Neuroendocrinology Letters Nos.1/2, Feb-Apr, Vol.24, 2003
 ISSN 0172–780X **www.nel.edu**

ORIGINAL ARTICLE

Responses of the HPA axis after chronic variable stress: Effects of novel and familiar stressors

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Submitted: *August 21, 2002*
Accepted: *September 13, 2002*

Key words: **stress; novelty; HPA axis; adrenocorticotrophin; corticosterone**

Neuroendocrinology Letters 2003; ***24****(1/2):97–103 pii: NEL241203A16*

Abstract

OBJECTIVES: We examined the role that novelty plays in determining interactions between chronic and acute stress, when both the chronic and acute stressors emphasize emotional processing (i.e. stressful stimuli that do not present immediate threats to somatic homeostasis, and are processed primarily by limbic and forebrain circuits).

METHODS: Rats were exposed to a chronic variable stress (CVS) regimen, and were subsequently tested to evaluate responses to novel and familiar acute stressors. One group was exposed to CVS that included restraint, and was then tested with this *familiar* stressor. Another group was exposed to CVS that did not include restraint, and were tested with restraint as a *novel* stressor. Additional rats were not chronically stressed. Plasma adrenocorticotrophic hormone (ACTH) and corticosterone (CORT) were assayed.

RESULTS: When the rats were exposed to familiar acute stress after CVS, ACTH responses were blunted. The ACTH responses were normal in the rats that were tested with novel acute stress – the responses resembled those of rats that had no prior stress experience. CORT responses did not differ between the groups, regardless of stress history.

CONCLUSIONS: Despite the fact that all the chronic and acute stressors emphasized emotional processing of aversive stimuli, and thus likely involved overlapping limbic and forebrain circuits, the hormonal responses differed depending upon familiarity with the acute stressor. Further research is required to identify the neuronal mechanisms that mediate these differing responses to novel and familiar emotional stressors.

Abbreviations:

adrenocorticotrophic hormone	(ACTH)
analysis of variance	(ANOVA)
arginine vasopressin	(AVP)
chronic variable stress	(CVS)
corticosterone	(CORT)
corticotrophin releasing hormone	(CRH)
glucocorticoid receptor	(GR)
hypothalamic pituitary adrenal axis	(HPA axis)
immunoradiometric assay	(IRMA)
mineralocorticoid receptor	(MR)
paraventricular nucleus	(PVN)
radioimmunoassay	(RIA)

Introduction

Stress exposure activates a variety of physiological coping systems, including the hypothalamic-pituitary-adrenal (HPA) axis. Activity of the HPA axis can be initiated by inputs from various limbic brain structures [1, 2], brainstem inputs [3], immune components (most notably cytokines) [4, 5], and sympathetic nervous system innervation [6, 7]. Communication between and within these inputs permits the HPA axis to be an integrative system that can alter and adapt its responses to the type and duration of stressors to which an organism is exposed.

In general, inputs to the HPA axis converge on the paraventricular nucleus (PVN) of the hypothalamus, where a cascade of neuronal and hormonal activities is initiated. Briefly, stress-induced release of corticotrophin releasing hormone (CRH) and/or arginine vasopressin (AVP) from hypothalamic neurons activates release of adrenocorticotrophic hormone (ACTH) from pituitary corticotrope cells, which ultimately increases synthesis and release of glucocorticoid hormones from the cortex of the adrenal gland. The resulting elevations in circulating glucocorticoid concentration activate widespread alterations in energy regulation and metabolism that promote acute coping with stressful challenges [8, 9]. In addition, these circulating glucocorticoids exert negative feedback modulation of the HPA axis through actions at the hippocampus, hypothalamus, pituitary, and other sites [7, 10–12].

Stressful stimuli that activate these responses have been described as belonging to two categories – emotional or "*processive*" stressors, and somatic or "*systemic*" stressors [1, 13]. Processive stressors require cognitive processing and appear to be relayed primarily through limbic forebrain inputs to the hypothalamus. These stressors do not generally constitute immediate threats to homeostatic regulation of bodily systems. Exposure to a novel environment is one example of an animal model of processive, limbic-mediated stress as lesions to pre-frontal cortex, hippocampus, or amygdala impair hormonal responses to this stressor [14, 15]. In humans, fear, worry, and anxiety are typical processive stressors in that they require cognitive processing but do not present an immediate physical threat. Conversely, systemic stressors may not require higher order cognitive processing, and they represent real and immediate physiological challenges to an organism. Systemic stressors are not initially relayed through limbic and forebrain processing, but rather are relayed primarily through more direct hypothalamic inputs from brainstem regions [1]. Food deprivation, exposure to heat or cold, nociceptive stimuli, and immune insults are all common systemic stressors for animals and humans.

