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Evidence for a lack of phasic inhibitory properties of habituated stressors on HPA axis responses in rats

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Abstract

MASINI, C.V., H.E.W. DAY, T. GRAY, L.M. CREMA, T.J. NYHUIS, J.A. BABB, AND S. CAMPEAU. Evidence for a lack of phasic inhibitory properties of habituated stressors on HPA axis responses in rats. *PHYSIOL BEHAV* 00(0) 000–000, 2010. – This experiment tested the hypothesis that habituation to repeated stressor exposures is produced by phasic inhibitory influence on the neural circuitry that normally drives the paraventricular nucleus of the hypothalamus and subsequently the adrenocortical hormone response to psychological stress. Such a process would be expected to lower the acute response to a novel stressor when experienced concurrently with a habituated stressor. Rats were exposed to restraint or no stress conditions for 14 consecutive days. On the 15th day, the rats were exposed to the control condition (no stress), acute restraint, loud noise, or restraint and loud noise concurrently. Blood was taken and assayed for ACTH and corticosterone and brains were collected to examine *c-fos* messenger RNA expression in several brain areas. As predicted, the rats that received the same (homotypic) stressor repeatedly and again on the test day displayed low levels of ACTH and corticosterone, similar to the control conditions (i.e., showed habituation). All rats that received a single novel stressor on the test day, regardless of prior stress history, exhibited high levels of ACTH and corticosterone. The rats that received two novel stressors also displayed high levels of ACTH and corticosterone, but little evidence of additivity was observed. Importantly, when a novel stressor was concurrently given with a habituated stressor on the test day, no reduction of HPA axis response was observed when compared to previously habituated rats given only the novel stressor on the test day. In general, *c-fos* mRNA induction in several stress responsive brain areas followed the same patterns as the ACTH and corticosterone data. These data suggest that habituation of the adrenocortical hormone response to psychological stressors is not mediated by phasic inhibition of the effector system.

Keywords

ACTH; *c-fos* mRNA; Corticosterone; Noise stress; Restraint

1. Introduction

Habituation is generally described as a decrease in the strength of a response to a stimulus after repeated presentations of that stimulus [1–3]. The response can be anything from a reflex to a more complex set of responses, such as the multidimensional responses induced by stress. Though historically thought of as the simplest form of learning, the specific neural mechanisms mediating habituation to stress have yet to be clearly defined. Activation of the

hypothalamo-pituitary-adrenocortical (HPA) axis is routinely used as an indicator of an acute response to stressors [4–6]. Plasma levels of adrenocortical hormones, specifically adrenocorticotrophic hormone (ACTH) and corticosterone decrease after repeated exposure to the same (homotypic) stressor [7–13]. The habituation of this neuroendocrine stress response may involve similar neural processes as does habituation of other responses [7,8,14].

The leading proposed neural mechanism mediating habituation in simpler nervous systems is long-term synaptic depression at sensory-motor synapses [3,15,16] because muscular fatigue, motor neuron fatigue, and sensory receptor adaptation can usually be ruled out following habituation [3,17]. With regard to habituation of neuroendocrine responses to repeated homotypic stress exposures in rats, the exact location of such sensory-motor synapses is more difficult to pinpoint. The anterior pituitary release of ACTH into the vasculature, which eventually reaches the adrenal cortex to in turn induce the production and release of corticosterone, is controlled by a small number of parvocellular neurons located in the paraventricular nucleus of the hypothalamus (PVN) [18–20]. Although this hypothalamic structure provides a likely site of sensory-motor integration important for habituation of adrenocortical hormone responses to repeated stress exposures, the PVN also receives information from multiple brain regions that could themselves provide the important sensory-motor interfaces responsible for HPA axis habituation to repeated stress [19,21,22]. These observations suggest that either the PVN or one or more of the brain regions projecting to the PVN could provide the functional sensory-motor interface where a process such as synaptic depression associated with habituation would be mediated.

