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13. ABSTRACT *(Maximum 200 Words)*

The purpose of this proposal is to test the hypothesis that chronic stress produces lasting changes in brain dopamine function leading to permanent neuronal damage through oxidative mechanisms. Certain subject populations may be uniquely susceptible to this pathological cascade through hyperresponsiveness of the corticotropin releasing factor stress response system. To test this hypothesis, two specific aims are currently in progress: 1) To examine the effects of chronic stress on behavioral, neurochemical and molecular measures of neuronal pathology to the brain dopamine system; 2) To develop a phenotype of hyperresponsiveness of the CRF hypothalamic pituitary adrenal axis by selective breeding. The results show evidence of CRF induced hypoactivity in the brain dopamine system as measured by an increase in catalepsy and a decrease in amphetamine-induced stereotyped behavior that last up to four months. In addition, the first phase of selective breeding of rats hyperresponsive to stress has been accomplished. The initial separation in HPA axis activity showed a two- to four-fold difference between low and high responders. These results should ultimately provide not only key information about neuronal dysfunction produced by chronic stress but critical knowledge of the genetic/molecular factors that contribute to individual vulnerability to stress and its pathological consequences.

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George Nash 13 July 2000

PI - Signature Date

Table of Contents

INTRODUCTION:

Enduring dysregulation of stress response systems has afflicted as many as one-third of combatants in major conflicts, and disorders of the stress response systems related to military operations produce substantial human and government cost. The purpose of the present proposal is to explore the effects of chronic stress on neuronal pathology by examining the interaction of neurochemical, molecular and genetic factors. The hypothesis under test is that chronic stress produces lasting changes in brain dopamine function. These changes may lead to permanent neuronal damage through oxidative mechanisms, and certain subject populations may be uniquely susceptible to this pathological cascade through hyperresponsiveness of the corticotropin-releasing factor (CRF) stress response system. To test this hypothesis two specific aims are currently in progress: 1) To examine the effects of chronic stress on behavioral, neurochemical and molecular measures of neuronal pathology to the brain dopamine system; 2) To develop a phenotype of hyperresponsiveness of the CRF hypothalamic pituitary adrenal (HPA) axis by selective breeding. The results show evidence of CRF-induced hypoactivity in the brain dopamine system as measured by an increase in catalepsy and a decrease in amphetamine-induced stereotyped behavior that last up to four months. In addition, the first phase of selective breeding of rats hyperresponsive to stress has been accomplished. The initial separation in HPA axis activity showed a tenfold difference between low and high responders. These results ultimately should provide not only key information about neuronal dysfunction produced by chronic stress but critical knowledge of the genetic/molecular factors that contribute to individual vulnerability to stress and its pathological consequences.

BODY:

Specific Aim 1: To measure the effects of stress on behavioral, neurochemical and molecular changes in neuronal pathology to the brain dopamine systems.

Experiment 3: Effect of chronic CRF on animal models sensitive to nigrostriatal DA system damage.

CRF chronic treatment

16 animals were implanted with intracerebroventricular (i.c.v.) cannulas (see Ahlers et al., 1992; Ahlers and Salander, 1993, and Menzaghi et al., 1994 for details) and subjected to 13-day exposure to chronic repeated CRF administration i.c.v. Rats received one injection per day of CRF at a dose of 1 microgram (5 microliter) per infusion. Controls received 5 microliter of saline i.c.v. The behavioral tests of DA function were administered as follows:

Behavioral Measures

Catalepsy: Catalepsy was measured using the bar test according to Pulvirenti and Koob, 1993. Rats were injected with the D-2 antagonist eticlopride and then both their forepaws were placed on a bar 9 cm from the floor. The time elapsed until the rats re-position both forepaws on the floor was recorded by an experimenter blind to the treatment condition. Control rats typically step down within a few seconds while eticlopride-treated rats remain with their paws on the bar significantly longer. To choose the right dose of eticlopride, four tests were conducted with different doses (0, 0.025, 0.050 and 0.1 mg/kg s.c.) of eticlopride using a Latin Square design. The dose of 0.05 mg/kg was chosen. At the time of the experiments, control and treated animals were injected with eticlopride 0.05 mg/kg and then tested 2 hrs after the injection.

Amphetamine-induced stereotyped behavior: Rats were habituated to the photocell test cages for 90 min during the light part of their light/dark cycle. After 90 min rats received a subcutaneous s.c. injection of 4 mg/kg of d-amphetamine and tested for an additional 3 hours. Locomotor activity was measured using the total number of photocell beam breaks and crossovers. Sterotyped behavior was rated according to the scale of Creese and Iversen (1973) and by categories of behavior (Fray et al., 1980; Koob et al., 1984).

