COMPARATIVE EFFECTS OF THE CRF AGONIST, OVINE CRF, AND CRF ANTAGONIST, ASTRESSIN, ON HOMECAGE BEHAVIOR PATTERNS AND DEFENSE IN THE MOUSE

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI'I IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER’S OF ARTS

IN

PSYCHOLOGY

MAY 2005

By
Catherine F. Borna Farrokhi

Thesis Committee:
Robert J. Blanchard, Chairperson
D. Caroline Blanchard
Edward Chronicle
University of Hawaii at Manoa
Abstract

COMPARATIVE EFFECTS OF THE CRF AGONIST, OVINE CRF, AND ANTAGONIST, ASTRESSIN, ON HOMECAGE BEHAVIOR PATTERNS AND DEFENSE IN THE MOUSE

by Catherine F. Borna Farrokhi

Chairperson of the Supervisory Committee: Professor Robert J Blanchard
Department of Psychology

Corticotropin-releasing factor (CRF) has been implicated in physiological and behavioral responsivity to stress and emotions including fear and anxiety. Baseline CRF elevation has been linked to depression and anxiety disorders and its effects include disruptions in sleep patterns and feeding behaviors in animals. This study attempted to characterize the effects of CRF agonists and antagonists over time by evaluating the behavior patterns of CD-1 mice in their homecage for a period of 3 hours following injection of the preferentially binding CRF1 receptor agonist, ovine CRF, or the CRF receptor antagonist, astressin. Furthermore, anti-predator responses in a new pharmacologically validated anxiety model, the Rat Exposure Test (RET), were used to assess the defensive behaviors of mice following administration of these CRF compounds. Intracerebroventricular administration of ovine CRF interrupted sleep patterns over the 3 hour time period but also suppressed active behaviors including eating, drinking, grooming, rearing, and locomotor activity during the first hour of testing. Crouching or freezing was enhanced following both low and high doses of ovine CRF. Ovine CRF also produced an anxiogenic response in the RET, decreasing
locomotor activity and contact time while increasing freezing and avoidance behaviors. As expected, the CRF antagonist, astressin, did not disrupt behavior patterns in the homecage, since baseline anxiety levels were presumably low under this test condition. However, contrary to expectation, astressin also failed to produce an anxiolytic effect in the RET. This result provides a parallel to others which claim that the anxiolytic efficacy of astressin can only be observed following a pre-stress condition elevating CRF levels prior to infusion. Analysis combining ovine CRF followed by astressin may provide information on the efficacy of the latter in reducing elevated CRF levels. Further analysis of selective CRF1/CRF2 agonists and antagonists may reveal differential roles individual CRF receptor subtypes play in modulating defensive behaviors.
TABLE OF CONTENTS

Title Page.................................................................i
Committee Approval..................................................ii
Abstract.................................................................iii
Table of Contents.......................................................v
List of Tables ..........................................................vi
List of Figures ..........................................................vii
Introduction..............................................................8
Experiment 1: Ovine CRF .............................................15
Experiment 2: Astressin ...............................................22
Discussion ..............................................................28
Future Directions .......................................................33
References ..............................................................34
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>List of Homecage Behaviors</td>
</tr>
<tr>
<td>2</td>
<td>Ovine CRF RET Table of Means</td>
</tr>
<tr>
<td>3</td>
<td>Astressin RET Table of Means</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-4</td>
<td>Ovine CRF Homecage Graphs. 49</td>
</tr>
<tr>
<td>5-10</td>
<td>Ovine CRF RET Graphs. 50</td>
</tr>
<tr>
<td>11-14</td>
<td>Astressin Homecage Graphs. 52</td>
</tr>
</tbody>
</table>
1. Introduction

Elevated corticotropin-releasing factor (CRF) levels have been identified in the physiological stress response abnormalities seen in depressive disorder (Bisette et al, 2003; Arborelius et al, 1999; Widerlov et al, 1988; Banki et al, 1987; Nemeroff et al, 1984) and some but not all anxiety disorders including posttraumatic stress (Arborelius et al, 1999; Bremner et al, 1996; Heim et al, 1997) and obsessive compulsive disorders (Fossey et al, 1996; Altemus et al, 1992). Frontline pharmacological intervention for these disorders generally involves selective serotonin reuptake inhibitors, monoamine reuptake inhibitors, or benzodiazepines. However, the inconsistent findings on efficacy and potential side effects of these compounds prompt further investigation into more effective pharmacological treatments.

CRF antagonists are considered potential pharmacological alternatives for treating stress related disorders (Holmes et al, 2003; Nemeroff 2003, 2002; O’Brien et al, 2001). The synthesis of novel CRF agonist and antagonist compounds is providing information on the role of this neuropeptide in modulating the hypothalamic-pituitary-adrenal-axis (HPA axis) and adrenocorticotropic hormone (ACTH) release, as well as behavioral responses to these endocrine changes.

Secretion of CRF from the hypothalamus activates biosynthesis and release of ACTH and glucocorticoids from the pituitary-adrenal axis which in turn evokes a cascade of endocrine, autonomic, and behavioral responses (Vale et al, 1981; Jones et al, 1998; Carrasco & Van de Kar, 2003). CRF receptors are found in abundance throughout the neocortex, with most in the somatosensory striate and entorhinal cortex, and a less defined distribution in the motor and cingulate cortex (De Souza & Insel, 1990). Further
evaluation of the rat brain reveals ample binding in the lateral thalamus, paraventricular nucleus of the hypothalamus, brain stem, olfactory system, cerebellum, and spinal cord (De Souza & Insel, 1990). The limbic system carries high density CRF receptor binding with greater expression in the nucleus accumbens, amygdala, bed nucleus of the stria terminalis, and hippocampus (De Souza & Insel, 1990). Specific limbic regions with exceptionally high receptor distribution are within the central, cortical, and medial amygdaloid nucleus and bed nucleus of the stria terminalis (Bissette, 1990). While CRF receptors are abundant within a number of non limbic structures, pathways and receptors within the limbic system are believed to mediate the influence of CRF on autonomic and emotional responses to stress (Gray, 1990).