Habituation of HPA axis responses to stressors does not appear to be due to modification of sensory systems per se. For example, several studies employing repeated loud noise or audiogenic stress report sizable and significant ACTH and corticosterone habituation [9,11,23]. As with other sensory systems, auditory functioning is not reliably modified after neuroendocrine response habituation to repeated loud noise exposures, as measured with auditory brainstem evoked potentials [11], the acoustic startle reflex or its prepulse inhibition [23], or *c-fos* mRNA induction in multiple brainstem auditory nuclei [11]. Likewise, motor fatigue of the “motor” neurons of the paraventricular nucleus of the hypothalamus does not appear to provide the mechanism for neuroendocrine response habituation because exposure to a novel (heterotypic) stressor following habituation induces normal, or an even greater (sensitized), neuroendocrine response [24,25]. These results not only argue against simple motor fatigue, but also minimize the possibility that stress habituation is mediated by the development of an active tonic inhibitory mechanism at the level of the PVN. One possibility, however, is that presentation of the habituated stimulus itself induces an active phasic inhibitory mechanism either at the level of the PVN, or in a central brain region or regions that control PVN activity. If such an inhibitory mechanism were active during exposure to a habituated stimulus, the HPA axis response to a superimposed novel stimulus would be expected to be smaller than to the novel stimulus alone under this active phasic inhibitory state. The present study was therefore designed to test the hypothesis that exposure to a habituated stimulus (restraint stress) induces a state of active phasic inhibition that would reduce the neuroendocrine responses and *c-fos* mRNA induction in selected brain regions to a novel heterotypic stressor (loud noise) when presented simultaneously. Under the tested conditions, the data provide no evidence of a habituated stimulus-induced active phasic inhibition of HPA axis responses, a mechanism which is important to test for our overall understanding of habituation to stress.

2. Method

This experiment was conducted to test the possibility that exposure to a habituated stimulus may induce phasic inhibition, as indexed by adrenocortical hormone release. Using rats that were repeatedly restrained for 14 days to ensure habituation, a 90 dBA white noise was concurrently administered with restraint stress on test day 15.

2.1. Subjects

Sixty-six male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 250–300 grams at the time of arrival were used. They were housed in a dedicated colony facility and grouped four to five in clear polycarbonate cages ($48 \times 27 \times 20$ cm) containing floor wood shavings, and covered with wire lids providing food (rat chow) and water *ad libitum*. Animals were housed for a period of at least 7 days after arrival from the supplier, before any experimental manipulations were conducted. They were kept on a controlled light/dark cycle (lights on 7:00 am - off at 7:00 pm) under constant humidity and temperature conditions. The rats were weighed when singly housed the day before the start of the experiment, and on days 3, 9 and 14 of the study. All procedures were performed between 9:00 am and 12:00 pm to reduce variability due to normal circadian hormonal variations. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Colorado and conformed to the United States of America National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.2. Wire Mesh Restrainers

Restrainers were constructed from 0.64 cm wire mesh. The mesh was formed into 7.6 cm diameter cylinders that were 30.5 cm long. A 5.1 cm wide, 0.64 cm thick piece of white painted wood was placed at the bottom of the mesh cylinder to form a platform for the rat to sit on. The mesh was stapled to the wood on the outside of the cylinder. The ends of the cylinders were plugged with 7.6 cm diameter plastic atrium grates. Sections of the grates were removed to allow the rats' tails to protrude from the cylinders. The grates were secured on both sides of the restrainers with small bungee cords. With the grates in place, the internal dimensions of the wire mesh restrainers were similar to those of standard Plexiglas restrainers (17.8 cm length and 6.4 cm diameter).

2.3. Noise Apparatus

The acoustic chambers used consisted of ventilated double wooden (2.54 cm plywood board) chambers, with the outer chamber lined internally with 2.54 cm insulation (CelotexTM). The internal dimensions of the inner box were 59.69 cm (w) $\times$ 38.10 cm (d) $\times$ 38.10 cm (h), inside of which a rat home cage could be placed. Each chamber was fitted with a single 15.24 cm $\times$ 22.86 cm Optimus speaker (#12-1769-120 W RMS) in the middle of the ceiling. Lighting was provided by a fluorescent lamp (15W) located in the upper left corner of the chamber. Noise was produced by a General Radio (#1381) solid-state random-noise generator with the bandwidth set at 2 Hz-50 kHz. The output of the noise generator was amplified (Pyramid Studio Pro #PA-600 $\times$) and fed to the speakers. The speaker characteristics allowed relatively flat delivery between 20 and 27,000 Hz, rolling off quickly (20 dB/octave) at both ends. Noise intensity was measured by placing a Radio Shack Realistic Sound Level Meter (A scale; #33–2050) in the rat's home cage at several locations and taking an average of the different readings. The noise intensity was checked daily before and after each session. Intensity was set at 90 dBA. The ambient/background noise level inside the chamber was approximately 60 dBA.

