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The purpose of this project is to explore the effects of chronic activation of the brain corticotropin releasing factor (CRF) stress system and to investigate individual susceptiblity to this pathological cascade. It is our hypothesis that sustained central CRF activation results in damage to the brain dopamine system through oxidative mechanisms and that certain populations may be uniquely susceptible. In Specific Aim 1, the goal is to selectively breed rats for high and low response to stressors on the basis of their hypothalamic pituitary adrenal axis response to footshock. Two independent lines (replications) are being bred (the fifth generation of Line 1 is currently in utero and the third generation of Line 2 is nearing weaning). The lines appear to be separating at a steady, although moderate, rate, possibly reflecting a locking in of the original selection. In Specific Aim 2, the goal is to explore the effects of chronic hyperactivity of the brain CRF stress system on dysregulation of brain monoamine systems with a focus on dopamine. Chronic hyperactivity of the CRF system by repeated central administration of CRF produced a temporary dysfunction of the mesocortical and nigrostriatal dopaminergic systems possibly mediated by oxidative damage.

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# Chronic stress and Neuronal pathology: Neurochemical, Molecular and Genetic Factors

The hypotheses being tested in this project are that chronic stress produces enduring changes in brain stress systems that ultimately result in permanent neuronal damage to brain dopamine system through oxidative mechanisms and that certain subject populations may be uniquely susceptible to this pathological cascade through hyper-responsiveness of the CRF stress response system.

**Specific Aim 1: To continue the selective breeding of rats for high stress responsiveness (HSR) and low stress responsiveness (LSR).**

The goal of this aim is to selectively breed rats for high and low response to stressors using the hypothalamic pituitary adrenal axis response as the dependent variable. When exposed to stressors, rats of the genetically heterogeneous NIH stock show significant individual differences in their adrenocorticotrophic hormone (ACTH) response to a mild footshock stressor. Capitalizing on this variability between animals, we have been selectively breeding rats showing high and low ACTH response to mild footshock to create the HSR and LSR rat lines, respectively.

Two independent replications of these two lines are being bred. Rats of line 1 are referred to as HSR1 and LSR1 and line 2 rats are HSR2 and LSR2. The establishment of a second independent line allows for the validation of the findings of the first line. Such duplicate breeding is an established standard procedure in selective breeding programs to ensure the trait specificity of the selective line, and to eliminate selection artifacts that are not related to the trait under investigation. The fifth generation of Line 1 and the third generation of Line 2 are currently in utero (Line 1) or nearing weaning (Line 2). Cumulative ACTH levels (pg) taken at 10, 30, 45 and 60 minutes following 0.25 mA footshocks (1 sec shock duration, 2 shocks per minute for 60 minutes) in the HSR and LSR rats of generations 1 through 4 are shown in Figure 1. The lines appear to be separating at a modest rate, and may simply be locking in the original selection. Data from the parents of these rats are shown in Figure 2. We continue to select very divergent responders for breeding.

As mentioned above, Line 2 generation 3 rats will be tested in the next month or so. As of generation 2, the separation of the HSR2 and LSR2 lines was comparable to generation 2 HSR1 and LSR1 rats. In addition, we are experiencing no problem selecting breeders that show divergent ACTH responses to footshock to continue this line.

The original shock paradigm utilized a 0.5 mA shock session, followed three days later by a 0.25 mA shock session. It became clear after three generations of Line 1 breeding that it was difficult to select consistently high and low extremes for breeding based on both shock intensities. Further analysis revealed poor correlations between the ACTH responses to these two shock intensities. Responses to 0.25mA were more variable in the original outbred population, making this intensity the more optimal choice for selective

breeding. Therefore the paradigm was changed for Line 1, generation 4 and Line 2, generation 2 such that two 0.25mA sessions were used. However, again, the correlation between the results obtained from these two sessions was very poor. This is likely due to the fact that the first shock test influences the response to the second shock test in a variable manner. For example, some rats may change their behaviors during the second test in an attempt to lessen the pain from the shocks, while other rats may become sensitized to the procedure and show an increase in responsiveness. It has been decided that only a single 0.25mA shock session will be used in subsequent breeding of both replicate rat lines.