Results:

The results show that chronic CRF administration produced an increase in catalepsy one day after administration of chronic CRF treatment (Table 1). Catalepsy continued to increase with repeated testing but also increased in the control group, presumably reflecting increased experience with the repeated testing. Future experiments require independent groups or repeated testing at only one time point at monthly intervals.

The results of testing of amphetamine-induced stereotyped behavior show a pattern of dopamine hypoactivity that lasted up to three months post-CRF. Amphetamine at a dose of 4 mg/kg produces pronounced stereotyped behavior for approximately 2-3 hours post-injection. This stereotyped behavior was attenuated in the CRF-treated animals at 24 hours, 1 week, 1 month, and 4 months post-CRF (Figures 1-3). These data suggest that there is some hypodopaminergic function in CRF-treated animals that can be exposed by pharmacological probes. Current ongoing studies are examining neurochemical and histological measures of dopamine functioning.

Experiment 2: The effect of chronic physical stress on animals models sensitive to nigrostriatal DA system damage.

Two groups of 8 rats were subjected to either three minutes of ether-stress (N=8) or identical handling without ether-stress (N=8). Catalepsy testing after eticlopride administration and stereotyped behavior following d-amphetamine administration were conducted as in Experiment 3. The results show no significant difference between the groups of 24 hours and one week post-stress exposure for either catalepsy or stereotypy (Figure 2, Table 2). However, there is a tendency for an increase in catalepsy and a decrease in stereotyped behavior as observed in the CRF-treated animals. This experiment is currently in progress.

Experiment 5: Effects of chronic stressors on measures of oxidative stress and pathology.

The purpose of this experiment is to examine the effects of stressors and chronic CRF on neurochemical measures of dopamine dysfunction. Progress to date has focused on establishment of the measures of oxidative stress. The immunohistochemical techniques proposed for the detection of the presence and distribution of oxidative damage in histological preparations were implemented by use of antibodies to malonaldehyde (MDA) developed by Drs. W. Palinski, and J.L. Witztum of UCSD. This approach utilizes monoclonal antibodies specific for MDA-adducts which afford the immunohistochemical localization of sites of oxidative damage in brain sections. The technique was optimized using as positive controls transgenic animals overexpressing the cytockine IL6, which induces oxidative tissue damage in the CNS. A very high degree of cellular localization was observed with good signal-to-noise ratio. Current work is aimed at investigating the distribution of oxidative cell damage in animals chronically treated with CRF as proposed in the application.

Specific Aim 2: To selectively breed rats for hyper- and hypo-responsiveness of the HPA axis.

Experiment 2: Selective breeding of rats for high response to stress (HRS) and low response to stress (LRS) using the ACTH response to stressor exposure as the selected phenotype.

The purpose of this experiment is to develop a phenotype of hyper- and hypo-responsiveness of the corticotropin-releasing factor (CRF) hypothalamic pituitary adrenal axis by selective breeding with the ultimate goal of elucidating the relationship between chronic stress and neuronal pathology.

Behavioral Measures

Male and female breeding stock rats (n=10 each) were obtained from the NIH (N/NIH rats). They were implanted with indwelling jugular intravenous (i.v.) cannulae and allowed to recover for 2-3 days. On experimental day 1, rats received electroshocks to the paws: 0.5 milliamps, 1-sec shock duration, 2

shocks per min, 60-min session. Blood samples were taken at 0 min (basal) and at 10, 30, 45, and 60 min following the initiation of shocks. Three days later, rats received shocks of reduced intensity (0.25 milliamps), and blood samples were collected according to the same protocol. Plasma ACTH levels (pg/ml) were measured by radioimmunoassay. Following shock testing, I.V. cannulae were cauterized shut and internalized. Opposite-gender rats of similar ACTH responses (high or low) then were paired and transferred to a facility for breeding.

Results

On each of the two experimental shock days, male and female rats were rank-ordered according to their total ACTH response (the sum of ACTH levels determined 0, 10, 30, 45, and 60 min following shock initiation) (Figures 3 and 4). The rank number was averaged over the two test days, which provided an overall ranking of stress response within each gender. Based on the mean rank and the consistency in rank at the two shock intensities, the following was observed:

Males Animals # 3 and 6 were low responders; # 5, 7, and 10 were high responders.
Females Animals # 1 and 7 were low responders; # 2, 6, and 10 were high responders.

These data were used to pair low and high responding rats for breeding and subsequent shock testing of offspring.

