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Effect of unexpected stressor duration on the expression of stress response habituation

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EFFECT OF UNEXPECTED STRESSOR DURATION ON THE
EXPRESSION OF STRESS RESPONSE HABITUATION

by

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Effect of unexpected stressor duration on the expression of stress response habituation

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The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.
ABSTRACT

While habituation develops to repeated predictable psychological stress, manipulating certain parameters of the stress experience may lead to disruption of a stressor’s predictability and subsequent dishabituation of the stress response. In this experiment, we investigated whether behavioral, endocrine, and neural responses (indicators of activity of the Limbic-Hypothalamic-Pituitary-Adrenal-, or LHPA-, axis) to psychological stress (restraint) differ when the duration of stress given on test day violates expectations based on prior stress experience. Rats experienced 10 min of daily restraint on Days 1-4 followed by either the same duration (10 min) or a longer duration (30 min) of restraint on Day 5. Video recordings were collected for each restraint episode (Days 1-5) and trunk blood and brains (for immediate early gene expression analysis [c-fos mRNA]) were collected following restraint on Day 5. Struggling behavior was manually scored as active attempts to escape the restraint device. Rats experiencing the same duration of repeated restraint showed decreased plasma corticosterone (CORT) compared to 10 min acute restraint group (habituation). In addition, these rats showed decreased active struggling over repeated restraint trials. Conversely, the rats experiencing a longer duration of restraint on Day 5 showed an increased CORT response (dishabituation). These rats showed a habituated behavioral
response during the first 10 min of restraint, however struggling behavior increased once the duration of restraint exceeded the expected duration (with a peak at 12 min). This peak in struggling behavior did not occur during 30 min acute restraint, indicating that the effect was related to memory of previous restraint experience and not simply due to a longer duration of restraint. In contrast, the animals still showed habituated c-fos mRNA expression in the paraventricular nucleus (PVN), lateral septum (LS), and medial prefrontal cortex (mPFC) in response to the increased stressor duration. Thus, there is dissociation between c-fos mRNA expression in key stress responsive brain regions and the behavioral and endocrine response to increased stressor duration. In conclusion, habituation of the endocrine and behavioral stress responses occurred when the duration of the stressor matches previous experience, while dishabituation occurred (with remarkable temporal precision) following an unpredicted increase in stress duration.
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CHAPTER I

INTRODUCTION

Stress plays a predisposing and exacerbating role in a number of physical and psychological dysfunctions, such as impaired immunity, cardiovascular disorders, major depressive illness, and chronic anxiety [1-3]. However, the influences of stress on physiological and psychological disorders are often difficult to analyze because an individual's perception of a stressor and subsequent responses differ based on prior experience [4-6]. For example, habituation of a variety of stress-related endpoints (struggling behavior [7], HPA-axis activity [8], and sympathetic adrenomedullary activity [9]) occurs after repeated exposure to the same, or homotypic, stressor, and dysregulation of the neural circuitry that supports habituation may be involved in the etiology of these disorders.

Restraint is widely used as an animal model of psychological stress, which is more emotionally- and perceptually-processive than physical stress, such as foot shock [10]. Manipulating certain parameters of the perceptual experience of a psychological stressor can violate expectations based on prior experience of a stressor and subsequently disrupt habituation of the stress response [11]. Grissom et al. (2007) document the importance of novel contextual cues in disrupting habituation to repeated restraint, however, changing multiple sensory cues between restraint experiences can potentially confound results when observing changes in neural activity, since it is
unclear whether the change reflects a violation of expectations versus simply a response to a novel sensory stimulus. Although many experiments have used cues to address the predictability of both the onset and termination of a physical stressor[12-15], these experiments do not address the extent to which rats are generating expectations of a stressor outcome themselves, without developing associations with external cues. This is a psychological dimension of stress that is largely untested, and one that may play a very important role in the development of habituation to repeated psychological stress.