2.4. Procedure

Seven days after arrival from the supplier, the rats were individually housed and acclimated to the noise chambers 30 min / day for 3 consecutive days. The rats were always placed in the same chamber. After the 3 day acclimation period, the rats were placed in the chambers for 30 min / day for 14 days and then an additional test day (day 15). On days 1 – 14, the home cages of half the rats were placed in the acoustic chambers (without noise) for 30 min. The other half of the rats were restrained in wire mesh restrainers for 30 min within their home cages in the acoustic chambers (without noise). On the test day (day 15), the rats were placed in the acoustic chambers under the following conditions: no noise or restraint (control; $n = 15$), 90 dB noise ($n = 17$), restraint ($n = 17$), or restraint + 90 dB noise ($n = 17$). After 30 min, the rats were rapidly decapitated and blood and brains collected. The choice of the 30 min time period was dictated by our previous findings that indicated peak ACTH and corticosterone release in responses to loud noise 30 min after the onset of the stressor [26].

2.5. Corticosterone ELISA

The corticosterone assay was performed according to the manufacturer's instructions (kit #ADI-901-097 – Enzo Life Sciences, Ann Arbor, MI) except for an adaptation for using a smaller volume of plasma as follows. The steroid displacement reagent (provided with the kit) was added to the assay buffer at a concentration of 0.5 $\mu\text{l/ml}$. Plasma (10 μl) was diluted 1:50 with the amended assay buffer. The diluted plasma sample (100 μl) was then processed as described in the kit directions. This method used equivalent final concentrations of steroid displacement reagent, and was determined to result in equivalent assayed corticosterone levels as the standard method, in which 2.5 μl steroid displacement reagent was added to 97.5 μl plasma, and the plasma diluted with standard assay buffer (data not shown). All samples were run in the same assay. Levels were then quantified on a BioTek Elx808 microplate reader and calculated against a standard curve generated concurrently.

2.6. ACTH Radioimmunoassay

The ACTH assay was performed according to the manufacturer's instructions (ACTH IRMA Ref 27130 - Diasorin, Stillwater, MN). Two hundred μl of plasma was used for this assay. The plasma was incubated overnight with a ^{125}I -labeled monoclonal antibody specific for ACTH 1–17, a goat polyclonal antibody specific for ACTH 26–39, and a polystyrene bead coated with a mouse anti-goat antibody. Only ACTH 1–39 in the sample bound both antibodies to form an antibody complex. Beads were washed to remove unbound radioactivity, counted with a gamma counter, and the concentrations of ACTH values were quantified and calculated against a standard curve generated concurrently. The sensitivity of the assay ranged from 1.5 to 1400 pg/ml. All samples were run in the same assay.

2.7. In Situ Hybridization Histochemistry

After rapid decapitation, brains were removed and frozen in isopentane chilled to -30°C , and stored at -80°C . Twelve micron sections were then cut on a cryostat (Leica model 1850, Wetzlar, Germany), thaw mounted onto polylysine coated slides, and stored at -80°C until further processed. Slides were fixed in a buffered 4% paraformaldehyde solution for 1 hour, and rinsed in 3 changes of $2\times$ standard saline citrate (SSC) buffer. The slides were then acetylated in 0.1 M triethanolamine (pH 8.0) containing 0.25% acetic anhydride for 10 min, rinsed for an additional 5 min in H_2O , and dehydrated in a progressive series of alcohols.