CRF receptor subtypes have also been identified. CRF receptor subtype 1 and CRF receptor subtype 2 have been identified in the mammalian brain and have a predominantly heterogeneous distribution (Chalmers et al, 1995). Analysis of the rat brain shows that CRF 1 receptors (CRFR1) dominate in the cortex, cerebellum, and sensory and motor regions (Chalmers et al 1995; Van Pett et al, 2000). CRF 2 receptors (CRFR2) are exclusively found in subcortical regions including the medial amygdala, ventromedial nucleus of the hypothalamus, lateral septum, and the entorhinal cortex (De Souza, 1995; Chalmers, 1996; Primus, 1997). The expression of CRFR1 and CRFR2 within other subcortical regions which include the hypothalamus, amygdala, hippocampus, septum, pituitary, and olfactory regions, exhibits less distinct distribution of receptor subtypes but some heterogeneity can be observed within structures (De Souza, 1995; Chalmers, 1996; Primus, 1997; Van Pett et al, 2000). Splice variants divide
CRFR2 into CRFR2α which is the type found mostly in brain tissue and CRFR2β which is found in the brain and peripheral tissue (Lovenberg et al, 1995).

CRF agonists and antagonists reveal potentially distinct roles played by these receptor subtypes in behavioral and endocrine responses. CRFRI is well established as mediating a direct response to stress, including anxiety (Griebel et al, 2002), fear (Takahashi, 2001), aggression (Farrokhi et al, 2004), and sleep pattern disruptions in animals (Jones et al, 1998; Lancel et al, 2002) and humans (Held et al, 2002). CRFRI is also believed to modulate the HPA axis response to stress (Pellymounter et al, 2002). A CRFRI antagonist viable for human research, R121919, has already been developed and tested in patients with major depression and appears to reduce symptoms with minimal side effects (Held et al 2004; Kunzel et al 2003; Zobel et al, 2000).

The role of CRFR2 in mediating the stress response is less evident. Current developments of CRFR2 agonists and CRFR2 antagonists are allowing better characterization of CRFR2 functions by isolating the effects of this receptor. CRFR2 is believed to play a role in behavior but does not appear to modulate the ACTH response through activation of the HPA axis (Jones, et al 1998; Takahashi et al, 2001; Bakshi et al, 2002; Pellymounter et al, 2002, 2003). However, studies using CRFR2 agonists or antagonists to evaluate behavior have yielded inconsistent results. Central administration of the CRFR2 agonist, urocortin II, has shown to increase anxiogenic responses in the EPM, open field test, and marble burying task (Pellymounter et al, 2002, 2004). Urocortin II administered icv was also shown to reduce the acoustic startle reflex following enhancement of the acoustic startle response with pretreatment of CRF (Risbrough et al, 2003). However, other studies have shown that urocortin II and
urocortin III, another highly selective CRFR2 agonist, produce increases in open arm time in the EPM when infused icv, indicating that these compounds produced an anxiolytic-like effect (Valdez et al, 2002, 2003).

Studies testing the behavioral effects of CRFR2 antagonists have also yielded inconsistent results. Takahashi et al (2001) demonstrated that the CRFR2 antagonist, antisauvagine, produced an anxiolytic effect in the conditioned freezing, EPM, and defensive-withdrawal tests. However, Radulovic et al (1999) found that same compound infused into the lateral septum produced an anxiogenic effect enhancing context dependent fear conditioning. Development of CRFR2 knockouts or mutant mice supports the latter finding. CRFR2 knockout mice are believed to exhibit increased anxiety-like and depressive-like behavior (Bale et al, 2000, 2002) indicating that CRFR2 activation may reduce the stress response. However, the role of CRFR2 on behavior cannot be fully interpreted through evaluation of knockouts since the effects are yet to be understood as directly related to the absence of CRFR2 or a consequence of other developmental anomalies attributed to the genetic expression of these knockouts.

Understanding and isolating CRF receptor subtypes are important for characterizing the physiological and behavioral functions of CRF. However, non-selective CRF compounds are important for understanding the effects of fluctuating central and circulating CRF levels since natural activation of CRF initiates the functioning of both receptor subtypes and these effects are not fully understood. The present series of experiments evaluated two non-selective CRF compounds to better characterize the behavioral effects of activating or inhibiting both CRFR1 and CRFR2 subtypes using a CRF agonist, ovine CRF, and a CRF antagonist, astressin.
Ovine CRF is a frequently used CRF agonist which has been evaluated extensively for its anxiogenic profile in humans and in animal models. While it preferentially binds to CRFR1, it has also been shown to bind to CRFR2 (Eckhart et al, 2001). In humans with depression, ACTH and cortisol levels are typically elevated; intravenous administration of ovine CRF increased plasma cortisol levels but blunted the ACTH response in these individuals (von Bardeleben & Holsboer, 1990; Nerozzi et al, 1998, Heim, 2002). Interestingly, although plasma cortisol levels were further elevated, anxiogenic responses were not observed, an indication that behavioral consequences of CRF are central rather than resulting from the circulating endocrine response (von Bardeleben & Holsboer, 1990).

In animal models, ovine CRF produced behavioral changes including anxiogenic responses in the elevated plus maze, increased startle response, locomotor activity, and reduced feeding behaviors (Jones et al, 1998; Valdez et al, 2002). Chronic ovine CRF treatment also produced anxiogenic responses in addition to increased adrenal weight and reductions in thymus volume (Buwalda et al, 1997, 1998) demonstrating a nonadaptive behavioral and physiological consequence to elevated CRF levels.

Astressin is a CRF antagonist with a nonselective affinity for both CRF 1 and 2 receptor subtypes. It has been tested in a variety of animal models. Immunohistochemical analysis demonstrates astressin's capability of inhibiting stress-induced ACTH secretion at efficacy rates up to 100 times more potent than the commonly used antagonist, α-helical CRF when tested in vitro, and 10 times more potent in vivo (Kinzig et al, 2003; Pelleymounter et al, 2002; Rivier et al, 1996; Gulyas et al, 1995). In behavioral analysis done by Pelleymounter et al (2002), astressin reduced anxiety-like behavior in the EPM
and compulsive burying in the marble burying task, which is suggested to serve as a potential model of obsessive compulsive disorder (OCD) (Njung’e and Handley, 1991a). This test is sensitive to compounds commonly used to treat OCD including diazepam and fluoxetine (Eddy & Walbroehl, 1998; Njung’e and Handley, 1991b).