It appears that the LSR lines have diverged from the base population to a greater extent than the HSR lines (see Figure 1 for Line 1 results). This suggests that there may be selective pressure against increased responsiveness to stress. Certainly we have seen evidence of an effect of low responsiveness to stress borne out by LSR1 females. LSR1 females chosen to be breeders from the third generation of offspring had problems in the delivery of their litters. In fact, two of these females died during delivery, presumably due to a decreased response to the stress of delivery. While this is of great concern to us, it suggests that the selective breeding of LSR1 rats has resulted in a physiologically relevant phenotypic change of low circulating corticosterone. We will very closely watch the fourth generation females who will give birth within the next week or two. If the problems with delivery continue, corticosterone supplementation will be considered.

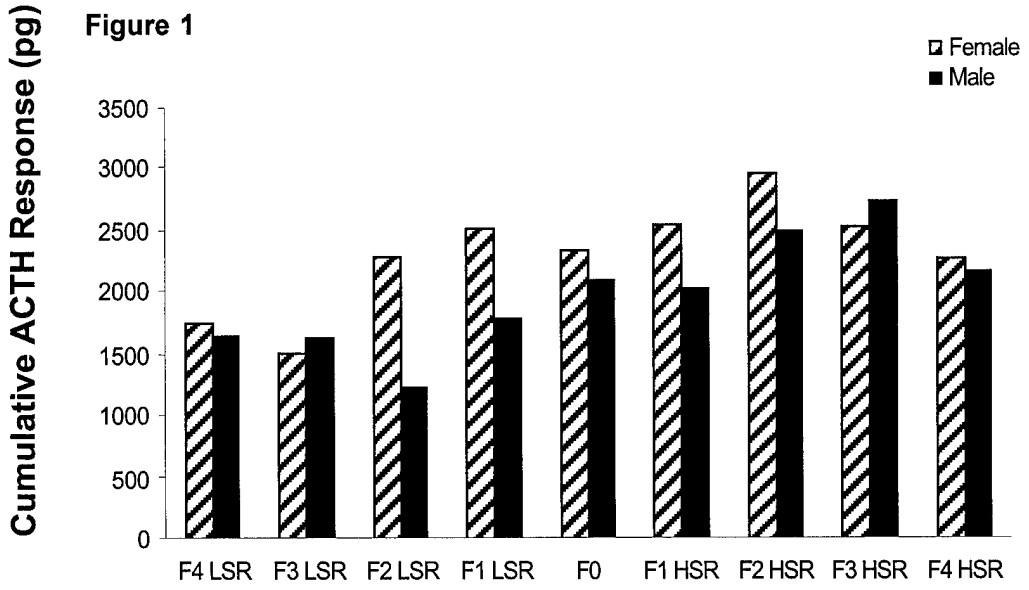


**Figure 1. Selective breeding of HSR1 and LSR1 rats.** Mean cumulative ACTH responses (pg) following a series of 0.25mA footshocks are shown in female and male rats. The base population of genetically heterogeneous n/NIH rats is denoted as F0.

Selected generations 1-4 of HSR rats are shown to the right of the F0 generation, whereas LSR generations are shown to the left of F0 from right to left.