The breeding pairs for selected generation 1 are:

Low Responding Line:	High Responding Line:
Male 3 x Female 7	Male 5 x Female 2
Male 6 x Female 1	Male 10 x Female 6
	Male 7 x Female 10

Current Breeding Status: The rats have been bred and there are currently 12 offspring plus an as yet uncounted new large litter from the Low Response to Stress (LRS1) breeders and 16 offspring plus an as yet uncounted new large litter from the High Response to Stress (HRS1) breeders. It is estimated that 10 male and 10 female first generation (S1) LRS1 and 10 male and 10 female first generation (S1) HRS1 rats will be tested as described above for ACTH response to shock and then bred in a similar manner to produce the second selected generations (S2) of LRS1 and HRS1 rats. In addition, an independent cohort of N/Nih rats will be obtained from NIH to initiate the LRS2 and HRS2 replicate selected lines. These rats will be tested and bred in an identical fashion to the first set of selected lines and serve as a control for phenotypes which segregate between the LRS and HRS lines which have no relationship to the selected trait.

Discussion

The results to date on the project provide preliminary support for two of the hypotheses from the proposal. First, chronic administration of corticotropin-releasing factor (CRF) produces changes in neuropharmacological responses that reflect a decrease in striatal dopaminergic function. There is an increase in cataleptic behavior produced by a dopaminergic receptor antagonist and there is a decrease in stereotyped behavior associated with administration of a dopaminergic indirect agonist, d-amphetamine. This profile of results resembles that of a modest lesion to the nigrostriatal dopaminergic system (Koob et al., 1984). Chronic ether stress for 10 days did not produce a significant change in these measures suggesting that the duration or intensity of the stressor may not have been sufficient to observe changes.

The second hypothesis that has been supported is that rats can be selected for breeding based on their hypothalamic pituitary adrenal response to stressor exposure as measured by blood levels of ACTH. The NIH (N/NIH) strain of rats showed a 2-4 fold difference in response to a modest footshock stressor and these animal have been successfully bred. Low and high responding rats have been bred and the offspring are in sufficient number to continue the breeding and selection program. The selective breeding project is being followed regularly by the consultants to the project (Dr. Catherine Rivier- The Salk Institute and Dr. T.K. Li- Indiana University Medical Center). These animals will become a major resource for future studies for exploring the basis for individual differences in the vulnerability of the brain dopaminergic systems to stressor exposure. The studies underway in this proposal are providing a basic research foundation for identifying the specific phenotype that will predict enduring dysregulation of brain stress response systems in service personnel.

In addition, histological measures of oxidative stress have been implemented. This approach utilizes monoclonal antibodies specific for MDA-adducts which afford the immunohistochemical localization of sites of oxidative damage in brain sections. Current work is aimed at investigating the distribution of oxidative cell damage in animals chronically treated with CRF as proposed in the application.

KEY RESEARCH ACCOMPLISHMENTS:

- Chronic administration of CRF produces changes in neuropharmacological responses that reflect a decrease in striatal dopaminergic function.

- A selective breeding program for hypothalamic pituitary adrenal responsivity to stressors has been initiated with the NIH (N/NIH) strain of rats

- Biochemical and histological measures of dopaminergic function and oxidative stress have been set up and will be used to measure brain changes associated with chronic stressor and chronic CRF exposure.

REPORTABLE OUTCOMES:

No manuscripts or presentations have yet emanated from this body of work. It is anticipated that a manuscript will be prepared this coming funding period from the neuropharmacological studies and a preliminary report of the selective breeding will be prepared following the third generation of breeding. The development of a line of rats selectively bred for their hypothalamic-pituitary-adrenal response to stressors will be a major resource for the scientific community and should result from continuing support of this program.

CONCLUSIONS:

The work accomplished to date provides preliminary support for the hypothesis that the brain stress systems when highly activated can compromise functioning in the brain dopaminergic systems. This is of significance because the brain dopaminergic systems not only are involved in the initiation of movement but also have a major role in motivational processes. Drugs that activate the brain dopaminergic systems sustain performance in situations of fatigue and at a minimum one could extrapolate that a hyperresponsive brain stress system may make an individual particularly vulnerable to exhaustion and unable to function in sustained combat missions.

The selective breeding program has been successfully launched using the outbred NIH strain of rats. This same strain of rats has been used extensively for other selective breeding programs and thus is a validated model for selective breeding. These animals, with continued breeding, will provide a major resource for identifying the neurobiological basis for individual differences in stress responsivity that could lend a combatant particularly vulnerable to brain dopaminergic dysfunction.

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APPENDICES:

Table 1. Cataleptic effects of the dopaminergic D-2 antagonist in CRF and control exposed animals.

Table 2. Cataleptic effects of the dopaminergic D-2 antagonist in ether-stressed and control animals.