More interesting and profound results are demonstrated in studies which have shown the anxiolytic-like effectiveness of astressin infusion after elevating baseline CRF levels. This approach appears more reflective of the potential efficacy of CRF antagonists on analogous disorders in humans where elevated basal CRF levels are observed. Spina et al (2000) demonstrated that i.c.v. infusion of astressin reduced avoidance behaviors following pre-exposure to social stress and rat/humanCRF (h/rCRF) in the EPM. Astressin was also effective in increasing social interaction time in the social interaction test (SI) following injection of the CRF agonist, urocortin, which characteristically reduced interaction time when administered alone (Sajdyk & Gehlert, 2000). Direct hippocampal infusion of astressin prior to h/rCRF also blunted the enhanced fear conditioning of footshock (Blank et al 2002; Radulovic et al 1999). However, some studies with prior stress or CRF conditioning reported that astressin administered alone was ineffective at producing an anxiolytic-like effect (Sajdyk & Gehlert, 2000; Spina et al, 2000), while others report the anxiolytic profile of astressin administered alone (Pelleymounter, 2002). The potency of astressin’s CRF receptor binding capability has been evaluated. A comparison study of astressin and α-helical CRF following ovine CRF agonist infusion was demonstrated both in vitro and in vivo (Brauns et al, 2001). In vitro analysis demonstrates astressin’s superior binding capability compared to α-helical CRF (Gulyas et al, 1995; Brauns et al, 2001). However, α-helical CRF, but not astressin,
successfully reversed the effects of ovine CRF and its anxiogenic-like action in vivo. It is unclear whether these incongruent behavioral findings reflect astressin’s CRF antagonizing capabilities. This prompts further investigation of astressin using alternative behavioral tests to demonstrate its potential anxiolytic-like profile.

The purpose of the present series of experiments was to characterize of the role of CRF agonists and antagonists in modulating behaviors in a non-manipulated environment, the subject’s homecage, followed by analysis of these effects in an ethologically relevant threat situation, the Rat Exposure Test (RET). The homecage test should establish the effects of acutely administered CRF agonists and antagonist on characteristic behavior patterns in a relatively non threatening situation over an extended three hour time span. While homecage tests have been conducted with a variety of CRF compounds (Jones et al, 1998; Valdez et al, 2002) none have done a fine analysis of changes over time following a single injection accounting for initial responses to the CRF disruption as well as long term effects. Behaviors to be evaluated include feeding, sleep patterns, locomotion, as well as potentially anxiety-like or defensive behaviors, which are not characteristically observed in the homecage.

Following the homecage test, the role of CRF in modulating defensive behaviors was assessed using the RET. This test is a model of defensive behavior (Yang et al, 2004) that elicits a series of ethologically based responses. The focus of this test is on the defensive behaviors of mice toward a potential predator (a rat) where an ambiguous and threatening situation elicits distinct proximal and postural changes (procedure described in Yang et al, 2004). Defensive behaviors of mammalian species are believed to serve as a valid index of anxiety (Blanchard et al 2003, 2001, 1990) as they are sensitive to a
variety of anxiolytic and anxiogenic compounds (Blanchard et al, 1993a, 1993b, 1990; Griebel 1995a, 1995b). This model is employed to determine whether administration of ovine CRF will increase the expected anti-predatory response and if astressin can reduce the defensive response.

This research was divided into two experiments. The first evaluated the role of CRF agonist, ovine CRF, in the homecage followed by the RET. The second evaluated the CRF antagonist, astressin, in the homecage followed by the RET. The following hypotheses were formulated and evaluated in these studies:

- **Hypothesis 1**: Ovine CRF disrupts characteristic behavior patterns in the homecage
- **Hypothesis 2**: Ovine CRF produces an anxiogenic effect in the RET
- **Hypothesis 3**: Astressin does not disrupt characteristic behavior patterns in homecage
- **Hypothesis 4**: Astressin produces an anxiolytic effect in the RET

The main goal of this study is to evaluate this pair of tests as an effective way to determine CRF agonist and antagonist effects on a non-threatening and a threatening situation. This information will provide a means for finer analysis of the CRF receptor subtypes in modulating behaviors.

2. Experiment 1

2.1 Subjects
Male CD-1 mice between the ages of 12-15 weeks obtained from Charles River Laboratories (Wilmington, MA) were used as subjects. All animals were single-housed under a 12 hour light-dark cycle. Testing was conducted during the light cycle between the hours of 1 p.m. and 5 p.m.

2.2 Surgical Procedure

Right-side unilateral i.c.v. cannulas were implanted using the following coordinates from bregma: AP -.2mm, ML -1.0mm, DV -2.3mm. Animals were anesthetized using .4 ml pentobarbital (.65mg/kg), .04 ml glycopyrolate, and isoflurane gas as needed. Animal were given 7-10 days of recovery prior to testing. During this recovery period, cannulas were cleaned and animals were handled daily to habituate and reduce baseline stress levels during drug infusion.

Cannula placements were verified by means of perfusion and histological analysis following the conclusion of all behavioral testing. Only animals with proper cannula placement were included in the studies.

2.3 Drugs

Ovine CRF was obtained from Max-Planck Institute for Experimental Medicine (Gottingen, Germany). Original concentration of the compound was .2μg/μl diluted in 200 μl 5mM acetic acid in artificial cerebrospinal fluid solution (CH3COOH/1xCSF). A two-fold dilution of ovine CRF was administered as the low dose for a final concentration of .1 μg/μl solution (n = 10). The high dose of ovine CRF was .2 μg in a volume of 1.0 μl (n = 10). 1.0 μl dose of the CH3COOH/aCSF solution was used as the vehicle (n = 8).
Single unilateral infusions were conducted for 30 seconds and the injector remained in the cannula for an additional 60 seconds to help prevent back flow. All doses were administered in a volume of 1.0 µl.

2.4 Homecage Test

The homecage apparatus consisted of a clear polycarbonate cage measuring 17.8 cm x 28 cm x 12.8. Animals were single housed prior and during testing with food and water provided as needed. The homecage test was conducted to follow potential changes in characteristic daily behavior patterns over a 3 hour time period during the inactive light phase. The purpose was to characterize all behaviors observed in the homecage thus consisted of exhaustive parameters which included sleep, feeding, grooming, locomotor activity, standing, rearing, and risk assessment or fear related behaviors as indicated by stretch attend and crouch immobile postures. Table 1 lists the behaviors and provides a brief description of each category. Locomotor activity, standing, feeding, and rearing were collapsed to form an active behavior category for analysis. However, locomotor activity was also analyzed alone for comparison with studies showing increased activity in rats treated with ovine CRF (Buwalda et al, 1997, 1998; Jones, 1998; Valdez, 2002).

Animals were habituated to the testing room 24 hours prior to the start of the experiment. On the test day, animals were injected and immediately placed back in the homecage and recorded while undisturbed for the 180 minute duration of the test. Testing was conducted between the hours of 1 pm and 5 pm.

2.4.1 Behavior analysis
Behaviors were scored by a trained observer blind to drug doses using the timesampling method. Behaviors were observed and scored every minute for 3 hours. Upon completion of scoring, the 180 minutes were collapsed into 20 minute time bins in which the individual behaviors were tallied. As a result, 9 time periods covering 20 minute durations for each were created.

2.4.2 Statistical Analysis

Repeated measures analysis of variance (ANOVA) was used to analyze the homecage data. Subsequent Newman Keuls post hoc analysis was used to verify differences between groups and changes between each 20 minute time bin.