One parameter of restraint experience that can be easily manipulated without changing the perceptual experience of restraint is the duration. Therefore, to test whether rats generate expectations of a stressor’s outcome based on prior experience, we gave rats a consistent duration of restraint (10 min) for the first four days of repeated restraint experience, and then increased the duration to 30 min on the last day of restraint experience. Through behavioral, neuroendocrine, and immediate early gene analyses (used as indicators of activity of the Limbic-Hypothalamic-Pituitary-Adrenal-, or LHPA-, axis), we investigated the hypothesis that habituated responses to repeated restraint are disrupted when the duration of restraint on the test day violates expectations based on prior stress experience. We expected that rats would show increased struggling behavior in response to an unexpected increase in restraint duration, and that this increase in anxiety behavior would be paralleled by increased secretion of stress hormones, as well as increased immediate early gene expression in stress responsive brain regions.
A number of immediate early genes are rapidly induced in select brain regions by stress experience and show significant habituation to repeated stress [16, 8], however c-fos mRNA is the best characterized. Therefore, we chose c-fos as an indicator of changes in activation of transcription factor proteins to initiate transcription and/or repression of other genes involved in altering the neural (and subsequently behavioral and endocrine) response to a stressful stimulus [17]. We chose three key stress-responsive brain regions to measure changes in c-fos mRNA expression: the paraventricular nucleus of the hypothalamus (PVN), the lateral septum (LS), and the medial prefrontal cortex (mPFC, both prelimbic and infralimbic subregions) to determine which of these regions might be involved in dishabituation of the stress response.

Activation of the paraventricular nucleus of the hypothalamus (PVN) represents the first step in a physiological cascade of signals referred to as the Hypothalamic-Pituitary-Adrenal- (HPA-) axis response to stress. Neural activation of the PVN represents the convergence of signals from a number of limbic brain regions projecting both directly and indirectly to the PVN [18-20], which are ultimately responsible for the perception of the stressfulness of an experience. Therefore, if an unexpected increase in restraint duration is perceived by the animal as stressful, and this results in increased secretion of stress hormones, this increase should also be reflected as an increase in expression of c-fos mRNA in the PVN.

Significant research has focused on which brain regions may be involved in perception of stress and dysregulation of the stress response. In particular, the lateral septum (LS) is an important mediator of stress-related behaviors. Stimulation of CRF₁ receptors in the LS reduces food intake and promotes stress-related behaviors [21], and
stimulation of histaminergic neurons in the LS show an anxiogenic behavioral response in elevated plus maze [22]. Although these studies well-characterize the role of the LS in long-term modulation of stress-related behavior in rodent models of anxiety disorders, there are no investigations showing the role of the LS in the behavioral response to a psychological stressor. We expect that an unexpected increase in restraint duration will cause an increase in struggling behavior that will also be reflected as an increase in c-fos mRNA in the LS.

Structural remodeling in the medial prefrontal cortex (mPFC) is evidenced in imaging studies of human mood and anxiety disorders, therefore altered function of this regulatory circuitry is likely to affect an animal’s ability to adapt normally to repeated stress experiences [23-25]. In particular, temporary inactivation of the mPFC during repeated restraint experience increases hormone responses to future restraint challenge [26], and inhibition of the prelimbic (PL) subregion of the mPFC has specifically been shown to increase response to acute restraint [27]. In contrast, inhibition of the infralimbic (IL) subregion of the mPFC decreases the response to acute restraint [28], which indicates that these subregions may play opposing roles in regulating the response to repeated restraint experience. We examined two subregions of the mPFC, the IL and PL subregions, which show differential projection patterns [29, 30] that may contribute to variation in their roles of regulating the HPA-axis responsiveness to stress [31, 28]. We expect that the prefrontal cortex would be necessary to detect an unexpected increase in stress duration, therefore we expect to see increased expression of c-fos mRNA in the mPFC in response to increased stress duration, and this increase should be distinct between the PL and IL subregions. If the
mPFC does show a *c-fos* mRNA expression profile that parallels an unexpected increase in stress duration, then future experiments will transiently inactivate mPFC subregions to further elucidate whether mPFC activity is necessary and sufficient for comparing past and present stress experience to drive the dishabituation response.
CHAPTER II
MATERIALS AND METHODS

Animal Procedures

Male Sprague-Dawley rats (285-320g at time of experimentation) were obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN, USA) and were housed 2 per cage in polycarbonate tubs. All animals were given ad lib water and rodent chow and were given at least one week of acclimation after arrival to the animal facilities at the University of Colorado at Boulder. The colony room lights were maintained on a 12-h light/dark cycle, with lights on at 0700 h. Procedures for ethical treatment of animals conformed to the guidelines found in the “Guide for the Care and Use of Laboratory Animals,” DHHS Publication No. (NIH) 80-23, revised 2010 8th ed. and were approved by the University of Colorado Institutional Animal Care and Use Committee.

Experimental Design

Rats were divided into four treatment groups (n=12, N=48) according to restraint experience on Days 1-4 (repeated 10 min restraint vs. home cage acute stress) and duration of restraint on Day 5 (test day, 10 min vs. 30 min; see Table 1).
Table 1 - Experimental Design, with restraint experience on Days 1-4 (home cage vs. repeated restraint) and test day restraint challenge duration on Day 5 (10 min vs. 30 min) for a total of 4 treatment groups. 2x2 factorial design; n = 12, N=48.