Activation and negative feedback regulation of the HPA axis are modified by exposure to recurring or chronic stress [16]. Indeed, acute stress responses are generally considered important and necessary for healthy coping with challenges, whereas overexposure to stress and the resulting alterations in functioning of the HPA axis lead to a variety of deleterious consequences [17, 18]. In humans, chronic psychological stress has been has been reported to lead to disease, neurological damage, psychopathology, and even premature death [19–22]. Accordingly, it is important to understand and explain the mechanisms involved in chronic psychological stress.

It is well established that repeated homotypic stress exposure (i.e. repeated exposure to a single type of stressor, such as repeated exposure to cold) generally leads to habituation in glucocorticoid hormonal responses to that familiar stressor [23–28]. In contrast, homotypic stress can enhance both the ACTH [29] and corticosterone (CORT) [29, 30] responses to a *novel* acute stressor, such as acute restraint after repeated cold stress (but see [31, 32]). It has been proposed that adaptations of the HPA axis are stressor-specific, so that chronic homotypic stress will differentially alter HPA responses to an acute stressor depending upon whether that acute stressor is familiar or novel [29, 30]. In summary, these observations indicate that cross adaptations between chronic stress and acute stress depend upon whether the acute stressor is novel or familiar. However, the potential for cross adaptation between novel and acute stressors after chronic stress could also be influenced by the natures of the chronic and acute stressors. Although the neuronal circuits that mediate systemic and processive stressors have not been fully characterized, the fact that they differ (at least partially) in their recruitment of limbic and brainstem systems [1] suggests that combinations of these differing types of stressors could differ in their potential for cross-adaptation. For example, the response to an acute stressor after repeated systemic stress might depend not only on whether the acute stressor is novel or familiar, but also on whether the acute stressor is systemic (i.e. mediated primarily by brainstem inputs to the hypothalamus that may overlap with the inputs from the chronic systemic stressor), or processive (i.e. mediated primarily by limbic inputs to the hypothalamus that may not overlap extensively with the inputs activated by the chronic systemic stressor).

In the present study we examined interactions between chronic and acute stressors in a rodent model of chronic variable stress (CVS). In this model we repeatedly exposed rats to stressors that we believe are primarily processive in nature (e.g. novel environment, restraint, switching cage mates), and we avoided use of systemic stressors (e.g. food and water deprivation,

cold). At the end of the CVS regimen, the rats were tested with an acute processive stressor (restraint) that was either familiar (i.e. experienced during the CVS regimen) or novel. HPA axis responsiveness was evaluated by measuring plasma ACTH and CORT concentrations.

Materials and Methods

Animals

Male Long-Evans rats weighing 250–300 grams (Harlan Inc., Indianapolis, IN, USA) were pair-housed in standard plexiglas cages. The rats were kept on a 12:12 h light/dark cycle in a room maintained at 20°C and given *ad libitum* access to food and water. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Florida, and all procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experimental Methods

The rats were randomly assigned to 5 groups that differed in chronic and acute stress administration. The rats in Group 1 (n = 6, controls) were not exposed to chronic or acute stress. The rats in Group 2 (n = 6) were exposed to the CVS regimen (including restraint) without acute stress. The rats in Group 3 (n = 18) were not exposed to chronic stress, but they were exposed to restraint stress (acute stress) immediately prior to decapitation. The rats in Group 4 (n = 18) were exposed to a CVS regimen that included 4 exposures to restraint stress. These rats were exposed to restraint stress (*familiar* acute stressor) immediately prior to decapitation. The rats in Group 5 (n = 18) were exposed to a CVS regimen in which 4 exposures to a brightly lit open field substituted for restraint. These rats were exposed to restraint stress (*novel* acute stressor) immediately prior to decapitation. The group assignments are summarized in Table 1. Independent groups of rats were killed at different time points of exposure to restraint stress so that in each of the groups that were exposed to acute restraint stress (Groups 3, 4, and 5), 6 rats were decapitated after 5 minutes of restraint, 6 rats were decapitated after 15 minutes of restraint, and 6 rats were decapitated after 30 minutes of restraint.