2.5 Rat Exposure Test (RET)

The RET was conducted 1 week following the homecage test.

2.5.1 Apparatus

Testing was conducted in a 46 x 24 x 21 cm clear polycarbonate cage covered with a metal lid. The exposure cage was divided into two equal sized compartments by a wire mesh screen. The home chamber was a 7cm x 7cm x 12cm box made of black Plexiglas on three sides and clear Plexiglas on one side to facilitate videotaping. The home chamber was connected to the exposure cage by a clear Plexiglas tube tunnel (4.4 cm in diameter, 13 cm in length, elevated 1.5cm from the floor of the two chambers).

2.5.2 Predatory stimuli

Adult male Long Evans rats (average weight, 450 grams) from breeding colonies maintained at the University of Hawaii were used as predatory stimuli. In order to keep
the stimulus rats uniformly active during and across test sessions, they were systemically injected with 5.0 mg/kg of d-amphetamine 20 minutes prior to testing. A new rat was used after every five trials or if cessation of movement or stereotypy was observed.

2.5.3 Procedure

The apparatus and procedure are as described in Yang et al, 2004. All tests were conducted during the light phase of the light/dark cycle under the illumination of a 100-watt red light to help reduce possible elevation of baseline anxiety levels under white light. They were recorded using a horizontally-mounted video camera. Prior to the start of each trial, home cage bedding of each subject was placed on the floor of the home chamber as well as in the exposure cage area not partitioned for the rat, referred to as the surface area. Each part of the apparatus was cleaned with 5% alcohol and dried with paper towels between trials. The testing procedure consisted of two phases.

Phase 1: Habituation. Each subject was allowed 3 days of habituation in the apparatus. The mouse was placed in the center of the surface and was allowed to explore freely for 10 minutes with no rat present.

Phase 2: Exposure Test. On the fourth day, an amphetamine treated male Long-Evans rat was introduced behind the wire mesh. 30 minutes after receiving a single dose of injection, the mouse subject was placed in the center of the surface area.

2.5.4 Behavior analysis

Behaviors were scored by a trained observer blind to drug condition using the ethological analysis software “Hindsight” (Developed by Dr. Scott Weiss). The parameters consisted of proximal and behavioral measures. The proximal measures
assessed relative to the location of the stimulus rat were the duration of time spent in the home chamber, tunnel, and on the surface. Locomotor activity was comprised of the number of transits between these locations. Total contact time included the frequency and time spent in contact with the wire mesh. The behavioral measures were frequency and duration of stretch attend postures (SAP, a posture in which the body is stretched forward and the animal is motionless), freezing, (complete cessation of movement except breathing), grooming, and defensive burying (sawdust pushed from the chamber into tunnel opening).

2.5.5 Statistical Analysis

One way analysis of variance (ANOVA) was used to analyze the RET data. Subsequent Dunnett's post hoc analysis was used to make comparisons between groups.

3. Results

3.1 Ovine CRF Homecage Results

3.1.1 Sleep (graph 1)

Sleep patterns were evaluated by means of lying frequency observed for each time bin. For vehicle controls, lying was increased over a 60 minute period following injection (p<.001) and remained steady thereafter. Both dose groups showed a reduction of lie or sleep compared to the vehicle group [F(16, 200)=5.0664, p<.00001]. Neither dose group showed a significant change in sleep over the 180 minute test duration.

3.1.2 Crouch Immobile (graph 2)

ANOVA revealed a significant difference between the three groups in crouch immobile (freezing) F(16, 200)=2.5597, p<.01. The vehicle group showed a frequency of
less than 2 observations of crouch immobile at each 20 minute time bin. The levels for this group remained unchanged for the 180 minute test duration. Post hoc analysis demonstrated that crouch immobile increased for the low dose during the 2\textsuperscript{nd} time period ($p<.001$) and declined by the 4\textsuperscript{th} time period approximately 80 minutes into the observation ($p>.05$). Crouch significantly increased for the high dose during the 3\textsuperscript{rd} time period ($p<.001$) and declined by the 6\textsuperscript{th} time period, approximately 120 minutes into the session ($p>.05$).

3.1.3 Locomotor Activity (graph 3)

Locomotor activity was significantly different across the 3 groups $F(16, 200)=3.4682$, $p<.0001$. Locomotor activity for the vehicle group was increased for the first 40 minutes of testing. Neuman Keuls revealed locomotor activity was suppressed during the first 20 minutes for both the low ($p<.05$) and high dose groups ($p<.01$). Suppression of locomotor activity for the high dose group was maintained 40 minutes into testing ($p<.01$) after which it stabilized across the three groups.

3.1.4 Active Behaviors (graph 4)

Eating, drinking, grooming, rearing, and locomotor activity were combined in an active behavior category. Analysis revealed a significant difference between groups $F(16, 200)=4.4397$, $p<.00001$. Both low ($p<.05$) and high doses ($p<.0001$) exhibited suppression of active behaviors during the first 20 minutes in comparison to the vehicle group. The high dose group maintained suppression of active behavior 40 minutes into the session ($p<.01$). Following the 2\textsuperscript{nd} time bin, active behavior stabilized across the three groups.

3.1.5 Other Behaviors Measured
Risk assessment, as measured by stretch attend and stretch approach postures, was not significantly different for the dose groups. With the exception of locomotor activity, individual behaviors in the active behavior category could not be analyzed across time bins as the occurrences of observations were too infrequent to be analyzed as single categories.

3.2 Ovine CRF RET Results

Table 2 presents a table of means for the ovine CRF RET test. Ovine CRF reduced the amount of time spent on the surface area (graph 5) \([F(2, 25)=8.3046, p<.01]\) and significantly increased the amount of time spent in the chamber (graph 6) \([F(2, 25)=10.243, p<.001]\). Locomotor activity, which was measured as the number of transits between the surface, tunnel, and chamber, was reduced in the ovine CRF groups (graph 7) \([F(2, 25)=3.7852, p<.05]\). However, Dunnett’s post hoc test revealed this effect was significant only for the high dose group \(p<.05\). Total duration of freezing was increased in both drug dose groups (graph 8) \([F(2, 25)=4.0951, p<.05]\); subsequent post hoc analysis revealed this effect was significant only for the low dose group \(p<.05\). Mesh contact (graph 9) \([F(2, 25)=8.1229, p<.01]\) and climb duration (graph 10) \([F(2, 25)=5.1518, p<.05]\) were significantly lower in both dose groups in comparison to controls.