A ^{35}S -labeled cRNA probe was generated for *c-fos* from a cDNA subclones in transcription vector using standard in vitro transcription methodology. The rat *c-fos* cDNA clone (courtesy of Dr. T. Curran, St. Jude Children's Research Hospital, Memphis, TN) was

subcloned in pGem3Z and cut with HindIII to yield a 680 nt cRNA probe. Riboprobes were labeled in a reaction mixture consisting of 1 µg linearized plasmid, 1× T7 transcription buffer (BioLabs), 125 µCi ³⁵S-UTP, 150 µM NTPs (CTP, ATP, and GTP), 12.5 mM dithiothreitol, 20 U RNase inhibitor, and 6 U RNA polymerase (T7). The reaction was allowed to proceed for 120 min at 37° C, and probe was separated from free nucleotides over a Sephadex G50-50 column. Riboprobes were diluted in hybridization buffer to yield approximately 1.5×10^6 dpm/70 µl buffer. The hybridization buffer consisted of 50% formamide, 10% dextran sulfate, 3× SSC, 50 mM sodium phosphate buffer (pH=7.4), 1× Denhardt's solution, and 0.1 mg/ml yeast tRNA. Diluted probe (70 µl) was applied to each slide and sections were coverslipped. Slides were placed in sealed plastic boxes lined with filter paper moistened with 50% formamide in distilled water, and were subsequently incubated overnight at 55° C. Coverslips were then removed, and slides were rinsed several times in 2× SSC. Slides were then incubated in RNase A (200 µg/ml) for 60 min at 37° C, washed successively in 2×, 1×, 0.5× and 0.1× SSC for 5–10 min each, and washed in 0.1× SSC for 60 min at 65° C. Slides were subsequently rinsed in fresh 0.1× SSC, dehydrated in a graded series of alcohols, and exposed to BioMax MR X-ray film (Eastman Kodak, Rochester, NY).

Two to three slides for a given brain region from each rat included in the study were processed simultaneously to allow direct comparisons in the same regions. Multiple *in situ* hybridizations were thus performed at different levels of the brain with all animals represented to reduce the effects of technical variations within regions. Sections of all rats in the same region were all exposed on the same X-ray film to further minimize variations. Semi-quantitative analyses were performed on digitized images from X-ray films in the linear range of the gray values obtained from our acquisition system (Northern Light lightbox model B 95, a SONY TV camera model XC-77 fitted with a Navitar 7000 zoom lens, connected to an LG3-01 frame grabber [Scion Corp., Frederick, MD] inside a Dell Dimension 500, captured with Scion Image version 4.03 for Windows). Signal pixels of a region of interest were defined as being 3.5 standard deviations above the mean gray value of a cell poor area close to the region of interest. The number of pixels and the average pixel values above the set background were then computed for each regions of interest, and multiplied, giving an integrated mean gray value measure. An average of four to eight measurements were made on different sections (which included bilateral counts made in all cases), for each region of interest, and these values were further averaged to get a single integrated mean gray value per region for each rat. Templates were created for each brain region analyzed using anatomical landmarks according to a rat brain atlas [27]. This method reflects both the number of cells expressing mRNA and the expression level per cell, as determined by cell and grain counts of emulsion-dipped slides [28].

2.8. Data Analysis

Body weight data were analyzed using a repeated measures analysis of variance (ANOVA) with day as the repeated measure and group (restraint or no restraint) as the between subjects factor. Plasma ACTH, corticosterone, and *c-fos* mRNA induction data from day 15 were analyzed using 2×4 ANOVAs with habituation condition (restraint days 1 – 14 or no restraint (control) days 1 – 14) and test day conditions (control, restraint, noise, or noise + restraint) as the between-subjects factors (a more stringent significance level was set for *c-fos* mRNA data, $p < 0.01$, to reduce the possibility of type II errors). Tukey's honestly significant difference (HSD) multiple means comparisons were used to analyze *post hoc* differences ($p < 0.05$ for weight, ACTH, corticosterone, and *c-fos* mRNA data).

3. Results

3.1. Body Weight

Rats in both the control and restraint groups started the experiment at the same weight and all gained weight over the experiment. However, rats in the restraint groups put on less weight over the same time (significant day $\times$ group effect, $F(2,108) = 30.74$, $p = 0.0001$, see Figure 1), indicating a significant effect of this stressor.

