the noradrenergic nucleus, locus ceruleus (LC), and serotonergic dorsal and median raphe nuclei, there were on average twice as many Foslike positive neurons in the desipramine groups as in the saline group, but the differences were not statistically significant because of large group variances.

DISCUSSION

Behavior

The chronically treated animals that received desipramine on the last session exhibited more exploratory behavior and less freezing than did the saline group. The behavior of this desipramine-treated group differed both from that of the group treated acutely with desipramine and from the animals treated with desipramine on all but the last session. Because of this, neither acute same-day treatment nor chronic treatment alone accounted for the effect. The relative efficacy of chronic vs. acute antidepressant administration in reversing shock-induced immobility has support in previous accounts of the failure of acute imipramine treatment to restore the ability to escape following uncontrollable shock (41,57). Inactivity in an animal induced by uncontrollable shock is a good predictor of failure to learn an escape response (32,33). The attempts to escape by the animals chronically administered desipramine on all including the last day have a parallel in the increased duration of swimming in animals treated with antidepressants in the forced swim test (43).

To the authors' knowledge, the present study is the first to directly compare the effects of chronic desipramine treatment with vs. without treatment on the final day of testing. The results agree with previous findings on the efficacy of treatment on the last day in chronically treated animals (5,28, 40,41,43,55,61 but disagree with the positive effect reported following treatment with desipramine on all but the last day (25). Our negative results also run counter to what would be expected from the clinical literature (37).

The failure of our results to replicate those of Leshner et al. (25) may have been due to administering less drug, differences in the number of shocks, and/or the behavioral measure. We gave the chronic animals 20 injections of desipramine, 5 mg/kg over 10 days (two per day). This is less than the effective doseage of Leshner et al., which was one injection of 20 mg/kg on 1 day and 14 injections (two per day) of 10 mg/kg over 7 days. Thus, doseage could have been a factor in the failure to replicate. On the other hand, 10 mg/kg per day has been found to be an effective dose in other chronic desipramine studies with same-day administration (43,55).