Figure 1. Effects of d-amphetamine on stereotyped behavior in CRF and control exposed animals

Figure 2. Effects of d-amphetamine on stereotyped behavior in ether stressed and control exposed animals

Figure 3. Plasma ACTH response in male NIH strain rats at two intensities of footshock stress

Figure 4. Plasma ACTH response in female NIH strain rats at two intensities of footshock stress

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TABLE I

Eticlopride (0.05 mg/kg sc)-Induced Catalepsy (sec)

Time after CRF chronic treatment	*Control (n=8)*	*CRF (n=8)*
1 day	12.51 ± 2.29	43.62 ± 10.44 **
1 week	63.60 ± 34.38	79.87 ± 22.33
1 month	114.63 ± 39.26	83.54 ± 19.61
3 months	156.37 ± 47.49	76.98 ± 23.07
4 months	137.12 ± 37.7	114.58 ± 31.60

**$p<0.05$ (t-test)

TABLE II

Eticlopride (0.05 mg/kg sc)-Induced Catalepsy (sec)

Time after Ether chronic treatment	*Control (n=6)*	*Ether (n=8)*
1 day	14.78 ± 3.08	22.58 ± 10.06
1 week	25.03 ± 6.43	66.7 ± 32.06

Values represent mean ± SEM of n animals

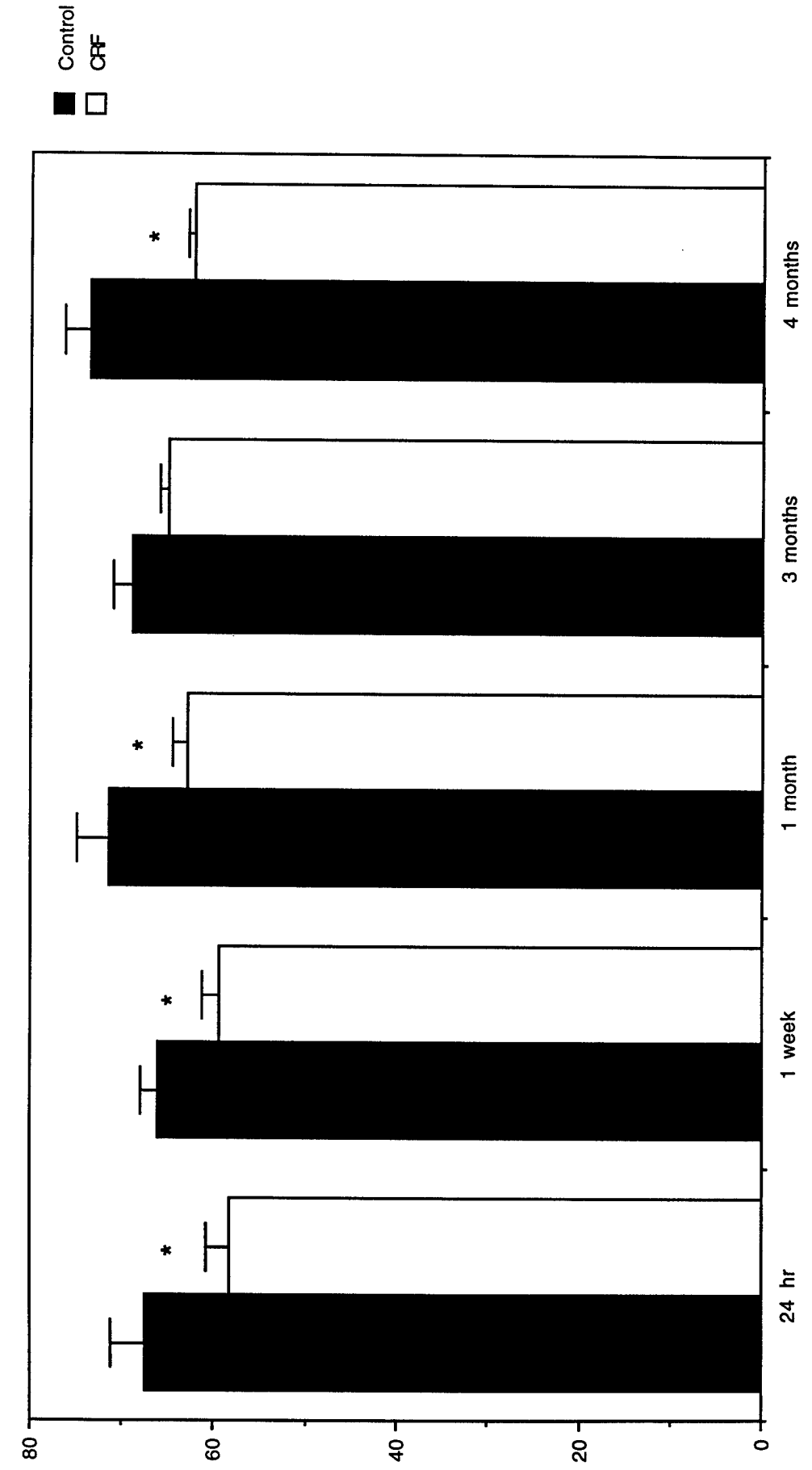


Figure 1: Effects of d-amphetamine on stereotyped behavior in CRF and control exposed animals. Data represent the total stereotyped behavior scores using the Creese-Iversen scale (0-6) in CRF (ICV) and vehicle (ICV) treated rats (N=8/group) *$p<0.05$ vs control Student's t test.