The CVS regimen is summarized in Table 2, and it consisted of the following stressors:

1) **novel environment** – Each rat was removed from its home cage and placed into a different environment, consisting of a circular corridor (10 cm x 170 cm) with standard bedding on the floor, lit from above. After 120 minutes exposure, each rat was returned to its home cage.
2) **switch cage mate** – Each rat was removed from its home cage and placed into a new home cage with one other rat from the group. Pairings were scheduled throughout the course of the experiment such that each rat was paired with every other rat in the group three times. Each rat remained in the new pairing until the next scheduled change of cage mates.
3) **swim** – Each rat was removed from its home cage and placed into a tank of water at 28 °C for 10 minutes on the first trial, and for 20 minutes on each subsequent trial. After the swim, each rat was towel dried and returned to its home cage.
4) **restraint** – Each rat was removed from its home cage and placed into a restraining tube for 30 minutes. After exposure to restraint stress, each rat was returned to its home cage.
5) **light open field** – Each rat was removed from its home cage and placed an open field with standard bedding on the floor, in a brightly illuminated room (approximately 700 lux). The open field consisted of a 70 x 45 x 38 cm rectangular container with no top. After 30 minutes exposure to the open field, each rat was returned to its home cage.
6) **intermittent white noise** – The rats were moved in their home cages from the standard housing room and placed into a room that was isolated from the rest of the rats for 30 minutes. White noise was applied on a pseudo-random schedule in 30-second bursts at 90dB with a mean inter-stimulus interval of 60 seconds. After exposure to the noise stress, the rats were returned in their home cages to the standard housing room.
7) **intermittent footshock** – Each rat was removed from its home cage and placed into a 24 x 22 x 18 cm

Group	Treatment			
	Chronic Stress		Acute Stress	
	CVS w/ restraint	CVS w/o restraint	familiar restraint	novel restraint
1 (controls)				
2	■			
3				■
4	■		■	
5		■		■

Table 1: Assignment of rats to groups that were differentially exposed to chronic variable stress (CVS) and familiar or novel acute stress. These group assignments are summarized as follows: **Group 1** - (controls) no CVS, no acute stress; **Group 2** - CVS (including restraint), no acute stress; **Group 3** - no CVS; acute restraint stress; **Group 4** - CVS (including restraint), acute familiar restraint stress; **Group 5** - CVS (without restraint), acute novel restraint stress.

Chronic non-habituating stress regimen

DAY	Time	Stressor
1	8:00 AM	novel environment (2 hours)
	6:00 PM	switch cage mates
2	11:00 AM	switch cage mates
	5:00 PM	forced swim (10 minutes)
3	10:00 AM	restraint **or** light open field (30 minutes)
	12:00 PM	switch cage mates
4	9:00 AM	switch cage mates
	11:00 PM	intermittent white noise (80 dB/ 30 minutes)
5	11:00 AM	intermittent footshock (.8 mV/ 30 minutes)
	6:00 PM	switch cage mates
6	9:00 AM	switch cage mates
	1:00 PM	forced swim (20 minutes)
7	10:00 AM	restraint **or** light open field (30 minutes)
	3:00 PM	switch cage mates
8	11:00 AM	switch cage mates
	6:00 PM	intermittent footshock (.8 mV/ 30 minutes)
9	10:00 AM	switch cage mates
	5:00 PM	forced Swim (20 minutes)
10	11:00 AM	switch cage mates
	1:00 PM	intermittent white noise (80 dB/ 30 minutes)
11	9:00 AM	restraint **or** light open field (30 minutes)
	5:00 PM	switch cage mates
12	11:00 AM	forced swim (20 minutes)
	6:00 PM	switch cage mates
13	10:00 AM	restraint **or** light open field (30 minutes)
	1:00 PM	switch cage mates
14	11:00 AM	switch cage mates
	3:00 PM	intermittent footshock (.8 mV/ 30 minutes)
15	8:00 AM	novel environment (2 hours)
	6:00 PM	switch cage mates
16	9:00 AM	decapitation, without or with acute 5, 15, or 30 minute exposure to acute restraint

Table 2: Chronic Variable Stress Regimen (CVS): Chronically-stressed rats were exposed to two stressors per day across 15 days.

chamber with a grid floor over standard bedding. In the chamber, 0.8 mA shocks were delivered to the feet on a pseudo-random schedule in 30-second bursts at 0.8 mA with a mean inter-stimulus interval of 60 seconds. After exposure to the footshock stress, each rat was returned to its home cage.