4. Experiment 2

4.1 Subjects

Male CD-1 mice between the ages of 12-15 weeks obtained from Charles River Laboratories (Wilmington, MA) were used as subjects. All animals were single-housed
under a 12 hour light-dark cycle. Testing was conducted during the light cycle between the hours of 1 p.m. and 5 p.m.

4.2 Surgical Procedure

Right-side unilateral i.c.v. cannulas were implanted using the following coordinates from bregma: AP -.2mm, ML -1.0mm, DV -2.3mm. Animals were anesthetized using .4 ml of pentobarbital (.65mg/kg), .04 ml glycopyrolate, and isoflurane gas as needed. Animals were given 7-10 days of recovery prior to testing. During this recovery period, cannulas were cleaned and animals were handled daily to habituate and reduce baseline stress levels during drug infusion. Cannula placements were verified by means of perfusion and histological analysis following behavioral testing. Only animals with proper cannula placement were included in the studies.

4.3 Drugs

Astressin was obtained from Max-Planck Institute for Experimental Medicine (Gottingen, Germany). Original concentration of astressin was .9μg/μl diluted in 200 μl 5mM CH3COOH/aCSF. The two doses administered were .9μg for the high dose (n = 8) and 1.0 μl of a two fold dilution (.45 μg/μl final) as the low dose (n =10). The CH3COOH/aCSF solution administered at a volume of 1.0 μl was used as the vehicle compound (n = 8). Single unilateral infusions were conducted for 30 seconds and the injector remained in the cannula for an additional 60 seconds to help prevent back flow. Cannula placements were verified following testing and only animals with proper cannula placement were included in the studies.
4.4 Homecage Test

The homecage apparatus consisted of a clear polycarbonate cage measuring 17.8 cm x 28 cm x 12.8. Animals were single housed prior and during testing with food and water provided as needed. The homecage test was conducted to follow potential changes in characteristic daily behavior patterns over a 3 hour time period during the inactive light phase. The purpose was to characterize all behaviors observed in the homecage thus consisted of exhaustive parameters which included sleep, feeding, grooming, locomotor activity, standing, rearing, and risk assessment or fear related behaviors as indicated by stretch attend and crouch immobile postures. Table 1 lists the behaviors and provides a brief description of each category. Locomotor activity, standing, feeding, and rearing were collapsed to form an active behavior category for analysis. However, locomotor activity was also analyzed alone for mean comparison of results found in experiment 1.

Animals were habituated to the testing room 24 hours prior to the start of the experiment. On the test day, animals were injected and immediately placed back in the homecage and recorded while undisturbed for the 180 minute duration of the test. Testing was conducted between the hours of 1 pm and 5 pm.

4.4.1 Behavior analysis

Behaviors were scored by a trained observer blind to drug doses using the timesampling method. Behaviors were observed and scored every minute for 3 hours. Upon completion of scoring, the 180 minutes were collapsed into 20 minute time bins in which the individual behaviors were tallied. As a result, 9 time periods covering 20 minute durations for each were created.
4.4.2 Statistical Analysis

Repeated measures analysis of variance (ANOVA) was used to analyze the homecage data. Subsequent Newman Keuls post hoc analysis was used to verify differences between groups and changes between each 20 minute time bin.

4.5 Rat Exposure Test (RET)

The RET was conducted 1 week following the homecage test.

4.5.1 Apparatus

Testing was conducted in a 46 x 24 x 21 cm clear polycarbonate cage covered with a metal lid. The exposure cage was divided into two equal sized compartments by a wire mesh screen. The home chamber was a 7cm x 7cm x 12cm box made of black Plexiglas on three sides and clear Plexiglas on one side to facilitate videotaping. The home chamber was connected to the exposure cage by a clear Plexiglas tube tunnel (4.4 cm in diameter, 13 cm in length, elevated 1.5cm from the floor of the two chambers).

4.5.2 Predatory stimuli

Adult male Long Evans rats (average weight, 450 grams) from breeding colonies maintained at the University of Hawaii were used as predatory stimuli. In order to keep the stimulus rats uniformly active during and across test sessions, they were systemically injected with 5.0 mg/kg of d-amphetamine 20 minutes prior to testing. A new rat was used after every five trials or if cessation of movement or stereotypy was observed.

4.5.3 Procedure
The apparatus and procedure are as described in Yang et al, 2004. All tests were conducted during the light phase of the light/dark cycle under the illumination of a 100-watt red light to help reduce possible elevation of baseline anxiety levels under white light. They were recorded using a horizontally-mounted video camera. Prior to the start of each trial, home cage bedding of each subject was placed on the floor of the home chamber as well as in the exposure cage area not partitioned for the rat, referred to as the surface area. Each part of the apparatus was cleaned with 5% alcohol and dried with paper towels between trials. The testing procedure consisted of two phases.

**Phase 1: Habituation.** Each subject was allowed 3 days of habituation in the apparatus. The mouse was placed in the center of the surface and was allowed to explore freely for 10 minutes with no rat present.

**Phase 2: Exposure Test.** On the fourth day, an amphetamine treated male Long-Evans rat was introduced behind the wire mesh. 30 minutes after receiving a single dose of injection, the mouse subject was placed in the center of the surface area.

4.5.4 Behavior analysis

Behaviors were scored by a trained observer blind to drug condition using the ethological analysis software “Hindsight” (Developed by Scott Weiss). The parameters consisted of proximal and behavioral measures. The proximal measures assessed relative to the location of the stimulus rat were the duration of time spent in the home chamber, tunnel, and on the surface. Locomotor activity was comprised of the number of transits between these locations. Total contact time included the frequency and time spent in contact with the wire mesh. The Behavioral measures were frequency and duration of
stretch attend postures (SAP, a posture in which the body is stretched forward and the animal is motionless), freezing, (complete cessation of movement except breathing), grooming, and defensive burying (sawdust pushed from the chamber into tunnel opening).

4.5.5 Statistical Analysis

One way analysis of variance (ANOVA) was used to analyze the RET data. Subsequent Dunnett’s post hoc analysis was used to make comparisons between groups.

5. Results

5.1 Astressin Homecage Results

Main effects of dose were not significant for any of the behavioral parameters in the astressin homecage test. Graphs of sleep, crouch immobile, locomotor, and active behaviors are provided for comparison to results found in experiment 1 of characteristic behavioral patterns. Sleep patterns significantly and systematically increased for all 3 groups by the third time period (p<.01 vehicle, p<.0001 low and high doses) and stabilized for the duration of the test (graph 11). Crouch immobile remained unchanged throughout the session (graph 12). Locomotor activity (graph 13) and active behaviors (graph 14) were reduced and stabilized for all 3 groups (p<.05 all groups) after the first time period.