Rats who experienced 10 min restraint on Days 1-4 and Day 5 were compared to rats who experienced 10 min acute restraint challenge for the first time to test for habituation. Conversely, rats who experienced 10 min restraint on Days 1-4 but experienced a longer duration (30 min) on Day 5, were compared to rats who experienced 30 min acute restraint challenge for the first time to test for dishabituation.

Stress Procedure and Behavioral Recording

Rats were removed from their home cage and placed into a restrainer on a black tabletop in a room adjacent to their home cage room. Restainers were cylindrical, adjustable length plexiglass tubes (15.5 ± 2.5 cm long and 6.3 cm diameter with air holes in the front, top and back). This version of restraint is considered to be primarily
psychological in nature because it does not produce pain or direct physical insult [10]. Struggling behavior during restraint was recorded via a ceiling-mounted video camera. Light and heavy mobility were blindly scored in seconds and divided into 1 min bins using manual event recording software (courtesy of J. Christianson) according to criteria described by Grissom, Kerr, and Bhatnagar [7]. Since there were no treatment group differences in light mobility scores, only heavy mobility scores are reported as “active struggling.” All behavioral manipulations were performed between 8:00 and 14:00 with time of day counterbalanced between treatment conditions.

Tissue Preparation and Processing

Rats were sacrificed 30 min after restraint onset on Day 5 (10 min restraint groups were placed back in their home cages for 20 min before sacrifice, see Figure 1).

**Figure 1** - Timeline of Test Day Stress Challenge. Rats experiencing 10 min restraint on Day 5 were returned to their homecage for 20 min so that all treatment groups are sacrificed 30 min after initial restraint onset. SAC=time of sacrifice.

Brains were flash frozen (isopentane bath maintained between -30 °C and -20 °C) and stored at -80 °C. Trunk blood was collected in ethylenediaminetetraacetic acid (EDTA)-coated tubes, placed on wet ice, and centrifuged for 15 minutes at 4 °C to
collect plasma for hormone assays. Plasma aliquots were then snap frozen on dry ice and stored within 45 minutes of sacrifice.

### Plasma Hormone Assays

Measurement of plasma corticosterone (CORT) was conducted in duplicate on 20 µl of plasma with an enzyme immunoassay kit (Assay Designs, Ann Arbor, MI, USA) according to manufacturer's instructions. Sensitivity for the corticosterone assay was 130 ng/100 ml and all samples were run in a single assay with a coefficient of variability of 7%. Plasma concentrations of adrenocorticotropic hormone (ACTH) were determined in duplicate (125 µl plasma) by competitive radioimmunoassay procedures previously described [32]. Radio-labeled $^{125}$I ACTH tracer was obtained from DiaSorin (Cat # 20515, Stillwater MN) and primary ACTH antiserum (rabbit antibody Rb7) was provided courtesy of Dr. Bill Engeland, University of Minnesota. The detection limit for this assay was 15 pg/ml and all samples were run in the same assay with an intra-assay coefficient of 10%.

### In situ Hybridization

We used *in situ* hybridization to examine c-fos mRNA expression in the brain. Coronal brain sections (12 µm) were cut on a cryostat (Leica Microsystems model 1850), thaw-mounted onto Colorfrost® plus microscope slides (Erie Scientific) and stored at -80 °C. Series of sections were collected at the approximate rostral-caudal
levels that contain the following brain regions as indicated in Paxinos and Watson [33]:
(1) prefrontal cortex (3.2 mm anterior to bregma), (2) lateral septum (0.7 mm anterior to
bregma), and (3) paraventricular nucleus of the hypothalamus (PVN; -1.8 mm posterior
to bregma). In situ hybridization for c-fos mRNA was performed as described previously
and utilized 35-S labeled riboprobes [8].

Autoradiographic Image Analysis

Semi-quantitative analyses of autoradiographs were performed on digitized images from X-ray films (Scion Image) as described by Campeau, Akil, and Watson [34]. All analyses were performed with the aid of a rat brain atlas (Paxinos and Watson [33]) for guidance in determining proper anatomical placement of regions of interest (ROI) on digitized images with the following specifications. For prefrontal cortex, a square was centered within the dorsal medial PFC (dmPFC; approximate location of prelimbic cortex) or ventral medial PFC (vmPFC; approximate location of infralimbic cortex). For the lateral septum and PVN, the ROI was drawn around the perimeter of the visibly discriminable brain structure.