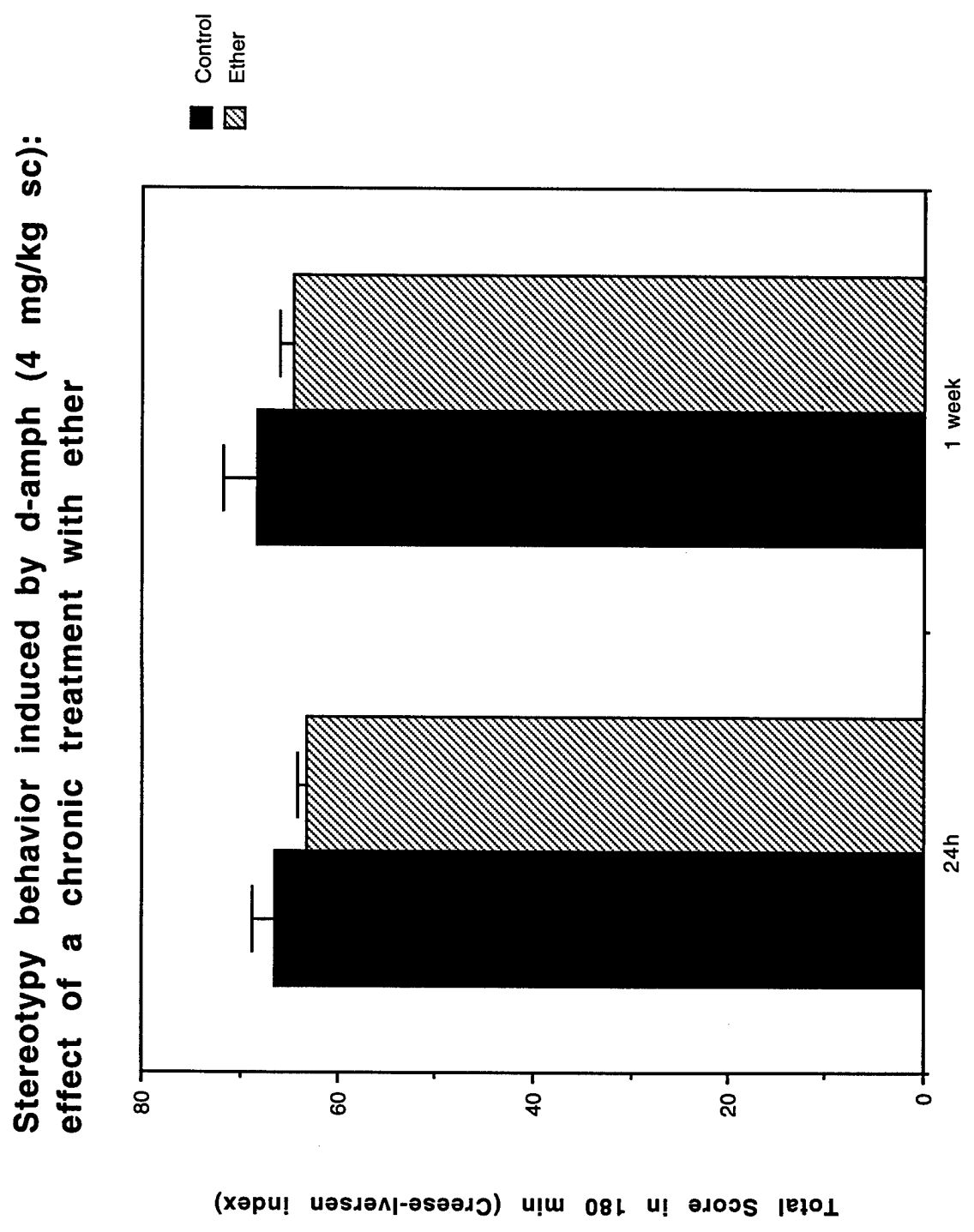
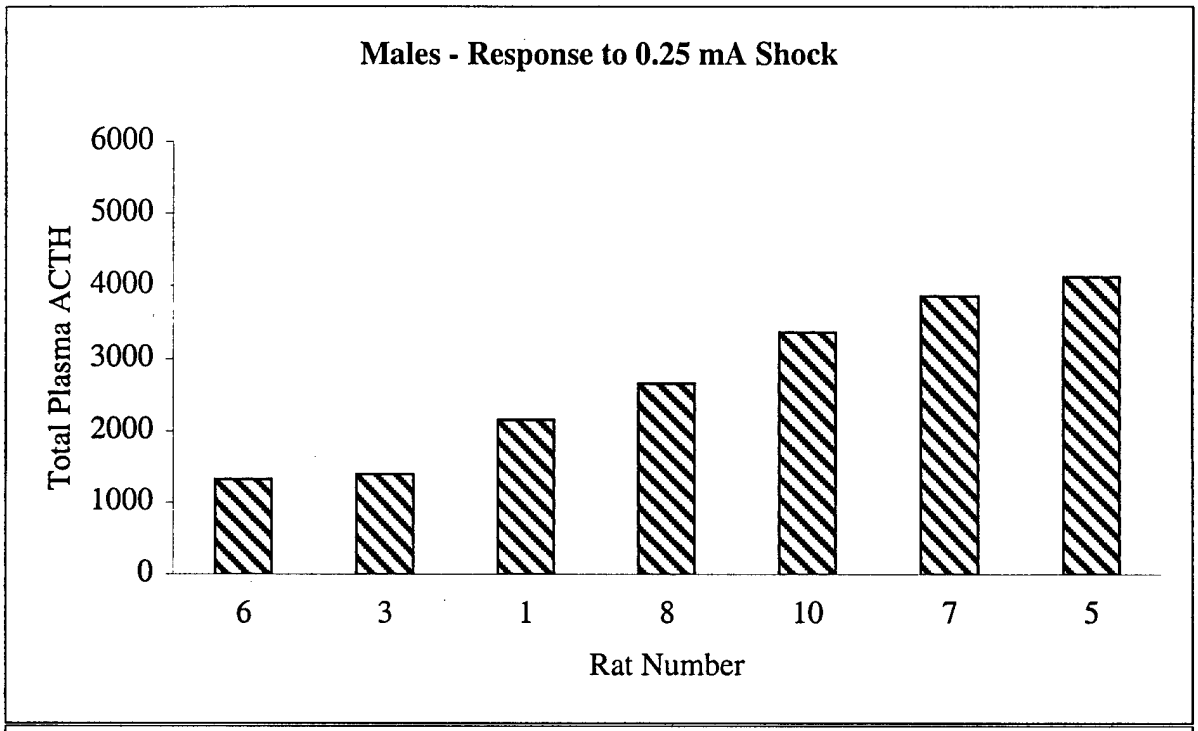