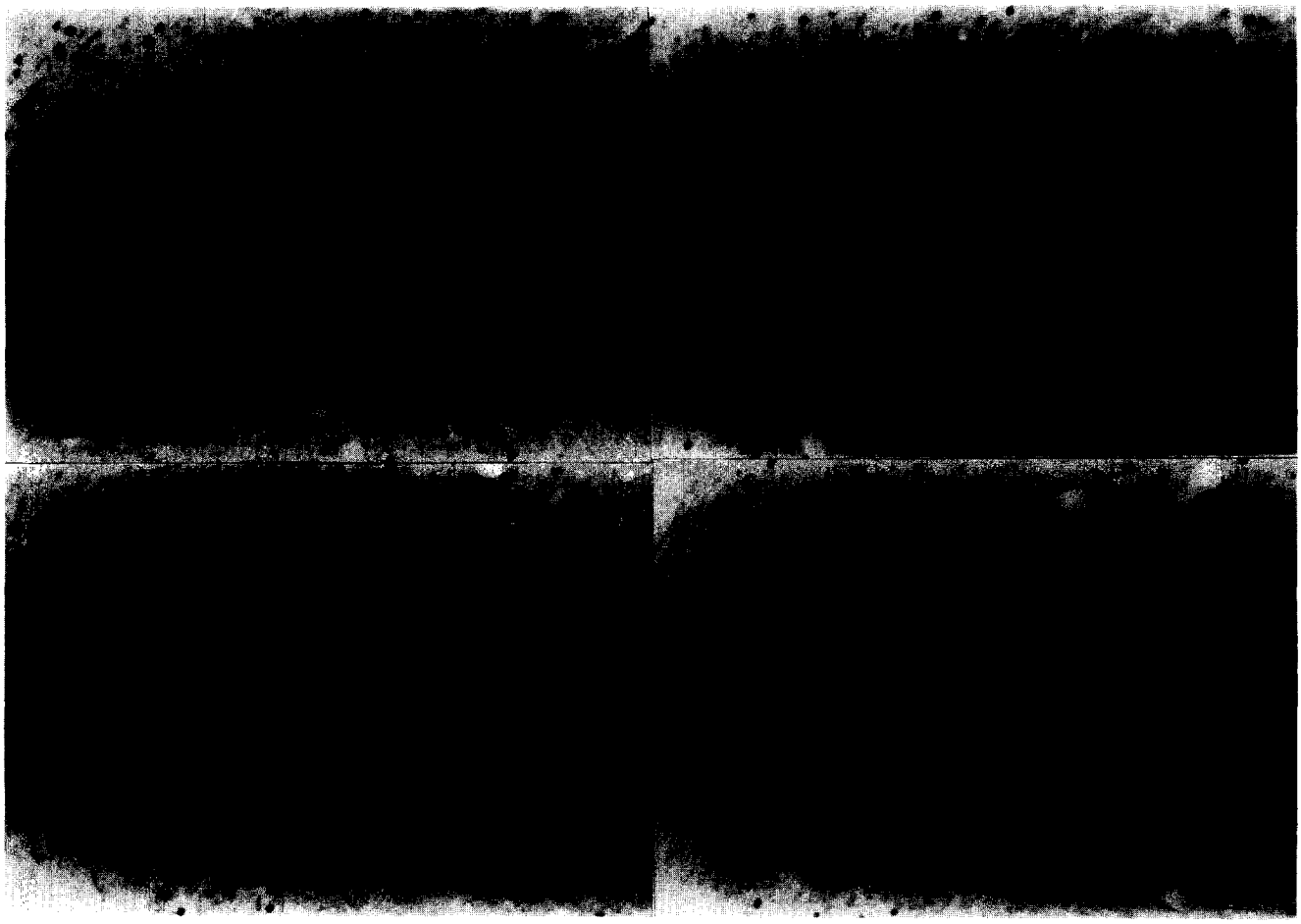

FIG. 2. Foslike immunoreactivity in the anterior cingulate cortex after pretreatment with vehicle (A), acute desipramine (B), chronic desipramine excluding the last day (C), and chronic desipramine treatment including the last day (D). Bar = 200 μm.

Increasing the number of the uncontrollable shocks increases the severity of the deficit (34). It is possible that the greater number of uncontrollable shocks used in the present experiment—150 shocks over three sessions—compared to 60 shocks to the rats of Leshner et al. (25) in one session may have produced a deficit that was insensitive to treatment on all but the last day. Finally, we measured the frequency and duration of freezing and investigative behaviors in the original box, with no shock on the test day. By contrast, Leshner et al. measured the acquisition of an escape response to foot-shock in an unfamiliar apparatus. Although freezing duration is a good predictor of performance on a test of learned helplessness (32), some or all of these factors may have contributed to the difference in results.

Immunohistochemistry

The principal finding of the study was that in response to conditioned stress, animals treated chronically with desipramine, including on the last day, altered *c-fos* expression only in five of the 60 structures assayed. In all five regions the experimental group exhibited decreased as opposed to increased numbers of Foslike immunoreactive neurons. The five structures were the anterior cingulate cortex, anterior claustrum, central amygdaloid nucleus, dentate gyrus of the dorsal hippocampus, and paraventricular nucleus of the thalamus. The medial frontal cortex (anterior cingulate) and the hippocampus have been implicated previously in antidepressant-induced reversal of stress effects (39,41,56,57). The claustrum, amygdala, and paraventricular thalamus have not been previously studied in this regard. However, indirect evidence supports the plausibility of the involvement of the latter structures. There are relatively high levels of labelled imipramine binding in the amygdala and paraventricular thalamus of unstressed rats (18). Similarly, following treatment of rats with a 5-HT neurotoxin, imipramine binding decreases in the cingulate cortex, amygdala, hippocampus, and thalamus (11). The claustrum was not assessed.