White noise and footshock stressors were presented on an intermittent variable schedule within the sessions, further contributing to the variable and unpredictable nature of the stress exposure.

Endocrine Assays

On the final day of the experiment (day 16), after exposure to the acute restraint stress (or at the equivalent time for acutely unstressed groups) each rat was rapidly decapitated. All decapitations were performed between 9:00 and 10:00 a.m. Trunk blood was collected from each rat (6 ml per rat) into polyethylene tubes on ice containing 600 µl (Na_2-EDTA) at 20 µg/µl. Blood samples were centrifuged at 4 °C for 5 min at 2800 rpm. The plasma fraction was isolated, aliquotted, and frozen at –80ºC. The adrenal and thymus glands were collected and weighed from each rat in the groups that were not chronically stressed, and from each rat in the groups that were chronically stressed and then exposed to thirty minutes of acute restraint stress. Plasma ACTH concentrations were determined by immunoradiometric assay (IRMA) using a kit from Nichols Institute Diagnostics (California, USA). Plasma CORT concentrations were determined by radioimmunoassay (RIA) using a kit from Diagnostic Products Inc. (Los Angeles, CA).

Statistical Analyses:

Potential impacts of the CVS regimen on basal functioning of the HPA axis were evaluated by assessing between-groups differences in basal plasma ACTH and CORT concentrations for the groups that were not exposed to acute stress (unstressed controls of Group 1, and chronically-stressed rats of Group 2). These potential between-groups differences in ACTH and CORT concentrations were each evaluated with a *t-test*.

Between-groups differences in plasma ACTH and in plasma CORT concentrations after acute restraint stress were compared between the group that was not chronically stressed (Group 3), the group that was chronically stressed with repeated exposure to restraint (Group 4), and the group that was chronically stressed without exposure to restraint (Group 5) using two-factor (3 groups x 3 time points) analyses of variance (ANOVAs). All significant effects were further analyzed with Newman-Keuls post tests, comparing values for each chronically stressed group (Groups 4 and 5), with the corresponding value for the group that did not receive chronic stress (Group 3).

Adrenal and thymus weights were expressed as mg tissue per 100 g body weight. Between-groups differences in adrenal and thymus weights were each analyzed using one-way ANOVAs, comparing each glandular weight between the five treatment groups.

Results

Effects of chronic stress alone

Plasma ACTH and CORT concentrations did not differ significantly between the rats in Group 1 that were not exposed to any stress, and the rats in Group 2 that were exposed to CVS without acute stress (ACTH: $T_{(10)} = -0.96$, $p > 0.05$; CORT: $T_{(10)} = 0.68$, $p > 0.05$; see Figure 1). Accordingly, the CVS regimen did not significantly alter basal ACTH and CORT levels at their circadian nadir.

Effect of chronic non-habituating stress followed by acute restraint stress

There were significant group differences across trials in the plasma ACTH concentrations after exposure to acute restraint stress ($F_{(5,53)} = 3.15$, $p < 0.05$). These differences arose from a significant blunting of the ACTH response after 5 and 15 min of acute restraint stress exposure in the group that was familiar with restraint stress (Group 4). There was a statistically sig-

