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An unexpected increase in restraint duration alters the expression of stress response habituation

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HIGHLIGHTS

- CORT and struggling behavior increase in response to unexpected restraint duration.
- Timing of this increase suggests that restraint duration is a salient stressor memory.
- *C-fos* mRNA shows habituation in PVN, LS, and mPFC regardless of restraint duration.
- Dissociation highlights varying dynamics of *c-fos* mRNA, CORT/ACTH, and behavior.
- Expectations of duration are an important parameter of psychological stress.

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ABSTRACT

While habituation develops to a repeated psychological stressor, manipulating certain parameters of the stress challenge experience may lead to dishabituation of the stress response. In this experiment, we investigated whether the behavioral, endocrine, and neural responses (*c-fos* mRNA immediate early gene expression) to a psychological stressor (restraint) differ when the duration of the stressor given on the test day violates expectations based on prior stress experience. Rats experienced 10 min of daily restraint on Days 1–4 followed by a challenge with either the same duration (10 min) or a longer duration (30 min) of restraint on Day 5. Rats' behavior was video recorded during the Day 5 restraint episode, and trunk blood and brain tissue were collected 30 min following restraint onset. Struggling behavior was manually scored as active attempts to escape the restraint device. Rats who experienced the same duration of repeated restraint showed a significant decrease of plasma corticosterone (CORT) compared to the 10 min acute restraint group (habituation). In addition, these rats showed decreased active struggling over repeated restraint trials. Conversely, the rats showed an increased CORT response (dishabituation) when they experienced a longer duration of restraint on Day 5 than they had previously. 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 the memory of previous restraint experience and not due to a longer duration of restraint. In contrast, these animals 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 was a dissociation between *c-fos* mRNA expression in key stress responsive brain regions and the behavioral and endocrine response to increased stressor duration. This dissociation may have been due to a greater lag time for *c-fos* mRNA responses to reflect the impact of a dishabituation response. In conclusion, habituation of the endocrine and behavioral stress response occurred when the duration of the stressor matches the previous experience, while dishabituation of the stress response was triggered (with remarkable temporal precision) by an unexpected increase in stress duration.

1. Introduction

Stress plays a predisposing and exacerbating role in a number of pathological physiological and psychological conditions, such as impaired immunity, cardiovascular disorders, major depressive illness, and chronic anxiety [1–3]. However, the influence of stress on physiological and psychological disorders is often difficult to analyze because an individual's perception of a stressor and subsequent responses differs based on prior experience [4–6]. For example, habituation of a variety of stress-related measures (struggling behavior [7], HPA-axis activity [8], and sympathetic adrenomedullary activity [9]) occurs after repeated exposure to the same, or homotypic, stressor. Dysregulation of the neural

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circuitry that supports stress-response habituation may be involved in the etiology of some of these physiological and psychological disorders.

Restraint is widely used as a rodent model of psychological stress [10]. Manipulation of certain stimulus parameters associated with a stressor challenge condition may disrupt the expression of stress response habituation if a mismatch is detected between the current stressor situation and expectations surrounding that situation due to prior experience of that stressor. For example, Grissom et al. [11] document the importance of novel contextual cues in disrupting habituation to repeated restraint. Changing multiple sensory cues between restraint experiences, however, complicates the interpretation of observed associated changes in neural activity, since it is unclear whether the changes reflect 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 generate expectations of a stressor outcome itself, without developing associations with external cues. This is a psychological dimension of stress that is largely untested, and one that may play an important role in the development and expression of habituation to repeated psychological stress.

One parameter of restraint experience that can be easily manipulated without changing the sensory experience of restraint and the surrounding context is the restraint 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 an indicator of relative activity of the limbic-hypothalamic-pituitary-adrenal 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 behavior would be paralleled by increased secretion of corticosterone, as well as increased immediate early gene expression in stress responsive brain regions.

A number of immediate early genes are rapidly induced in some brain regions by stress experience and show significant habituation to repeated stress [16,8], however *c-fos* mRNA is the best characterized. The *c-fos* gene encodes a transcription factor protein that regulates the expression of other genes that may be involved in neural adaptation 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 PVN represents the first step in the hypothalamic–pituitary–adrenal (HPA) axis neuroendocrine 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 results in increased HPA axis activity, this increase should also be reflected by an increase in *c-fos* mRNA in the PVN [21].

A considerable amount of research has focused on which brain regions may be involved in perception of stress and dysregulation of the stress response [18–20]. We have chosen to focus on the mPFC and LS based on our recent study in which we found that transient inactivation of the mPFC during initial exposure to restraint can interfere with the subsequent expression of HPA axis stress response habituation [22]. Moreover, in our recent study we found that the subsequent impaired expression of stress response habituation was selectively associated with relative *c-fos* mRNA levels in the mPFC and LS. These findings are consistent with other studies that observe altered PFC neural activity in stress-related disorders [23–25]. The prelimbic and infralimbic subregions of the rat mPFC have also been shown to provide regulatory control over stress-induced HPA axis activity [26–30]. Less is known about the role of the LS in stress response adaptation, but there is some evidence that the lateral septum (LS) is an important mediator of stress-related behaviors [31,32].

2. Materials and methods

2.1. Animal procedures

Male Sprague–Dawley rats (285–320 g 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.

2.2. 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) and duration of restraint on Day 5 (test day; 10 min vs. 30 min; see Table 1). Rats who experienced 10 min restraint on Days 1–4 and Day 5 were compared to rats that experienced 10 min acute restraint challenge for the first time to test for habituation. Conversely, rats that experienced 10 min restraint on Days 1–4 but experienced a longer duration (30 min) on Day 5, were compared to rats that experienced 30 min acute restraint challenge for the first time to test for dishabituation.

2.3. Restraint procedures 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. Restrainers 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 0800 and 1400 with time of day counterbalanced between treatment conditions.

Table 1
Experimental design. 2 × 2 between groups factorial design: restraint experience on Days 1–4 (home cage vs. repeated restraint) by test day (Day 5) restraint challenge duration (10 min vs. 30 min) resulting in a total of 4 treatment groups ($n = 12$, $N = 48$).

$n = 12$ Treatment group	Repeated restraint experience (Days 1–4)	Restraint challenge (Day 5)
Acute stress 10 min challenge	–	10 min
Repeated restraint/10 min challenge	10 min	10 min
Acute stress 30 min challenge	–	30 min
Repeated restraint/30 min challenge	10 min	30 min