As noted in the introduction, previous studies have implicated serotonergic mechanisms in the action of antidepressants. It is therefore tempting to rationalize the regional specificity of the present results as a consequence of changes in serotonergic transmission. However, other neurotransmitter systems have also been implicated in antidepressant drug actions, including noradrenergic (29,62), dopaminergic (13,35, 66), GABAergic (15,27), corticotropin (20), and opiate systems (14,65). The neurochemical and receptor mechanisms by which chronic desipramine decreases psychological stress-induced *c-fos* expression in these structures are yet to be explained. Regardless of the precise nature of these mechanisms,

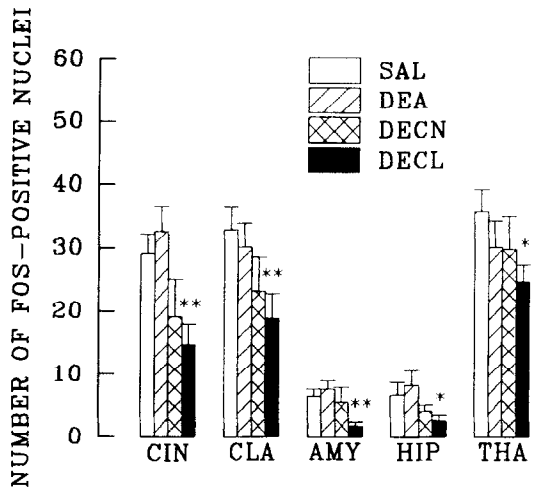

FIG. 3. The effect of treatment with saline (SAL; open bars), acute desipramine (DEA; single hatch), chronic desipramine treatment excluding the last day (DECN; crosshatch), and chronic desipramine treatment including the last day (DECL; filled bars), on the means and SEs of the number of Foslike positive neurons in the anterior cingulate cortex (CIN), anterior claustrum (CLA), central nucleus of the amygdala (AMY), dentate gyrus of the dorsal hippocampus (HIP), and paraventricular nucleus of the thalamus (THA). $*p < 0.05$; $**p < 0.01$ for the saline group.

the project results point to the anterior cingulate cortex, anterior claustrum, central nucleus of the amygdala, dentate gyrus of the dorsal hippocampus, and paraventricular nucleus of the thalamus as being potential substrates for the antidepressant actions of desipramine.

Two major caveats may be raised concerning the principal immunohistochemical finding of the present study. c-Fos activation in the paraventricular nucleus of the hypothalamus (PVN) and LC has been reported consistently in animals stressed acutely with unconditioned stimuli (2,4,8,9,16,42, 52,54,59). Therefore, it would have been reasonable to expect some effect in these structures in our study. However, the present study used stimuli conditioned to chronic stress, and these have equivocal effects. With conditioned stimuli, Pezzone et al. (42) found an effect on *c-fos* reactivity for PVN but reported none for LC, whereas Smith et al. (59) found a reaction for LC but reported that there was no change in PVN. The data of Pezzone and Smith are based on a comparison with unstressed controls. Judging from unpublished data on unstressed controls from other experiments in our laboratory, had we tested unstressed controls in this experiment, the effects in question would have been the same as those of Smith et al.

The second concern regarding the principal finding is the failure of the animals administered desipramine chronically on all but the test day to replicate the principal finding. This result was surprising given the preponderance of evidence that chronic antidepressant treatment initiates a neuronal cascade with enduring neurochemical effects (10,37). However, the effect of a 24-h antidepressant holiday on *c-fos* reactivity in the brain has not been previously reported. It might be argued that the failure to replicate the immunohistochemical finding is an artifact of the potentiation of the stress of exposure to the conditioned stressor by the stress of withdrawal from twice-daily desipramine treatment. However, there is no evidence for stressful withdrawal effects upon cessation of chronic antidepressant treatment (23). Conversely, it is possible that the principal finding is a consequence of desipramine acutely reversing a putative stressful effect of sodium pentobarbital. This appears unlikely because it is generally agreed that anaesthetics and sedatives inhibit rather than enhance *c-fos* immunoreactivity (30,31).

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