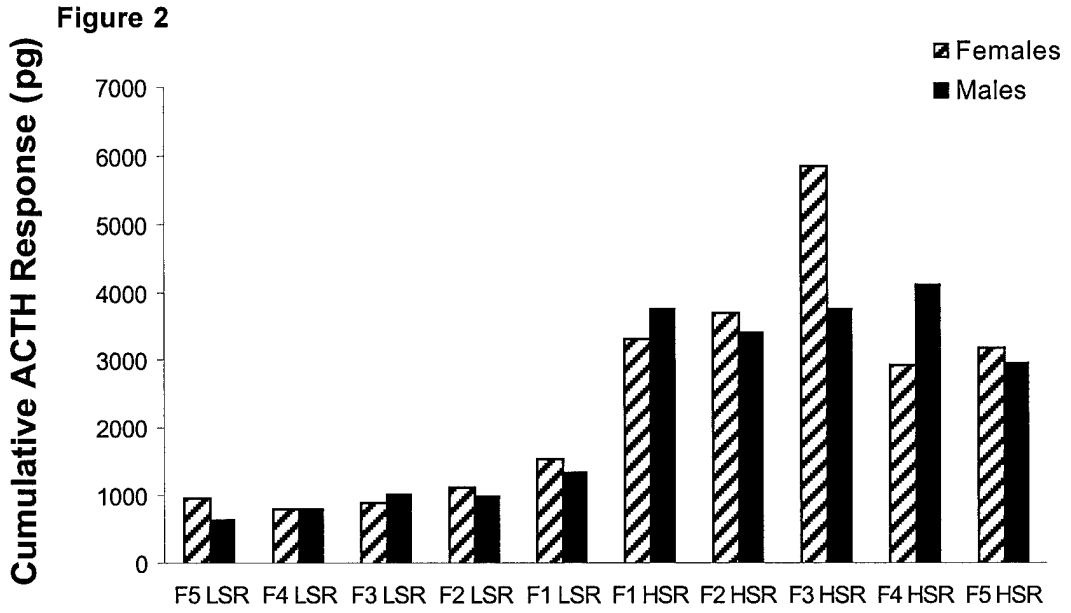


**Figure 2. Breeders chosen for the continuation of the HSR1 and LSR1 rat lines.** Mean cumulative ACTH responses (pg) following a series of 0.25mA footshocks are shown in female and male rats. F1 breeders for both lines were chosen from the original n/NIH population of rats. F2 breeders were then chosen from F1 offspring and so on. The F5 breeders are currently in the process of producing testable F5 offspring.

**Specific Aim 2. To explore the effects of chronic hyperactivity of the brain corticotropin releasing factor stress system on dysregulation of brain monoamine systems with a focus on dopamine and norepinephrine.**

**Effect of chronic CRF on behavioral dopaminergic parameters.** In order to test whether chronic activation of the brain corticotropin releasing factor (CRF) induces an impairment of the dopaminergic system, the effect of chronic CRF administration on d-amphetamine-induced stereotypy behavior and on eticlopride-induced catalepsy was measured. Rats were surgically implanted with a cannula intracerebrovetricularly (ICV) and after a period of recovery injected with 1μg/day of CRF for 13 days. The control group received the same volume of vehicle. Rats chronically ICV treated with CRF (1ug/day for 13 days) displayed a lower stereotypy response to amphetamine (4mg/kg sc) and an increased eticlopride (0.05mg/kg SC)-induced catalepsy. The differences in the response were significant at 1 day and the trend was present at 1 week after the CRF treatment but no differences were present after one month. These behavioral experiments

extend and complete studies conducted during the first funding year and support the notion that chronic CRF alters dopaminergic function.

**Effect of chronic CRF on biochemical parameters**

In parallel to the behavioral tests we evaluated dopamine and DOPAC malondialdehyde (MDA) levels in prefrontal cortex (PFC), striatum, nucleus accumbens and amygdala

*Dopamine and DOPAC levels*

Dopamine levels were significantly lower in the Prefrontal Cortex at 1 day and 1 week after the treatment and returned to control values 1 month after ($p<0.05$ vs control t-test). No significant differences in the levels of dopamine were found in the striatum at all time points (Fig. 3). However, the DOPAC/dopamine ratio was significantly increased at 1 week after the treatment both in PFC and Striatum (Fig. 3) ($p<0.05$ vs control t-test). Again no differences were present after 1 month.

*Malondialdehyde total level*

Malondialdehyde (MDA) total levels (a marker of oxidative damage) were evaluated using a colorimetric assay (OXIS International, Portland, OR) in different brain areas: prefrontal cortex (PFC), striatum and nucleus accumbens. The results revealed a non-significant trend to higher levels of MDA in striatum and prefrontal cortex after 1 day and 1 month of the treatment. No differences were present in the other brain areas tested. These experiments are being replicated and expanded.

**Effect of Ferrous-Citrate ICV infusion on stereotypy amphetamine behavior**

To validate whether oxidative stress in the brain induces an impairment of the dopaminergic system, a group of rats were acutely ICV injected with Ferrous-Citrate, an oxidizing agent, and tested after 7 days and 1 month in the stereotypy amphetamine test.
A group of 12 rats (6 per group) were injected ICV with 10 nmol of ferrous-citrate in saline, while the control group received saline. Rats were tested 7 days after ICV iron infusion.
Ferrous-citrate treated rats showed a lower stereotypy response to amphetamine either 7 days or 1 month after the infusion (Fig. 4). No differences in the basal locomotor activity were present between control and ferrous treated rats (not shown). These behavioral data indicate that oxidative stress impairs the dopaminergic system, as reflected in a blunted amphetamine response, and support the notion that the dopaminergic system is especially vulnerable to oxidative insults.