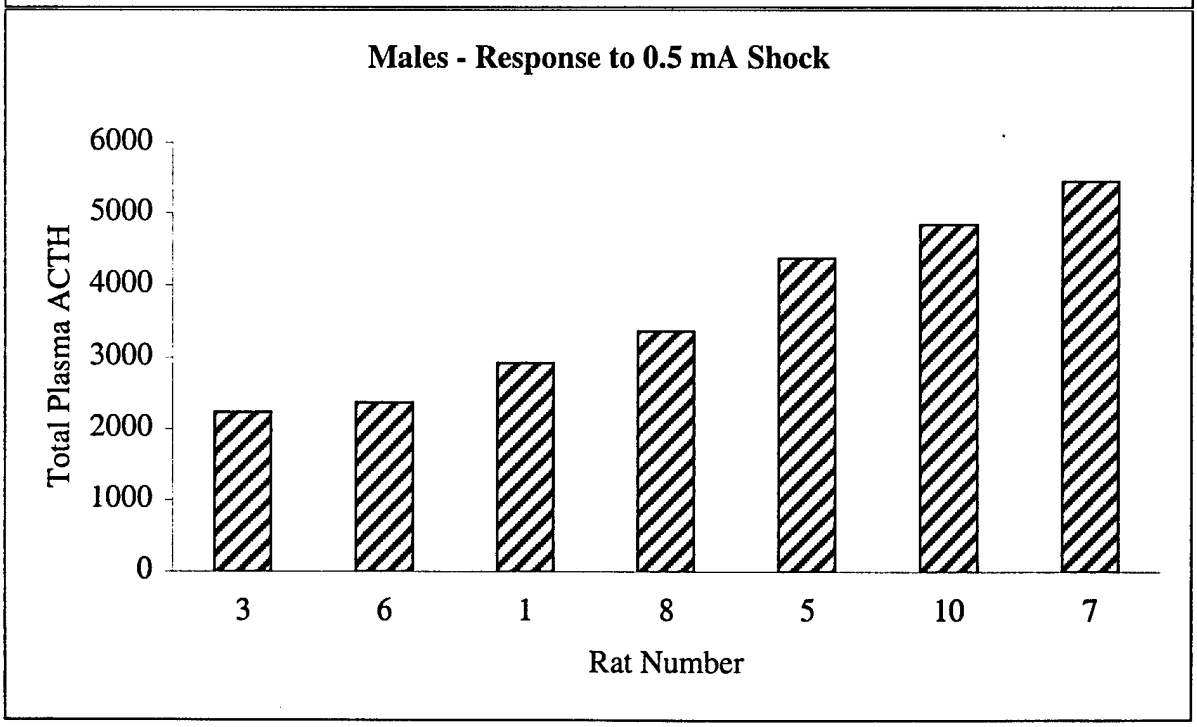