For all ROI analyses, an average gray level was determined for each rat by subtracting the ROI gray level from a background control reading from white matter in the same hemisphere. For each cortical ROI, at least six independent measurements across separate tissue sections were averaged. For each subcortical ROI, an average of at least three independent measurements were averaged. Average integrated density was expressed as an average percent difference from acute 30 min restrained rats, in
order to allow for direct comparison of relative c-fos mRNA expression levels across brain regions.

Statistical Analyses

Data from two cohorts (n=6 for each, n=12 total) were pooled and separate two-way ANOVAs were performed (SPSS 15) for each dependent measure (CORT, ACTH, c-fos). Three-way ANOVAs including cohort as a cofactor were also performed, however, c-fos in the PL and IL mPFC were the only measures to show significant cohort interaction with the other factors (restraint duration on test day and restraint experience; see discussion). For behavioral results, separate repeated measures ANOVAs were performed on the whole duration of restraint, plus the duration excluding the first minute, for both the 10 min and 30 min restraint groups. Post hoc analyses using one-tailed independent samples t-tests on each individual minute of restraint duration were performed to test for differences between groups based on restraint experience.

In cases where there were overall significant F-test results, post hoc pairwise comparison of group differences of interest using Fishers Least Significant Difference Test (FLSD) are indicated on the data figures (alpha level, P≤0.05). Small differences in within-group degrees of freedom within a given experiment are due to the loss of a few plasma and histological samples due to sample preparation or assay-related problems. Data presented represent group averages ±SEM.
CHAPTER III
RESULTS

Behavioral Results

Significant habituation of struggling behavior occurred both between trials due to restraint experience, as well as within the first 2 min of a 10 min trial of acute restraint. Comparisons of struggling behavior of both groups of rats who experienced 10 min restraint on Day 5 revealed that struggling behavior in the first two minutes of restraint habituated due to prior restraint experience (Figure 2). Repeated measures ANOVA of the entire restraint duration (minutes 1-10) revealed a significant main effect for time in restraint \[ F(9,198) = 7.06, p<0.001 \], as well as a significant interaction between time in restraint and restraint experience \[ F(9,198) = 5.071, p<0.001 \] due to the difference in behavior during the first minute between naïve and experienced rats. One-tailed independent samples t-test revealed a significantly higher response in the first minute [acute vs. repeated restraint, \( t = 6.33, p<0.001 \)] and second minute \( [t = 2.39, p<0.05] \), but not the third minute \( [t = -0.53, p=0.302] \), indicating that struggling behavior had habituated by the third minute of restraint. However, repeated measures ANOVA excluding the first minute from the analysis (minutes 2-10) did not show a significant main effect for time in restraint \[ F(8,176) = 1.87, p=0.68 \] or an interaction between time in restraint and restraint experience \[ F(8,176) = 0.826, p=0.581 \], indicating that there
was no difference in struggling behavior due to restraint experience during the last 9 minutes of restraint. Thus, the first minute of restraint experience was the only time point that showed a significant habituation effect.

Figure 2 - Struggling behavior of groups experiencing 10 min restraint on test day. Struggling behavior in the first two minutes of restraint habituates both within the 10 min acute restraint trial, as well as due to repeated restraint experience. Graph shows struggling behavior on Day 5 of rats experiencing 10 min restraint for the first time (acute restraint, solid line) versus 10 min of restraint for the 5th time (repeated restraint, dashed line). * indicates significant one-tailed independent samples t-test by restraint experience, p<0.05; error bars represent ± SEM, n=12.

We saw a similar habituation effect in struggling behavior in the first two minutes of restraint, both between trials due to restraint experience, as well as within the first 2 min of a 30 min trial of acute restraint. Comparisons of struggling behavior of both groups of rats who experienced 30 min restraint on Day 5 revealed that struggling in the first two minute of restraint habituated due to prior restraint experience (Figure 3).
Repeated measures ANOVA of whole duration of restraint (minutes 1-30) revealed a main effect for time in restraint [minutes 1 through 30, F(29,638) = 3.36, p<0.001] as well as an interaction between time in restraint and restraint experience [F(29,638) = 4.03, p<0.001]. One-tailed independent samples t-test revealed a significantly higher response in the first minute [acute vs. repeated restraint, t = 5.50, p<0.001] and second minute [t = 1.78, p<0.05], but not the third minute [t = 0.842, p=0.205], indicating that struggling behavior habituated by the third minute of restraint.