3.2. ACTH and Corticosterone

Univariate ANOVA revealed that there were significant differences in plasma levels between habituation conditions (restraint days 1 – 14 or no restraint days 1 – 14) for ACTH: $F(1,57) = 12.674$, $p = 0.001$ and corticosterone: $F(1,57) = 5.562$, $p = 0.022$, between test day conditions (ACTH, $F(3,57) = 17.717$, $p = 0.0001$; corticosterone, $F(3,57) = 23.462$, $p = 0.0001$), and an interaction effect (ACTH, $F(3,57) = 3.630$, $p = 0.018$; corticosterone, $F(3,57) = 5.037$, $p = 0.004$), as shown in Figure 2. Rats that were just placed into the noise chambers on day 15 (no noise or restraint) had low levels of ACTH and corticosterone, and did not differ regardless of restraint history (ACTH and corticosterone: $p = 1.00$). Rats that received another restraint exposure on the test day, displayed significantly lower ACTH and corticosterone levels compared to rats restrained for the first time (ACTH: $p = 0.003$; corticosterone: $p = 0.001$). Rats (both with and without a previous restraint history) that received noise on the test day also did not differ significantly (ACTH and corticosterone, $p = 0.998$). Rats that were exposed to both restraint and noise concurrently on the test day exhibited similar ACTH and corticosterone levels regardless of their restraint history (ACTH: $p = 0.218$; corticosterone: $p = 0.995$). And importantly, rats that were previously restrained and then exposed to both restraint and noise concurrently on the test day exhibited similar ACTH and corticosterone levels as rats previously restrained and then exposed to noise alone (ACTH: $p = 0.818$; corticosterone: $p = 0.811$).

3.3. *c-fos* mRNA Induction

The mean ($\pm$ SEM) levels and statistical results of the *c-fos* mRNA induction on the test day are presented in Table 1. Two-way ANOVAs revealed significant test day differences between groups for all the brain areas analyzed (smallest, $F(3,58) = 4.47$, $p = 0.007$, in the ventral posterior thalamus - PvTHAL). With the exception of all the auditory regions (inferior colliculus - IC, cochlear nuclei, dorsal ventral part of the medial geniculate nucleus - MGNdv, and auditory cortex), the medial preoptic area (MPOA), the basolateral amygdaloid complex (BLA), and the PvTHAL, all regions also demonstrated a significant effect of prior repeated restraint ($p < 0.01$), and significant interactions between prior treatment (no stress or repeated restraint) and test day conditions ($p < 0.01$). Additional post-hoc analyses revealed distinct patterns of *c-fos* mRNA distribution, as discussed below. The brain regions analyzed are shown in Figure 3.

As discussed above, the auditory areas analyzed (auditory cortex, cochlear nucleus, IC, and the MGNdv) all displayed a significant main effect of test condition on *c-fos* mRNA expression (all p 's < 0.01), especially in response to loud noise. The groups that were exposed to noise or restraint concurrently with noise on the test day that previously were not repeatedly restrained all exhibited significantly higher levels of *c-fos* mRNA induction compared to the control group, as determined by post-hoc analyses ($p < 0.05$, Table 1, and see example in cochlear nuclei, in Figure 4A). Additionally, the brainstem auditory areas (cochlear nuclei and IC) also had higher *c-fos* mRNA induction in the previously restrained rats that were exposed to noise or noise and restraint on the test day compared to controls (Tukey's post-hoc tests, $p < 0.05$). No significant differences were found between controls

and animals receiving restraint on the test day regardless of restraint history for any of these areas (all p 's > 0.05).

With regard to somatosensory processing brain regions, significant differences between groups were attained for the ventral posterior nucleus of the thalamus and the barrel field area of the somatosensory cortex (S1BF) on the test day (all p 's < 0.01, see Table 1). As exemplified in Figure 4B for the S1BF, these areas exhibited significantly higher levels of *c-fos* mRNA induction in the rats exposed to restraint or restraint and noise for the first time on the test day compared to control rats, as determined by post-hoc analyses (p < 0.05). The S1BF area also displayed significantly reduced levels of *c-fos* mRNA in the repeatedly restrained rats compared to acutely restrained rats (Tukey's post hoc test, p < 0.05). No significant difference in *c-fos* mRNA induction was observed between previously restrained rats receiving noise on test day and the previously restrained rats receiving both noise and restraint on the test day (Tukey's, p > 0.05).