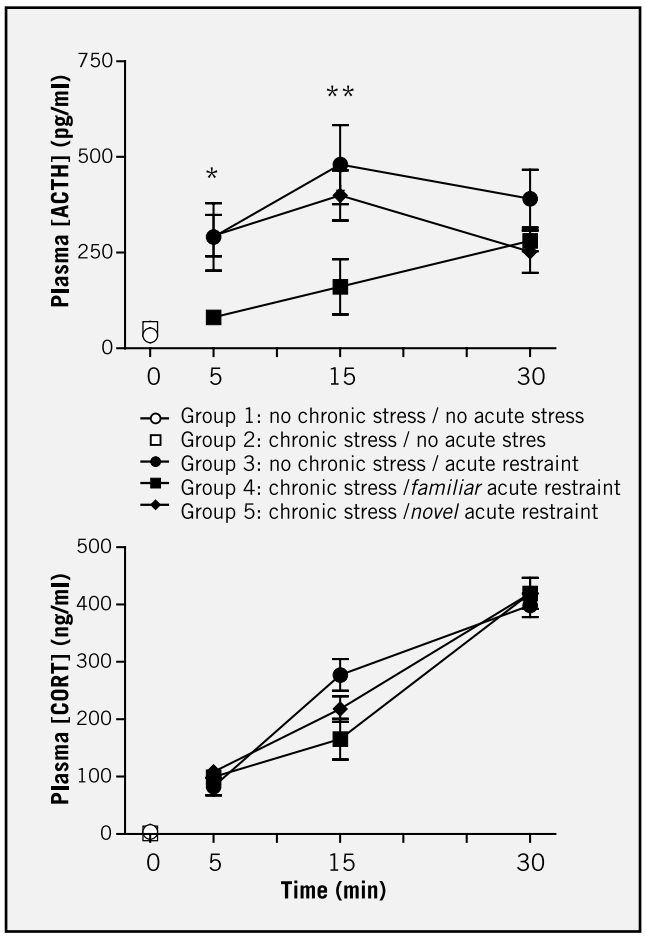

Fig. 1. The chronic variable stress regimen produced substantial alterations in hormonal responses to an acute familiar stressor (Group 4). When this group of rats was exposed to acute restraint after a chronic stress regimen in which they had experienced restraint, the ACTH response was blunted after 5 and 15 min exposure to the acute stress. The CORT response was normal in these rats. The ACTH and CORT responses in the group that was exposed to restraint without previous restraint experience (Group 5) did not differ from the responses in the rats that were not chronically stressed (Group 3). Values expressed are group means ± SEM (n=6 rats per group). Significant differences (Newman-Keuls tests) between chronically-stressed rats that were exposed to familiar acute restraint stress (Group 4) and rats that were not chronically stressed, but were exposed to acute restraint stress (Group 3) are depicted as follows: * $p < 0.05$, ** $p < 0.01$.

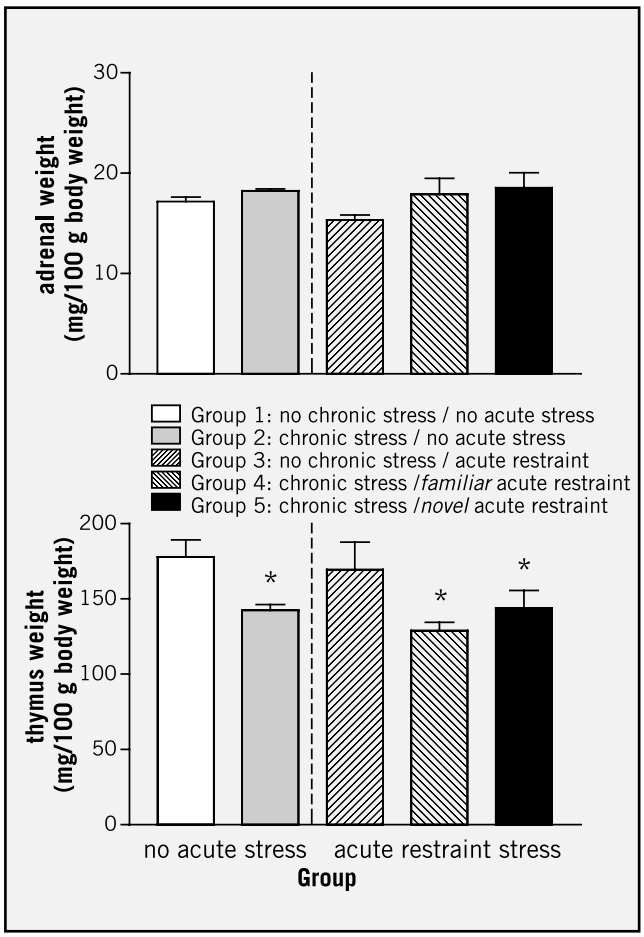

Fig. 2. The chronic variable stress regimen did not affect adrenal masses, but produced significant reductions in thymus gland masses. Thymus gland masses were significantly less in the chronically-stressed rats than they were in the unstressed controls. Values expressed are group means ± SEM (n=6 rats per group for unstressed controls, n=18 rats per group for stressed groups). Significant differences (Newman-Keuls tests) between chronically-stressed rats and appropriate unstressed controls are depicted as follows: * $p < 0.05$.

nificant increase in the plasma CORT concentrations across the 3 time points, but there were no significant between groups differences in these elevated CORT concentrations ($F_{(5,53)} = 1.084$, $p > 0.05$. The plasma ACTH and CORT concentrations are depicted in Figure 1.