![Figure 3](image.png)

**Figure 3** - Struggling behavior of groups experiencing 30 min restraint on test day. Struggling behavior in the first two minutes of restraint habituates both within the acute restraint trial, as well as repeated restraint experience, but increases when duration of restraint does not match previous experience (minutes 10 through 12). Struggling behavior of rats experiencing 30 min acute restraint (solid line) versus 10 min repeated restraint on Days 1-4, with 30 min restraint challenge on Day 5 (dashed line). Note: only struggling behavior on Day 5 is shown. Rats who experienced 10 min of repeated restraint on Days 1-4 show habituated struggling behavior during the first 10 min of restraint challenge on Day 5. However, between 10-12 min, when the duration exceeded what was previously experienced (dashed circle), the rats increase struggling, with a peak at 12 min. There is a trend for increased struggling for the remainder of restraint, with significantly higher struggling at minutes 17, 24, and 29. * Indicates significant one-tailed independent samples t-test by restraint experience, p<0.05. Error bars represent ± SEM, n=12.
An unexpected increase in restraint duration caused increased struggling behavior that peaks at 12 min. Excluding the first minute of restraint from the repeated measures ANOVA revealed a significant main effect for restraint experience over the duration of restraint [minutes 2 through 30, F(1,22) = 6.10, p<0.05]. One-tailed independent samples t-test for minutes 2 through 30 revealed a significantly higher response at minute 10 [t = -1.92, p<0.05], minute 11 [t = -2.02, p<0.05], minute 12 [t = -2.15, p<0.05], and minute 13 [t = -2.02, p<0.05], but not minute 14 [t = -0.21, p=0.418]. There is a trend for increased struggling for the rest of the duration of restraint, with significantly higher struggling at minute 17 [t = -1.95, p<0.05], minute 24 [t = -2.70, p<0.05], and minute 29 [t = -2.22, p<0.05].

Endocrine Results

Plasma CORT habituated to repeated 10 min restraint, and this habituation was disrupted following increased stressor duration (Figure 4). Two-way ANOVA of plasma CORT revealed significant main effects of both restraint experience [F(1,44) = 13.08, p<0.001] and duration of restraint on test day [10 min versus 30 min, F(1,44) = 42.60, p<0.001], with no significant interaction [F(1,44) = 1.70, p=0.199]. However, post hoc pairwise comparisons revealed no significant difference between groups experiencing 30 min restraint on test day (LSD p=0.109), supporting the presence of at least partial dishabituation of the response.
Figure 4 – Plasma CORT levels. Habituated plasma CORT responses to restraint challenge are disrupted following an unexpected increase in stress challenge duration. Solid bars = acute stress, white bars = repeated restraint experience. Rats experiencing 10 min of repeated restraint show significant habituation of plasma CORT when compared to acute 10 min restraint challenge (* indicates significant LSD pair-wise comparison between differing restraint experience with same test day duration, p<0.05). Rats experiencing 10 min repeated restraint on Days 1-4 with 30 min restraint duration on test day show increased CORT responses similar to experiencing 30 min acute restraint. Average concentrations of plasma CORT expressed in ng/ml, error bars represent + SEM, n=12.

Plasma ACTH did not show habituation to repeated 10 min restraint (due to the timepoint of sacrifice; see discussion), but prior restraint experience did decrease response to 30 min restraint on test day (LSD p<0.01, Figure 5). Two-way ANOVA of plasma ACTH revealed significant main effects of both restraint experience [F(1,44) = 6.20, p<0.05] and duration of restraint on test day [F(1,44) = 42.52, p<0.001], with no significant interaction [F(1,44) = 2.97, p=0.092].
Figure 5 – Plasma ACTH levels. Repeated restraint experience alters plasma ACTH concentrations in response to 30 min, but not 10 min, restraint duration on test day (Day 5). No significant difference exists between plasma ACTH after 10 min acute or repeated restraint, however previous experience of 10 min restraint on Days 1-4 significantly decreases plasma ACTH response to 30 min restraint duration on test day. * Indicates significant LSD pair-wise comparison between differing restraint experience with same test day duration, p<0.05. Average concentrations of plasma ACTH expressed in pg/ml, error bars represent ± SEM, n=12.

c-fos mRNA Expression

Each brain region included in the analysis showed a significant decrease in c-fos expression due to prior restraint experience, but each had a unique pattern of expression according to duration of restraint on test day (Figure 6).
Most brain regions did not show a significant cohort interaction (with the exception of the infralimbic subregion of the medial prefrontal cortex; see below), therefore the F values reported are from a two-way ANOVA collapsed across cohorts.