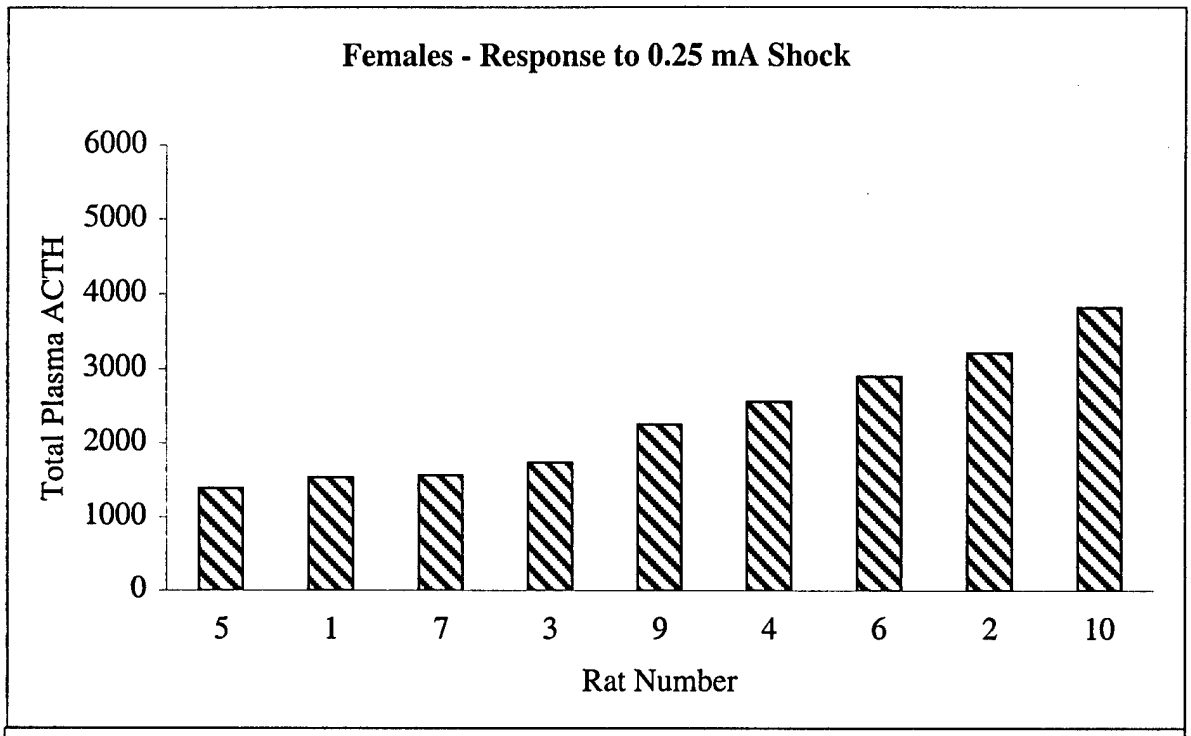
Figure 2: Effects of d-amphetamine on stereotyped behavior in ether-stressed and control exposed animals. Data represent the total stereotyped behavior scores using the Creese-Iversen scale (0-6) in ether-stressed and control treated rats (N=8/group).