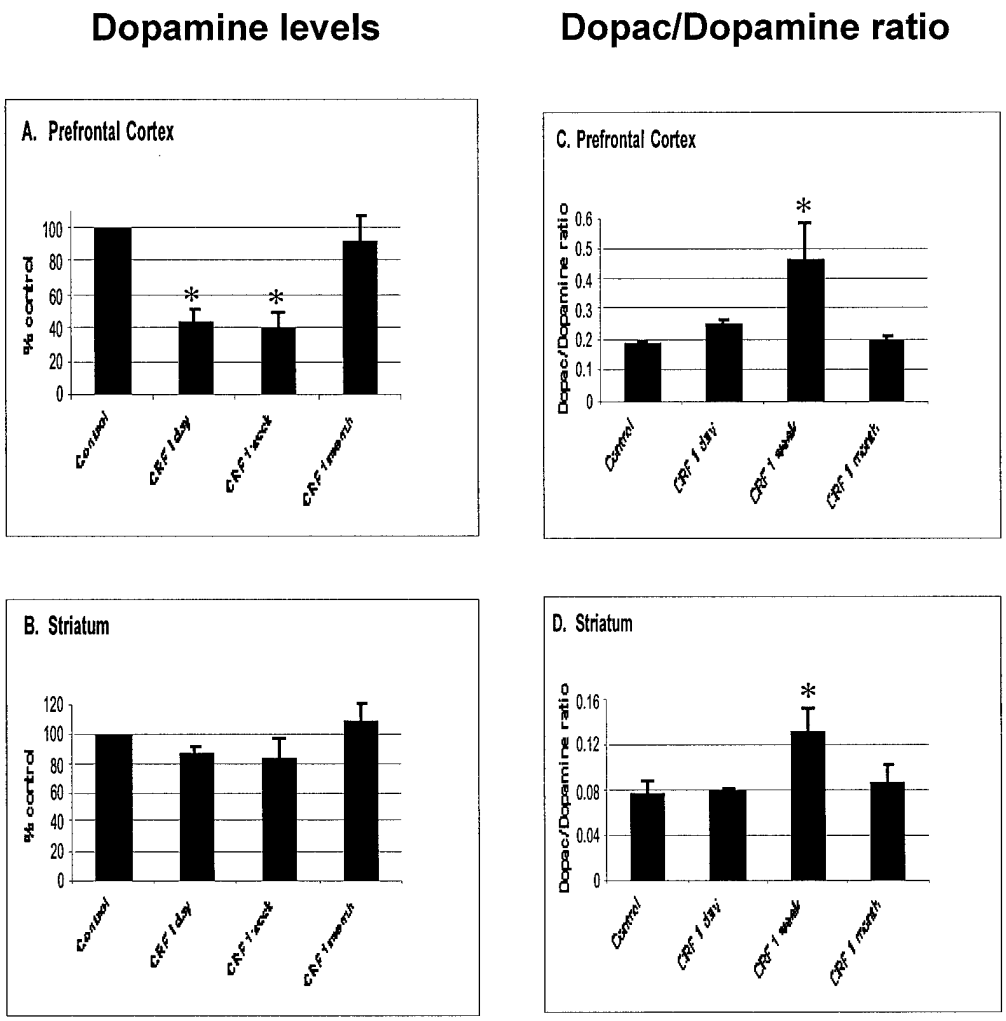


**Fig. 3. Effect of chronic (13 days) ICV administration of 1μg/day of CRF on Dopamine levels and Dopac/Dopamine ratios. A).** No significant differences in the levels of dopamine were seen in the striatum at 1 day, 1 week or 1 month following chronic CRF administration. **B)** Dopamine levels were significantly lower in the Prefrontal Cortex at 1 day and 1 week after chronic CRF administration and returned to control values 1 month after the treatment ($p<0.05$ *vs.* control t-test). The DOPAC/dopamine ratios were significantly increased at 1 week after the treatment both in PFC and striatum (A,B) ($p<0.05$ vs control t-test). Again, no differences were present after 1 month.