Habituation of c-fos mRNA expression still occurred in the paraventricular nucleus (PVN) regardless of increased restraint duration on test day. There was a significant main effect for restraint experience \([F(1,44) = 62.96, p<0.001]\) and duration of restraint on test day \([F(1,44) = 9.379, p<0.01]\), with no significant interaction \([F(1,44) = 1.163, p=0.287]\). Post-hoc pairwise comparison revealed that this main effect of duration of restraint on test day was significant between acute restraint (LSD p<0.01), but not repeated restraint (LSD p=0.168) groups, indicating that although PVN showed an
increased response to a longer duration of acute restraint, it did not show an increased response to a restraint duration that was longer than previously experienced.

Habituation of c-fos mRNA expression still occurred in the lateral septum (LS) regardless of increased restraint duration on test day. There was a significant main effect for restraint experience \[F(1,44) = 17.91, p<0.001\], but not for duration of restraint on test day \[F(1,44) = 2.283, p=0.138\], nor an interaction \[F(1,44) = 0.490, p=0.487\]. Therefore, LS did not show an increased response to a longer duration of acute restraint, nor did it show an increased response to a restraint duration that was longer than previously experienced.

Habituation of c-fos mRNA expression still occurred in the prelimbic subregion of the medial prefrontal cortex (PL mPFC) regardless of increased stressor duration on test day. There was a significant main effect for restraint experience \(F(1,44) = 27.796, p<0.001\), but not for duration of restraint on test day \(F(1,44) = 0.397, p=0.532\) or an interaction \(F(1,44) = 3.846, p=0.056\). Although there was a trend for an increased response to 10 min vs. 30 min acute restraint (LSD p=0.074), this difference was not significant. Therefore, PL mPFC did not show an increased response to a longer duration of acute restraint, nor did it show an increased response to a restraint duration that was longer than previously experienced.

The infralimbic subregion of the medial prefrontal cortex (IL mPFC) showed a significant cohort interaction with duration of restraint on test day [three-way ANOVA, \(F(1,40) = 4.976, p<0.05\], however this cohort interaction did not influence our ultimate interpretation and was not included in the results (see discussion for explanation of cohort effect). Habituation of c-fos mRNA expression still occurred in the IL mPFC
regardless of increased restraint duration on test day. There was a main effect for restraint experience \[F(1,44) = 20.829, p<0.001\], but not for duration of restraint on test day \[F(1,44) = 2.517, p=0.120\] or an interaction \[F(1,44) = 1.716, p=0.197\]. Therefore, IL mPFC did not show an increased response to a longer duration of acute restraint, nor did it show an increased response to a restraint duration that was longer than previously experienced.
CHAPTER IV
DISCUSSION

In conclusion, the duration of restraint experience is salient for rats experiencing repeated restraint experience, and manipulating restraint duration can result in negative behavioral and neuroendocrine responses to restraint challenge. Prior restraint experience decreases struggling response to repeated restraint, and an unexpected increase in restraint duration causes an increase of struggling behavior that may indicate a negative emotional state. Rats show remarkable temporal precision when perceiving an increase in restraint duration, and previous research shows that rats are comparable to humans in their perception of time. Time perception is supported by a cortical-striatal-thalamic-cortical loop and involves a three-step information processing model: 1) the clock stage, in which physical time is transformed into psychological time, 2) the memory stage, in which attentional processes guide whether information is temporally significant enough to be stored as memory, and 3) the decision stage, in which the stored time memory is compared to a sample value of the expected time of the event (stored in reference memory) [35, 36]. This timing ability, termed interval timing, possesses great flexibility, but is lacking in precision [37]; however, stress may play an important role in enhancing the attentional processes necessary for accurate memories of restraint duration. Therefore, the duration of restraint is an important
perceptual parameter that contributes to expectations generated based on previous experiences of psychological stress.

In support of the behavioral data, CORT shows some dishabituation to an unpredicted increase in restraint duration. However, changes in ACTH response to repeated restraint show no solid evidence supporting either the habituation or dishabituation effects seen in both the behavior and CORT data. Although this conclusion is surprising, since the ACTH response is expected to closely follow the CORT response as part of a tightly-coupled cascade of HPA-axis responses, the disparity is likely due to aspects of the experimental design that are not optimal for observing changes in ACTH secretion. For instance, rats who experienced 10 min restraint on test day (both acutely- and repeatedly-restrained groups) were returned to their home cage for 20 min before sacrifice, to allow consistency between time of sacrifice since the beginning of restraint between the treatment groups. During this 20 min period, any ACTH response that was initiated by the restraint experience has likely decayed, due to the shorter half-life of ACTH in the blood as evidenced by a faster return to baseline levels following the termination of restraint than CORT \[38\]. The ACTH response profile to 10 min acute restraint is less informative than expected due to this time consideration. Increased ACTH responses in the group that experienced a longer duration of restraint than expected could have simply been due to the increased duration of restraint on the test day, and not due to detection of mismatch between prior restraint experience and current restraint conditions. In addition, since there is only one timepoint in which plasma samples were collected for analysis of ACTH concentrations, it is uncertain during which timepoint in the entire duration of restraint that peak ACTH
secretion occurred. Therefore, whether or not ACTH secretion shows further support for disruption of habituation of the behavioral and endocrine stress response remains inconclusive. Further experiments including repeated blood sampling using indwelling jugular catheters are needed to tease apart the nuances of the timing of the ACTH response relative to an unanticipated increase in stress duration. In addition, measuring the response of the sympathetic nervous system would be another stress indicator that would provide greater temporal resolution than plasma hormone measures and would better characterize the emotional response surrounding an unexpected increase in stress duration.