5.2 Astressin RET Results

Table 3 presents the table of means for the Astressin RET test. Astressin did not produce any significant effects in the RET.
6. Discussion

The two experiments described here illustrate the behavioral effects of the CRF agonist, ovine CRF, and antagonist, astressin, in two distinct models. The homecage test was aimed at characterization of the time course of CRF mediated behavioral pattern changes without the introduction of additional stressors, while the RET evaluated the role of CRF agonists and antagonists in mediating the anti-predatory response in mice.

While studies have evaluated the acute effects of ovine CRF on locomotor activity in the homecage (Buwalda et al 1997, 1998; Jones et al, 1998; Valdez et al, 2002) this study is the first to combine other activity parameters including sleep pattern disruptions, crouch immobility, and potential risk assessment behaviors. Furthermore, this is the first to analyze the homecage effects of ovine CRF in mice.

The analysis indicated that ovine CRF did produce a disruption in characteristic homecage behavior patterns. Vehicle animals exhibited a stable sleep pattern approximately 40 minutes following injection. Ovine CRF appeared to suppress this pattern and facilitate wakefulness for the duration of the test. Interestingly, during the initial 40 minutes, a suppression of locomotor activity rather than an increase was observed in the ovine CRF groups which then stabilized for the remainder of the 3 hour time period. This is in contrast to studies which reported that ovine CRF increases locomotor activity. Buwalda et al (1997) found increases in locomotor activity in rats during active and inactive cycles up to 4 hours following ovine CRF infusion. Other studies using rats to analyze the effects of ovine CRF have verified an increase in locomotor activity up to 3 hours following acute injection (Jones et al, 1998; Valdez et al
2002). This difference in findings may be species related since mice exhibit greater baseline activity in comparison to rats. The difference might also reflect how locomotor activity was defined. In the rat studies, locomotor activity was defined as the absence of inactivity whereas the present study defined locomotion as movement about the homecage not accounted for by other active behaviors. The active behavior category included feeding, rearing, grooming, as well as locomotion. This category showed the same suppression pattern as locomotion alone during the first 20 minutes of testing for both low and high dose ovine CRF groups. For the high dose group, suppression of active behavior was maintained for the first 40 minutes of testing.

The predominant behavior induced by ovine CRF was crouch immobile, which often serves as an index of freezing. This could be distinguished from lying or sitting in terms of the crouching posture. Crouching is specifically associated with behaviors elicited by a threatening stimuli or situation (Blanchard, 1969). Crouching was observed for the low dose group for approximately 80 minutes following injection and maintained in the high dose group for 120 minutes after which both groups became indistinguishable from controls. These findings indicate that elevated CRF levels may produce anxiety-like behaviors under normal conditions without the presence of a stressor, providing a potential link to depression and anxiety disorders where maladaptive emotional responses may occur without the presence of an actual threat.

Follow-up analysis of the homecage test with the RET was employed to provide further evidence for the anxiogenic profile of ovine CRF. The RET, which targets innate fear responses, has shown to produce defensive anti-predator responses in CD-1 mice (Yang et al, 2004), and ovine CRF was expected to elevate fear- or anxiety-like behaviors
in this test. Animals which did not receive ovine CRF spent a substantial amount of time in contact or close proximity to the stimulus rat indicating a low level of avoidance toward the threatening stimulus. The anxiogenic effect of ovine CRF was demonstrated in both drug dose groups. These groups spent a significant amount of time freezing and also tended to avoid areas in closest proximity to the stimulus rat. The increased freezing exhibited by the ovine CRF groups supports the notion that crouch immobile observed in the homecage test may have been fear or anxiety related behavior. The suppression of locomotor activity observed in the RET drug dose groups also supports the homecage test findings and indicates that ovine CRF or other CRF agonists may not be increasing locomotion in mice as has been reported in studies using rats.

The homecage test and RET both provide robust evidence that ovine CRF produces or enhances anxiety-like behavior, supporting the first two hypotheses formulated for experiment 1.

A stressin has been characterized as an anxiolytic compound. It has been shown to reverse the effects of drug or social stress induced CRF elevation in the elevated plus maze (Spina et al, 2000) and to block enhanced context and tone dependent fear conditioning of hippocampal CRF infusions (Radulovic et al, 1999). In the second experiment, it was not expected that stressin would produce any characteristic behavioral disruptions in the homecage as this was an inherently low stress paradigm. In agreement with this expectation, sleep, locomotor activity, crouching and all other behaviors were indistinguishable from those observed in the homecage controls. Hence, it can be presumed that antagonism of CRF receptors does not alter behavior unless a stressor is presented. This is supportive evidence for the potential of CRF antagonists to
specifically target the abnormal CRF induced responses without disrupting other characteristic behaviors.

In the second portion of experiment 2, mice infused with astressin were introduced to the RET, with the expectation that a reduction in defense related behaviors would be observed for the drug dose groups. Contrary to expectation, astressin did not produce a clear anxiolytic effect in the RET. Neither proximal, nor behavioral measures were significantly different between vehicle and drug dose groups. The findings of this present study contradict a previous unpublished study in which the effects of astressin were also tested in the RET. In the previous study, astressin appeared to produce an anxiolytic effect, showing a reduction in SAP and freezing. However, results of this study may be inconclusive because atypical testing procedures were used which may have altered baseline anxiety or defensive measures. The animals in this previous study were tested in the Mouse Defense Test Battery one week prior to the RET. In this test, a mouse is placed in direct contact with an anesthetized rat and this elicits robust defensive responses. As a result, this preceding test may have elevated baseline CRF levels in the RET. Other potential problems with the previous astressin RET study was that drug doses were administered in different volumes rather than concentrations, habituation procedures were shortened to one day, and data analysis not performed blind to doses. Thus, it may be inappropriate to make a comparison between these two studies.

The present absence of an anxiolytic effect of astressin in the RET is in agreement with research showing that the effects of astressin and/or other CRF antagonists are not fully apparent until a pre-stressor elevating CRF levels is presented. In several previous studies in which pre-stressors were administered before the anxiolytic effect of astressin
could be observed (Radulovic et al., 1999; Sajdyk & Gehlert, 2000; Spina et al., 2000). That is, attempting to blunt the CRF response before CRF levels are elevated may be ineffective since baseline levels may not be robust enough for an observable CRF antagonist anxiolytic effect. However, elevating CRF levels prior to antagonist infusion may provide a mechanism against which antagonists may operate.