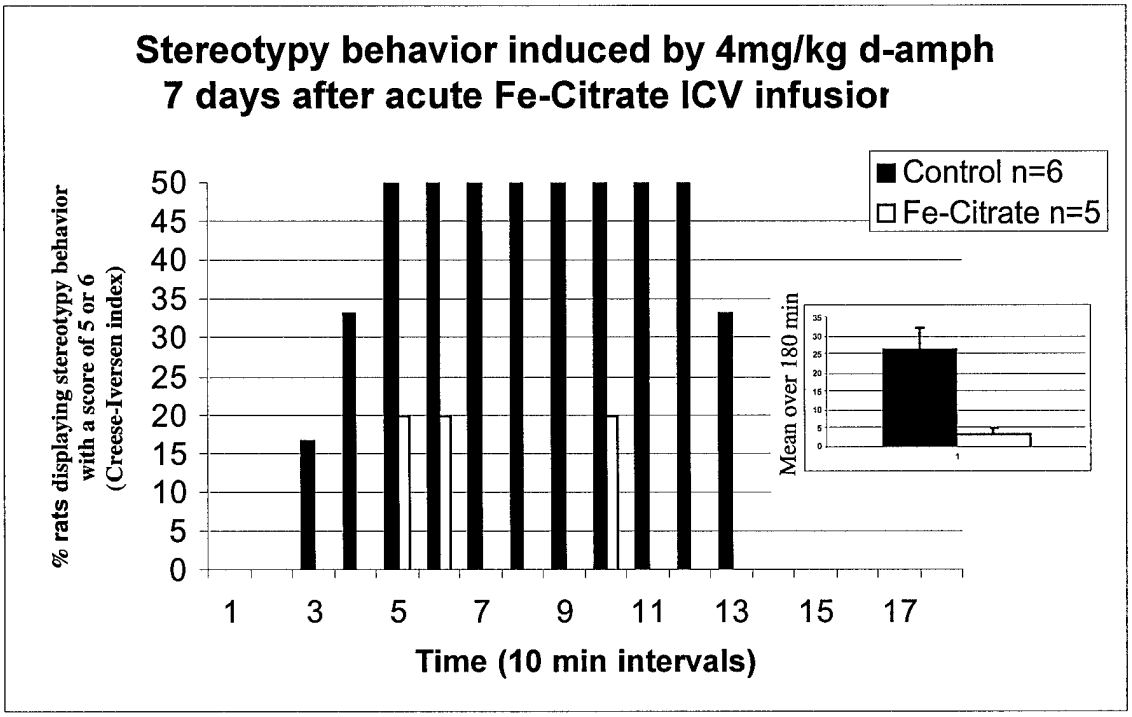


**Fig. 4 Effect of Ferrous-Citrate ICV infusion on stereotypy amphetamine behavior.** Rats were acutely injected ICV with the oxidizing agent Ferrous-Citrate. They were tested after 7 days and 1 month in the stereotypy amphetamine test. Ferrous-citrate treated rats showed a lower stereotypy response to amphetamine either 7 days or 1 month after the infusion. No differences in the basal locomotor activity were present between control and ferrous treated rats (not shown). Such a blunted amphetamine response suggests an impairment of the dopaminergic system and supports the notion that the dopaminergic system is especially vulnerable to oxidative insults.

**Conclusions.** In rats chronically treated with CRF, behavioral methods revealed alteration in dopaminergic function. Biochemical analyses showed that dopamine levels in the prefrontal cortex were decreased and DOPAC/dopamine ratios were increased in both the frontal cortex and striatum at 1 day and 1 week after the treatment. All of these parameters returned to basal values 1 month after the treatment. Levels of MDA, a marker of oxidative damage to membrane phospholipids, were increased 1 day after and 1 month after the treatment, although not significantly.

We have observed that these changes observed are reversible and that the PFC is the most sensitive brain area to chronic stress. The reversibility of the changes could be due to the dose of CRF used (1μg/day) and it is possible that a chronic treatment with higher doses of CRF would lead to permanent, more extensive and severe changes in the dopaminergic brain system. The involvement of the PFC is interesting in light of the role played by this region in cognition ("executive functions") and its involvement in stress, Parkinson's disease-associated cognitive impairments and depression. In addition deficits in

monoamine function in the PFC can trigger subsequent compensatory dysregulation in the striatum.

Taken together these data show that a chronic hyperactivity of the CRF system leads to a temporary dysfunction of the mesocortical and nigrostriatal dopaminergic systems probably mediated by oxidative damage. Such dysfunction may become more prominent or even irreversible when the stress exposure is more severe and/or protracted.