We investigated changes in the immediate early gene, c-fos mRNA, to determine the neural response profile underlying the negative behavioral and endocrine response to an unexpected increase in restraint duration. In general, the patterns of c-fos mRNA expression in our chosen regions of interest do not support the dishabituation effect seen in the behavior and CORT data. Although a transient increase in struggling behavior during restraint seems to be consistent with pronounced changes in CORT secretion, it may not be persistent enough to trigger changes in expression of the immediate early gene, c-fos, in our regions of interest. Most regions still showed habituation to repeated restraint, although the degree of this habituation varied between brain regions (Figure 7).
Figure 7 – Comparison of Varying Degrees of Habituation, expressed as the % difference in response between groups experiencing the same duration of restraint on test day (acute restraint group divided by repeated restraint group; arbitrary units). Solid bars = habituation between groups experiencing 10 min restraint on test day; White bars = habituation between groups experiencing 30 min restraint on test day. Plasma CORT shows much less habituation (dishabituation) following increased duration of restraint on test day, however, plasma ACTH shows more habituation to the increased restraint duration (30 min) than to the predicted stressor duration (10 min). C-fos mRNA expression profiles in all brain regions show habituation to repeated restraint, but to varying degrees. There is similar habituation in the PVN in response to increased stressor duration, however, in LS and PL/IL mPFC, there is less habituation in response to an increase in stressor duration. Therefore, in spite of the duration effect seen in the acute restraint groups in PL/IL mPFC, there may still be a supportive trend toward dishabituation.

Plasma CORT shows dishabituation (depicted as % habituation between acute and repeated restraint groups) following increased duration of restraint on test day, however, plasma ACTH shows more habituation to the increased restraint duration (30 min) than to the predicted stressor duration (10 min). There is similar habituation of c-fos mRNA in the PVN in response to increased stressor duration, however, in LS and PL/IL mPFC, there is less habituation in response to an increase in stressor duration that may be indicative of a supportive trend toward dishabituation. However, these data
remain speculative, and further experiments implementing intracranial cannula manipulations to transiently inactivate the mPFC during repeated restraint experience will help determine whether it is necessary for dishabituation of the behavioral and endocrine stress response.

In addition to a habituation effect, in some brain regions there was a significant effect of duration of acute restraint experience that occurred in the opposite direction as expected. In PVN, 30 min acute restraint shows higher c-fos mRNA expression than 10 min acute restraint, as expected, however it does not show the dishabituation effect of CORT, as would be expected. This may be due to the difference in time course of induction between CORT and c-fos (ACTH was previously discussed), where the c-fos mRNA induced around minute 12 did not have enough time to reach peak levels before the time of sacrifice (30 min from the beginning of restraint). However, c-fos mRNA is expressed in a number of other cells in the PVN that are not related to the release of CRH peptide (and thus, do not play a role in the initiation of the HPA-axis cascade of responses). CRH heteronuclear RNA (hnRNA) is another immediate early gene specific to the PVN that is tightly coupled to the HPA-axis stress response [39]. However due to the biochemical nature of hnRNA induction, its window of peak activity is much narrower than c-fos mRNA (c-fos mRNA peaks around 10-15 min, whereas CRH hnRNA peaks at 5 min) [40, 41] and a new experimental design would need to be implemented to optimize the time of sacrifice with its peak secretion.

We did not see a difference in c-fos mRNA expression in the LS between 10 min and 30 min acute restraint, which may indicate a ceiling effect of expression. However, we expected the c-fos expression profile to follow the behavioral dishabituation
response, since the LS plays an important role in regulating anxiety behavior [22, 21]. Changes in immediate early gene expression in LS may be dependent on the type of behavior observed, and may correlate more closely with other stress-related behaviors, such as stereotyped grooming [21], instead of with active attempts to escape the restrainer. Since grooming was included as “light” rather than “heavy” mobility in the analysis and there were no significant trends in light mobility, it was not included in the data presented in this study. A more detailed behavioral analysis separating behaviors into specific categories may reveal stronger correlations between IEG expression in the LS and behavioral effects.