The inability to find an astressin effect in the RET may also be due to the low level of defense behaviors exhibited by the controls. As observed, vehicle animals spent more than 60 percent of their time on the surface area, more than 30 percent in contact with the wire mesh, and very little time freezing indicating that a clear defensive response may not have been present. Vehicle control results in the ovine CRF RET study were similar. Consequently, an anxiolytic response could not be measured if an initial fear response was not apparent. However, strain comparison analysis by Yang et al. (2004) reported that CD-1 mice did show anxiety-like behavior toward the stimulus rat. Results of behavioral measures in the strain comparison study were similar to those found here, with the exception of surface time which was not reported. Thus, the levels reported here may appear low but not indicative of an absence of a defensive response. This prompts further investigation into the efficacy of astressin without the presence of a pre-stress condition.

The results of the second study demonstrate that astressin did not disrupt characteristic homecage behavior patterns of mice, but also failed to produce an anxiolytic effect in RET. Thus, the present RET astressin results, while not in agreement with hypothesis 4, do agree with previous findings that suggest it may be necessary to elevate CRF levels before the anxiolytic profile of astressin can be established.
7. Future Direction

The future direction for my dissertation research is to examine the efficacy of astressin in the homecage following baseline CRF elevation with ovine CRF or other agonists, in order to evaluate the efficacy of antagonists at reducing the response to CRF elevation. This affords a better parallel between pre-clinical research and clinical conditions such as depression and anxiety related disorders which characteristically involve initial elevations in basal CRF. To expand further on the role of CRF in modulating behavior, specific CRF receptor subtype agonists and antagonist can be used in the homecage and RET to better define the role of each subtype in modulating behavior. Since many previous studies have characterized the role of CRFR1 in the stress response, the focus of my dissertation will be on the role of CRFR2. This can be achieved by using ovine CRF or other agonists such as cortagine, a CRFR1 agonist, or urocortin II or III, which are CRFR2 agonists, in conjunction with the selective CRFR2 antagonists, anti-sauvagine-30. Other tests, including hormonal assays, may be added for better clarification and characterization of the role of CRFR2 on behavior. Finally, experiments infusing selective CRF compounds into specific brain regions may help describe the neural circuitry involved in the stress response.
8. References


Kunzel HE, Zobel AW, Nickel T, Ackl N, Uhr M, Sonntag A, Ising M, Holsboer F.


Njung'e K, Handley SL. Effects of 5-HT uptake inhibitors, agonists and antagonists on the burying of harmless objects by mice; a putative test for anxiolytic agents. Br J Pharmacol. 1991(b);104(1):105-112.


Sajdyk TJ, Gehlert DR. Astressin, a corticotropin releasing factor antagonist, reverses the anxiogenic effects of urocortin when administered into the basolateral amygdala. Brain Res. 2000;877(2):226-34.

Takahashi LK. Role of CRF(1) and CRF(2) receptors in fear and anxiety. Neurosci Biobehav Rev. 2001;25(7-8):627-36.


<table>
<thead>
<tr>
<th>Behavior/Postures</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sleep</td>
<td>Flat or curved back with weight and head on floor</td>
</tr>
<tr>
<td></td>
<td>Flat back with weight on floor, head up</td>
</tr>
<tr>
<td>Crouch Immobile (as an index of freezing)</td>
<td>Arch back, weight on floor, vibrissae movement</td>
</tr>
<tr>
<td></td>
<td>Flat back, forepaws extended</td>
</tr>
<tr>
<td></td>
<td>Flat back, forepaws extended, vibrissae movement</td>
</tr>
<tr>
<td>Risk Assessment (RA)</td>
<td>Stretch attend (SAP)</td>
</tr>
<tr>
<td></td>
<td>Stretch attend, sniffing</td>
</tr>
<tr>
<td>Active Behavior Categories:</td>
<td></td>
</tr>
<tr>
<td>Locomotor Activity</td>
<td>Flat back movement</td>
</tr>
<tr>
<td></td>
<td>Curved back movement, including cage top climbing</td>
</tr>
<tr>
<td>Rearing</td>
<td>Unsupported, immobile</td>
</tr>
<tr>
<td></td>
<td>Unsupported, movement</td>
</tr>
<tr>
<td></td>
<td>Supported, immobile</td>
</tr>
<tr>
<td></td>
<td>Supported, movement</td>
</tr>
<tr>
<td>Stand</td>
<td>Hind leg extension including any of the following:</td>
</tr>
<tr>
<td></td>
<td>Flat back, ears higher than back</td>
</tr>
<tr>
<td></td>
<td>Curved back, back higher than ears</td>
</tr>
<tr>
<td></td>
<td>Flat back sniffing</td>
</tr>
<tr>
<td></td>
<td>Curved back sniffing</td>
</tr>
<tr>
<td>Groom</td>
<td>Self grooming</td>
</tr>
<tr>
<td>Eat</td>
<td>As observed</td>
</tr>
<tr>
<td>Drink</td>
<td>As observed</td>
</tr>
</tbody>
</table>
Table 2. Ovine CRF RET Table of Means

<table>
<thead>
<tr>
<th>Location/Behavior</th>
<th>Dose</th>
<th>Vehicle</th>
<th>Low</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Error</td>
<td>Mean</td>
<td>Error</td>
</tr>
<tr>
<td>Contact_Freq</td>
<td>19.1250</td>
<td>4.00641</td>
<td>4.6000**</td>
<td>1.92758</td>
</tr>
<tr>
<td></td>
<td>4.0000**</td>
<td>2.48998</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contact_Duration</td>
<td>184.0184</td>
<td>49.94583</td>
<td>32.7312**</td>
<td>17.91588</td>
</tr>
<tr>
<td></td>
<td>30.3500**</td>
<td>19.97276</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chamber_D</td>
<td>221.5785</td>
<td>68.49402</td>
<td>502.8213**</td>
<td>38.61846</td>
</tr>
<tr>
<td></td>
<td>507.3235**</td>
<td>42.64144</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tunnel_D</td>
<td>56.4820</td>
<td>9.37916</td>
<td>29.1604</td>
<td>9.03648</td>
</tr>
<tr>
<td></td>
<td>28.7973</td>
<td>9.45589</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surface_D</td>
<td>309.3355</td>
<td>73.93551</td>
<td>65.6012**</td>
<td>31.25458</td>
</tr>
<tr>
<td></td>
<td>61.4039**</td>
<td>37.40069</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transits_F</td>
<td>46.8750</td>
<td>11.45400</td>
<td>21.0000</td>
<td>7.71578</td>
</tr>
<tr>
<td></td>
<td>16.5000*</td>
<td>5.32969</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stretch (SAP)_F</td>
<td>17.8750</td>
<td>5.30982</td>
<td>18.7000</td>
<td>2.80099</td>
</tr>
<tr>
<td></td>
<td>14.5000</td>
<td>2.66771</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAP_D</td>
<td>60.0676</td>
<td>19.89559</td>
<td>62.5337</td>
<td>9.81406</td>
</tr>
<tr>
<td></td>
<td>63.8706</td>
<td>9.54911</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Freeze_D</td>
<td>20.4225</td>
<td>9.40975</td>
<td>180.3621**</td>
<td>52.42165</td>
</tr>
<tr>
<td></td>
<td>148.7321</td>
<td>37.31328</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bury_F</td>
<td>12.7500</td>
<td>3.50382</td>
<td>15.9000</td>
<td>3.14272</td>
</tr>
<tr>
<td></td>
<td>16.1000</td>
<td>3.68013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bury_D</td>
<td>97.6499</td>
<td>37.99979</td>
<td>111.6410</td>
<td>27.72233</td>
</tr>
<tr>
<td></td>
<td>162.6331</td>
<td>42.49939</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Groom_F</td>
<td>3.6250</td>
<td>1.14856</td>
<td>2.7000</td>
<td>0.78951</td>
</tr>
<tr>
<td></td>
<td>1.5000</td>
<td>0.45338</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Groom_D</td>
<td>32.7108</td>
<td>14.88115</td>
<td>36.7879</td>
<td>19.46970</td>
</tr>
<tr>
<td></td>
<td>14.5516</td>
<td>4.60090</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Climb_F</td>
<td>6.3750</td>
<td>2.49855</td>
<td>0.5000**</td>
<td>0.40139</td>
</tr>
<tr>
<td></td>
<td>0.5000**</td>
<td>0.50000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Climb_D</td>
<td>51.0815</td>
<td>22.96847</td>
<td>3.3820*</td>
<td>3.32254</td>
</tr>
<tr>
<td></td>
<td>3.3663*</td>
<td>3.36630</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