In general, most of the stress responsive regions examined followed a similar pattern of *c-fos* mRNA induction. The areas that followed this pattern are the paraventricular nucleus of the hypothalamus (PVN), lateral septum, anteroventral part of the bed nucleus of the stria terminalis (BSTav), dorsomedial nucleus of the hypothalamus (DMH), basolateral amygdala (BLA), medial amygdala (MEA), prelimbic (PRL) cortex, infralimbic (IL) cortex, and orbitofrontal (OBF) cortex. These areas all displayed significantly higher *c-fos* mRNA induction in rats exposed to restraint or restraint and noise for the first time on the test day compared to control rats (Tukey's, all p 's < 0.05). These areas also had significantly reduced levels of *c-fos* mRNA in the repeatedly restrained compared to acutely restrained rats (Tukey's, all p 's < 0.05). Importantly, there were no significant differences in *c-fos* mRNA induction between previously restrained rats receiving noise on test day and the previously restrained rats receiving both noise and restraint on the test day (Tukey's, all p 's > 0.05). This pattern of *c-fos* mRNA expression was especially similar to the pattern of ACTH release in the various groups, as shown for the PVN in Figure 4C.

The control group and the previously restrained group that was exposed to control conditions on test day did not significantly differ for *c-fos* mRNA induction for any area examined (Tukey's, all p 's > 0.05). And, the previously restrained animals that received noise alone or noise concurrently with restraint on the test day did not display any significant difference between *c-fos* mRNA expression in any of the areas examined, as indicated in Table 1 (all p 's > 0.05).

4. Discussion

4.1. Wire restrainers

In this study, rats were exposed to restraint stress in wire restrainers or no stress (control condition) for 30 min / day for 14 days. Wire restrainers were constructed because Plexiglas restrainers lowered the intensity level of the noise that could be heard by the rats when they were exposed to noise while inside the restrainers, as measured in pilot studies. The newly constructed wire restrainers did produce ACTH and corticosterone responses that were significantly higher than no restraint controls and reached levels similar to acute restraint levels obtained with similarly sized Plexiglas restrainers. Importantly, pilot experiments indicated that rats in or out of the wire restrainers responded with ACTH and corticosterone to loud noise exposures equally. Acute noise also resulted in similar *c-fos* mRNA levels whether in or out of the restrainers in the auditory areas examined in this study. Additionally, the rats were weighed over the course of the experiment and the restrained rats gained significantly less weight over time compared to the no restraint control group.

4.2. Acute restraint or noise

On the test day (day 15) control animals had low ACTH and corticosterone levels and *c-fos* mRNA induction in all the brain areas examined, irrespective of stress history (restraint days 1–14 or control days 1–14). Rats exposed to restraint for the first time on the test day had significantly higher levels of ACTH and corticosterone than controls. All brain areas examined in these acutely restrained rats (except for the auditory areas: MGNdv, cochlear nucleus, inferior colliculus, and auditory cortex) also exhibited high levels of *c-fos* mRNA induction compared to control groups. Both groups of rats that experienced control or restraint conditions for 14 days had similar ACTH and corticosterone responses to moderate loud noise presented acutely. This was also found for *c-fos* mRNA induction in all the brain areas examined. This novel stressor (noise) led to a significant HPA axis activation, even in rats previously habituated to restraint. This is in agreement with multiple studies reporting that habituation is stressor dependent, such that later exposure to a heterotypic (different) stressor generally does not display evidence of HPA response habituation [24,25].

4.3. Repeated restraint

The acute responses to restraint habituated as evidenced by the rats that received restraint for all 15 days, which had significantly lower ACTH and corticosterone responses and lower *c-fos* mRNA induction to restraint on the test day. Besides the auditory areas (that had low levels of restraint stress induced *c-fos* mRNA), the only areas that were not significantly lower after repeated restraint stress were the medial preoptic area and ventral posterior thalamus.