There was an interesting effect of duration of acute restraint in mPFC that did not follow our expectations. In both PL and IL subregions, 10 min acute restraint showed higher c-fos induction than 30 min acute stress. This may be due to the fact that the rats who were given only 10 min of restraint were put back in their home cage for 20 min before sacrifice. The mPFC has wide-reaching projections that include subcortical motor regions [42], therefore it may play a role in the increased motor activity seen in rats who have been returned to their home cage immediately following restraint experience (unpublished observation). In addition, the IL subregion of the mPFC showed a significant cohort interaction with duration of restraint that might be responsible for this effect, since the increased response to 10 min acute restraint did not replicate between cohorts. Data between cohorts were pooled despite this interaction since the duration effect does not alter our ultimate conclusions, however it may affect our interpretation of the degree of habituation expressed (Figure 7).
Future experiments utilizing a longer duration of restraint or alternate immediate early genes, such as arc, in the analysis are needed to tease apart the relationship between neural activity and struggling behavior during restraint. Specifically, the immediate early gene, arc (activity-regulated cytoskeleton-associated protein), is distributed along dendrites, (rather than in the nucleus, as is the case for c-fos) where the protein is locally synthesized. Arc encodes a protein associated to actin, which is related to the N-methyl-D-aspartate receptor scaffolding involved in synaptic plasticity and learning [43]. Therefore, it may be closely related to the psychological dimensions we are interested in, since forming memories of past stress experience requires synaptic plasticity and learning. However, it is also likely that a transient increase in struggling behavior is not sufficient to activate dramatic changes in immediate early gene expression in the brain, since c-fos mRNA declines rapidly after induction due to self-inhibition, despite maintenance of exposure to stress [43]. In addition, the dynamics of both c-fos and arc expression vary dramatically between brain regions [43], therefore including additional timepoints for sacrifice is crucial to determine differences in peak response to an increased restraint duration.

This experiment presents a unique paradigm in which an emotional response is triggered without an explicit environmental event. This addresses an important psychological parameter of repeated restraint in which rats develop expectations based on memories of the duration of previous restraint experience. Although the results are inconclusive pending future experiments, this experimental design will prove useful in future studies investigating what aspects of memory support habituation to repeated, psychological stress.
CHAPTER V
CONCLUSIONS

In summary, rats can encode the duration of repeated homotypic stressor experiences and behave as if they have formed an expectation of the duration of that particular stressor upon subsequent exposure. In addition, they are able to perceive an extended duration of stressor exposure independent from external cues, and that perception leads to a behavioral response that may be consistent with a negative emotional response. While struggling behavior and plasma CORT show dishabituation in response to an unexpected increase in restraint duration, plasma ACTH concentrations and c-fos mRNA expression patterns show dissociated responses that do not support dishabituation (see Table 2).

<table>
<thead>
<tr>
<th>Dependent Measure</th>
<th>Dishabituation?</th>
<th>Next step…</th>
</tr>
</thead>
<tbody>
<tr>
<td>Struggling Behavior</td>
<td>YES</td>
<td>Other stress-like behaviors, eg. grooming</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Measuring autonomic stress response</td>
</tr>
<tr>
<td>Plasma CORT</td>
<td>YES</td>
<td>Repeated blood sampling (jugular catheters)</td>
</tr>
<tr>
<td>Plasma ACTH</td>
<td>NO</td>
<td>Repeated blood sampling (jugular catheters)</td>
</tr>
<tr>
<td>PVN (c-fos)</td>
<td>NO</td>
<td>Sac timepoints; CRH hnRNA in situ</td>
</tr>
<tr>
<td>LS (c-fos)</td>
<td>NO</td>
<td>Sac timepoints; other IEG in situ, eg. arc</td>
</tr>
<tr>
<td>PL/IL mPFC (c-fos)</td>
<td>NO</td>
<td>Sac timepoints; other IEG in situ, eg. arc</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cannula microinfusions</td>
</tr>
</tbody>
</table>

Table 2 – Summary of Results
Further experiments, including repeated blood sampling, additional timepoints for sacrifice, and additional immediate early gene analyses with different profiles of induction, are needed to determine which brain regions show responses that parallel an unpredicted increase in restraint duration. Once these regions of interest are identified, further experiments utilizing intracranial cannula microinfusions to temporarily suppress neural activity during repeated restraint experience will be explored to determine which regions are necessary for detection of mismatch between past and present restraint experience.
REFERENCES