Glandular Masses

The masses of the adrenal glands (corrected for body weight) did not differ significantly between groups ($F_{(4,25)} = 1.54$, $p > 0.05$). However, the masses of the rats' thymus glands were significantly lower in the groups that were exposed to CVS ($F_{(4,25)} = 3.22$, $p < 0.05$). The adrenal and thymus weights are presented in Figure 2.

Discussion

The CVS regimen was designed to focus on manipulations in which aversive stimuli are presented in an unpredictable and variable manner–a regimen that we believe resembles the unpredictability and loss of environmental control that constitute typical emotional stressors that are experienced by humans. Furthermore, the CVS regimen employed stressful stimuli that do not constitute severe and immediate threats to homeostasis–threats that we believe are not typically encountered by humans on a chronic basis outside of disease states. Accordingly, the stress regimen included presentation of aversive and emotionally-challenging stimuli at variable times of the day, and in an unpredictable order. This regimen of chronic unpredictable stress produced an interesting set of adaptations in the functioning of the HPA axis.

Thymus involution and adrenal hypertrophy are well-known consequences of chronic repeated stress [8, 33–36], and these glandular changes are associated with alterations in immunological and metabolic functions [8, 37–41]. In our experience, thymus masses are more sensitive to the effects of stress than are adrenal masses. Accordingly, the reductions that were observed in thymus mass (in the rats exposed to CVS, Figure 2) indicate that the chronic variable stress regimen had substantial effects on physiological regulation in these rats.

The hormonal adaptations that occurred appear to have been differentially sensitive to familiar and novel acute stressors. We found a blunting of the circulating ACTH response to acute stress exposure (restraint) after CVS, only if the acute stressor was familiar (Figure 1). This blunting was not observed in the rats that were exposed to restraint as a novel stressor after CVS (i.e. when restraint was not part of the CVS regimen). Accordingly, there appear to be stressor-specific alterations in pituitary responsiveness to acute stress after a regimen of variable emotional stressors. This difference in responsiveness to familiar and novel stressors is reminiscent of stressor specificity that has previously been reported (although the actual dynamics of the stress responses were quite different between these studies) when rats were exposed to a regimen of repeated systemic (cold) stress, and tested with acute processive (restraint) stress [29]. In other words, the present results demonstrate that stressor-specific adaptations occur even if the chronic and acute stressors are all emotional stressors that emphasize limbic processing. The mechanism(s) underlying this stressor-specific change in pituitary release of ACTH probably involves altered regulation of specific limbic inputs to the PVN. CVS is known to produce adaptations in expression of hippocampal mineralocorticoid receptors (MR), hippocampal, frontal cortical, and PVN glucocorticoid receptors (GR), as well as CRH and AVP in the PVN [42]. These central changes could produce the observed alterations in pituitary responses, either through altered initiation of PVN release of ACTH secretagogues (CRH and AVP), or by altered inputs from CORT-mediated negative feedback to the familiar stressor. In one previous study [31], it was reported that repeated immobilization produced a blunted ACTH response to an acute novel (tail shock) stressor, and this blunted response was normalized by adrenalectomy, indicating the importance of glucocorticoid feedback in this diminished ACTH response.

The history of CVS also produced an interesting interaction between pituitary and adrenocortical responses to the acute familiar stressor. The blunted ACTH responses during familiar stress were accompanied by CORT responses that were equivalent to the responses in the rats that were not chronically-stressed, and the rats that were exposed to novel acute stress (Figure 1). Accordingly, the initial ratio of ACTH to CORT differed significantly between these groups of rats. It is unclear at this time, whether this apparent dissociation between the magnitudes of circulating ACTH and CORT concentrations reflects a heightened sensitivity of the adrenal gland to circulating ACTH after chronic stress [43–45] or simply reflects the fact that less than maximal pituitary release of ACTH can produce very large circulating concentrations of CORT, a non-linear relationship between circulating ACTH and the adrenocortical response [46–48]. In either case, the dissociation between circulating ACTH and CORT concentrations after CVS could play an important role in HPA axis functioning during chronic stress and illness, wherein elevated CORT concentrations are known to greatly outlast elevations in circulating ACTH [49].

In conclusion, the CVS model of unpredictable emotional stress exposure may be important in understanding the effects of chronic emotional stress in animals and humans. This model coupled with familiar and novel acute stressors reveals that stressor-specific adaptations occur, even if all the manipulations emphasize processive, limbic-mediated stress. Further investigation will be necessary to tease apart the mechanisms underlying stressor-specific adaptations to familiar and novel stressors after CVS.

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