4.4. Simultaneous restraint + noise

Rats that experienced restraint concurrently with noise on the test day had similar ACTH and corticosterone responses and *c-fos* mRNA induction irrespective of stress history. Even with the dynamic ACTH responses measured in this study, and the deliberately weaker loud noise stimulus employed (as evidenced by both ACTH and corticosterone levels in response to this loud noise alone), no evidence of additivity of the two independent stressors alone was observed when they were superimposed together. This finding is relatively novel, and suggests that either near maximal ACTH and corticosterone release was induced by both challenges individually, thus reflecting a ceiling effect impossible to overcome, or that stress is not a state that can be characterized by linear additivity. The former possibility seems unlikely given that much higher ACTH and corticosterone levels are often reported in the stress literature. The alternative interpretation, that the stress state displays non-linear additive characteristics, does not appear to be supported by studies indicating intensity-related HPA axis responses to various stressor modalities [14,30,31], but these studies have always been carried out with single stimulus exposures, not superimposed exposures to stressors. A third alternative is that the combination of the two stressors may in some way be processed by the organism as an entirely different kind of stress than restraint alone or noise alone. Perhaps, presenting the homotypic (restraint) stressor simultaneously with the heterotypic (noise) stressor may change the perceptual context of the homotypic stressor and thus it is no longer perceived as a homotypic stressor. Though arguing against this, *c-fos* mRNA expression in the S1BF was similar in previously restrained rats and rats exposed to noise and restraint simultaneously on the test day. Additionally, recent studies with both restraint and loud noise have found that the adaptation of plasma ACTH and corticosterone responses to repeated stressor exposures is not significantly affected by contextual changes [32,33].

There was no inhibition of the adrenocortical hormone responses or *c-fos* mRNA induction to the moderate loud noise when concurrently exposed to restraint in the repeatedly restrained animals. The main comparison group is the repeatedly restrained group that

received 90 dB noise only on test day, and as can be seen in Figures 2 and 4, the superimposition of restraint to the same loud noise did not reduce ACTH or corticosterone responses or *c-fos* mRNA levels compared to the loud noise alone group. Importantly the hormonal responses to the novel loud noise were submaximal suggesting the lack of inhibition was not due to a ceiling effect. These interpretations rely on the observations of a lack of clear hormonal reductions by a habituated stimulus upon a novel stressor at a single time point (30 min), which might have missed differences earlier or later during the recovery process upon stressor termination. Likewise, the exact order of presentation of the superimposed stimuli might have been inadequate for the induction of a habituated stimulus-induced phasic inhibitory state. Additionally, it is also assumed that the brain areas involved in controlling the activity of the PVN are similarly involved for both restraint and noise stress as suggested by *c-fos* mRNA induction in multiple regions known to project to the PVN [19,20,34]. Additional studies are required to test these current limitations. Nonetheless, more generally these results might suggest that habituation to stress is not mediated via active inhibitory mechanisms, but that instead, habituation is the result of a process akin to a lack of excitation, which could be mediated by synaptic depression generally described to support habituation in lower organisms [3]. Immediate early gene expression studies have reported similar expression in lower sensory areas (*c-fos* mRNA in cochlear nuclei, nucleus of the trapezoid bodies, superior olivary complex, nuclei of the lateral lemniscus, and the external and central nuclei of the inferior colliculus between acute and chronic noise stress groups [11]), but reduced expression in thalamic nuclei (medial geniculate body after repeated noise stress [11] and ventroposteriomedial and dorsolateral geniculate nuclei after repeated restraint stress [35]). And, functional inactivation of different areas of the thalamus (posterior paraventricular thalamus for restraint stress and medial geniculate nucleus for loud noise stress) can block corticosterone habituation to repeated stress exposures [10, 36]. Perhaps the primary sensory information that an organism receives after repeated exposures is intact, but that information gradually gets gated at the level of the sensory thalamus. Studies testing this hypothesis are currently being conducted.

4.5. Conclusion

Rats that were previously restrained and then exposed to both restraint and noise concurrently on the test day exhibited similar plasma ACTH and corticosterone and *c-fos* mRNA expression in several stress responsive brain areas as rats previously restrained and then exposed to only noise. These results are in agreement with those previously reported in a study of the habituation of the arousal reaction to repeated tone exposures [37], in that no evidence of inhibition of the EEG arousal response was observed to a novel stimulus that was superimposed with a previously habituated stimulus. The present study strongly suggests that HPA axis habituation to psychological stress is not mediated by an active phasic inhibitory mechanism induced by the presence of the habituated stimulus directly at the level of the PVN or in structures controlling its activity.