# indicates p <.001, ** indicates p < .01, * indicates p < .05
Table 3. Astressin RET Table of Means

<table>
<thead>
<tr>
<th>Location/Behavior</th>
<th>Dose</th>
<th>Vehicle Mean</th>
<th>Vehicle Error</th>
<th>Low Mean</th>
<th>Low Error</th>
<th>High Mean</th>
<th>High Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contact_Freq</td>
<td>24.1250</td>
<td>1.83651</td>
<td>26.8000</td>
<td>3.18608</td>
<td>22.2500</td>
<td>1.55552</td>
<td></td>
</tr>
<tr>
<td>Contact_Duration</td>
<td>236.0265</td>
<td>35.10773</td>
<td>206.2777</td>
<td>27.30688</td>
<td>259.7901</td>
<td>38.87501</td>
<td></td>
</tr>
<tr>
<td>Chamber_D</td>
<td>135.3685</td>
<td>37.02308</td>
<td>141.9164</td>
<td>42.17112</td>
<td>88.6218</td>
<td>29.18896</td>
<td></td>
</tr>
<tr>
<td>Tunnel_D</td>
<td>51.6915</td>
<td>10.47248</td>
<td>44.2049</td>
<td>7.01401</td>
<td>44.3026</td>
<td>9.21940</td>
<td></td>
</tr>
<tr>
<td>Surf_D</td>
<td>406.3233</td>
<td>43.81958</td>
<td>410.1823</td>
<td>43.72814</td>
<td>466.0179</td>
<td>37.40652</td>
<td></td>
</tr>
<tr>
<td>Transits_F</td>
<td>54.8750</td>
<td>11.76168</td>
<td>57.4000</td>
<td>11.96495</td>
<td>49.0000</td>
<td>14.91045</td>
<td></td>
</tr>
<tr>
<td>Stretch (SAP)_F</td>
<td>11.5000</td>
<td>3.27872</td>
<td>8.5000</td>
<td>2.10950</td>
<td>6.6250</td>
<td>2.96971</td>
<td></td>
</tr>
<tr>
<td>SAP_D</td>
<td>36.9871</td>
<td>10.87982</td>
<td>37.3261</td>
<td>16.02389</td>
<td>17.5815</td>
<td>6.37907</td>
<td></td>
</tr>
<tr>
<td>Freeze_D</td>
<td>11.4165</td>
<td>5.46781</td>
<td>24.6995</td>
<td>9.58208</td>
<td>5.6883</td>
<td>2.83234</td>
<td></td>
</tr>
<tr>
<td>Bury_F</td>
<td>8.2500</td>
<td>3.21686</td>
<td>2.9000</td>
<td>0.88757</td>
<td>3.8750</td>
<td>2.08256</td>
<td></td>
</tr>
<tr>
<td>Groom_F</td>
<td>2.8750</td>
<td>0.44068</td>
<td>4.3000</td>
<td>1.08577</td>
<td>1.7500</td>
<td>0.36596</td>
<td></td>
</tr>
<tr>
<td>Groom_D</td>
<td>19.7976</td>
<td>3.99339</td>
<td>32.7563</td>
<td>10.18641</td>
<td>33.7399</td>
<td>22.82301</td>
<td></td>
</tr>
<tr>
<td>Climb_F</td>
<td>6.8750</td>
<td>2.34854</td>
<td>6.7000</td>
<td>2.03879</td>
<td>7.7500</td>
<td>2.28153</td>
<td></td>
</tr>
<tr>
<td>Climb_D</td>
<td>54.0655</td>
<td>22.13492</td>
<td>37.2790</td>
<td>13.23751</td>
<td>63.8738</td>
<td>21.71143</td>
<td></td>
</tr>
</tbody>
</table>

# indicates p <.001, ** indicates p < .01, * indicates p < .05
Graphs 1-4. Ovine CRF Homecage Test

Graph 1. Sleep Pattern

Graph 2. Crouched Immobile (freezing)

Graph 3. Locomotor Activity

Graph 4. Active Behaviors
(includes locomote, groom, eat, drink, rear)

# indicates p < .001, ** indicates p < .01, * indicates p < .05
Graphs 5-10. Ovine CRF RET Results

Graph 5. Ovine CRF RET: Surface Duration

Graph 6. Ovine CRF RET: Chamber Duration

Graph 7. Ovine CRF RET: Locomotor Activity

Graph 8. Ovine CRF RET: Freezing Duration

# indicates p < .001, ** indicates p < .01, * indicates p < .05
Graph 9. Cxine CRF: RET: Mesh Contact Duration

Graph 10. Oxine CRF: RET: Mesh Climbing Duration

# indicates p < .001, **indicates p < .01, * indicates p < .05
Graph 11-14. Astressin Homecage Test

Graph 11. Sleep Pattern

Graph 12. Crouch Immobile

Graph 13. Locomotor Activity

Graph 14. Active Behaviors

# indicates p < .001, **indicates p < .01, * indicates p < .05