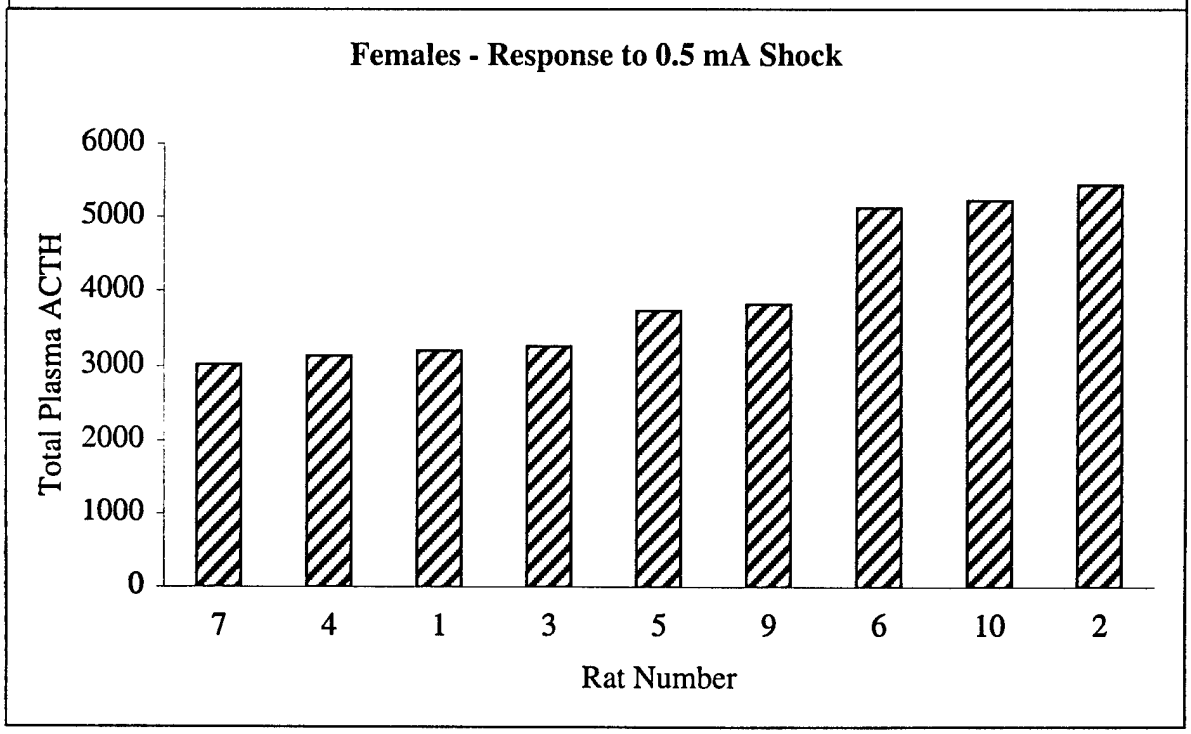




Low Response Breeders: 3 & 6
High Response Breeders: 5, 7 & 10

Figure 3: Plasma ACTH response in male NIH strain rats at two intensities of footshock stress. Values represent plasma ACTH response by individual male rats. Males #3 and 6 were designated as low responders and males #5, 7 & 10 were designated as high responders.





Low Response Breeders: 1 & 7
High Response Breeders: 2, 6 & 10

Figure 4: Plasma ACTH response in female NIH strain rats at two intensities of footshock stress. Values represent plasma ACTH response by individual female rats. Females #1 and 7 were designated as low responders and females #2, 6 & 10 were designated as high responders.

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Animal Research Committee [X] Grant [ ] Contract [ ] Fellow [ ] Other
The Scripps Research Institute [ ] New [ ] Competing continuation
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[ ] Original [X] Follow-up GRANT APPLICATION ID NO. FUNDING SOURCE

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2. TITLE OF APPLICATION OR ACTIVITY

3. PRINCIPAL INVESTIGATOR, PROGRAM DIRECTOR, OR FELLOW

George F. Koob, Ph.D.

POLICY:

Any research activity involving animals undertaken at The Scripps Research Institute (TSRI) must be reviewed and approved by our Institutional Animal Care and Use Committee (IACUC), the Animal Research Committee (ARC), in accordance with Public Health Policy regarding the care and use of laboratory animals. Applicants are required to submit to all granting agencies certification of ARC approval of compliance with the Guide for the Care and Use of Laboratory Animals, the American Association for the Accreditation of Laboratory Animal Care (AAALAC) standards, and the regulations set forth in the Animal Welfare Act (P.L. 91-579 and P.L. 94-279) and other applicable federal, state and local laws, regulations and policies. The Scripps Research Institute currently has an assurance (A3194-01) of compliance on file with the U.S. Department of Health and Human Services (HHS) which covers the institution's responsibility to monitor animal welfare. In the event that an applicant receives a pending approval from the ARC covering the proposed activity, certification of ARC review and approval must be submitted to the granting agency within 60 days of filing the grant application.

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[X] THE SCRIPPS RESEARCH INSTITUTE HAS AN APPROVED ASSURANCE OF COMPLIANCE OF FILE WITH HHS(A3194-01) which covers the care and use of laboratory animals and furthermore is accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

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[ ] The applicant has sought approval of the portions of this research activity which include laboratory animals from the ARC of The Scripps Research Institute in accordance with HHS recommendations and AAALAC standards.

JUN 27 2000 Date of ARC review and approval. (If approval is pending, write "pending". Follow up certification is required withi of filing grant application.)

(MO/DAY/YR)

[ ] This activity contains multiple projects, some of which involve other institutions(s) that have not been reviewed by the ARC. The ARC has granted approval on the condition that all projects involving laboratory animals will be reviewed and approved by an accredited institutional Animal Care and Use Committee (IACUC) before they are initiated and that appropriate certification will be submitted by the responsible institution.

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Norton B. Gilula

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