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Figure 1.

Graph displaying mean ($\pm$ SEM) body weight over the course of the experiment for rats in the control ($n = 31$) and restraint ($n = 35$) groups. * indicates a significant difference between groups ($p < 0.05$).

Figure 2.

Graphs showing mean ($\pm$ SEM) plasma levels of ACTH (Panel A) and corticosterone (Panel B) responses on test day. Black bars represent rats that were restrained days 1 – 14 and white bars represent rats that were not restrained (controls) days 1 – 14. The x-axis depicts the test day conditions. * indicates a significant difference from controls ($p < 0.05$). ^ indicates a significant difference from control 1–14 + restraint ($p < 0.05$). n.s. indicates that the noise exposed group that was previously restrained and the restraint + noise group that was previously restrained were not significantly different from each other.

Figure 3.

Representative photomicrographs displaying *c-fos* mRNA expression after 30 min of noise + restraint across the brain. Templates that were used for semi-quantitative analysis are shown for A: orbitofrontal cortex (OFC); B: prelimbic cortex (PRL) and infralimbic cortex (IL); C: lateral septum (LS); D: anteroventral bed nucleus of the stria terminalis (BSTav) and medial preoptic area (MPOA); E: paraventricular nucleus of the hypothalamus (PVN); F: barrel field area of the primary somatosensory cortex (S1BF), ventral posterior nuclei of the thalamus (VP), dorsomedial hypothalamus (DM), medial amygdala (MEA), and basolateral amygdala (BLA); G: dorsal and ventral parts of the medial geniculate nucleus of the thalamus (MGNdv) and auditory cortex (AC); H: inferior colliculus (IC); I: cochlear nuclei (COC).

Figure 4.

Graphs displaying mean integrated densities ($\pm$ SEM) of *c-fos* mRNA for cochlear nucleus (panel A), barrel field area of primary somatosensory cortex (S1BF; panel B), and paraventricular nucleus of the hypothalamus (PVN; panel C) on test day. Black bars represent rats that were restrained days 1 – 14 and white bars represent rats that were not restrained (controls) days 1 – 14. The x-axis depicts the test day conditions. * indicates a significant difference from controls ($p < 0.05$). ^ indicates a significant difference from control 1–14 + restraint ($p < 0.05$). n.s. indicates that the noise exposed group that was previously restrained and the restraint + noise group that was previously restrained were not significantly different from each other.

Table 1

Mean Integrated Densities / 100 ($\pm$ SEM) of *c-fos* mRNA Expression

Region	CON-CON	CON-REST	CON-NOISE	CON-N+R	REST-CON	REST-REST	REST-NOISE	REST-N+R
Forebrain:								
BSTav ^{1,2,3}	0.035(0.01)	3.3(0.8) *	0.7(0.2)	2.3(0.4) *	0.1(0.05)	0.2(0.04) ^	1.4(0.4)	1.0(0.3)
Lateral Septum ^{1,2,3}	0.1(0.01)	26.5(2.7) *	9.9(2.6)	25.8(4.3) *	1.7(1.1)	2.1(0.5) ^	9.2(2.0)	8.9(2.0)
Amygdala:								
Basolateral ²	0.6(0.1)	3.7(1.1) *	3.6(0.5)	4.0(0.8) *	1.6(0.2)	1.1(0.3) ^	2.7(0.4)	3.4(0.5)
Medial ^{1,2,3}	0.4(0.1)	3.8(1.4) *	1.4(0.3)	5.0(0.8) *	1.0(0.2)	0.6(0.05) ^	1.4(0.6)	1.3(0.4)
Cortex:								
Auditory ²	74.1(9.7)	135.4(11.2)	154.0(16.0) *	171.0(17.6) *	114.0(10.8)	107.5(13.8)	138.6(11.5)	128.4(17.6)
Infralimbic ^{1,2,3}	0.6(0.2)	6.8(1.2) *	1.7(0.5)	6.8(1.6) *	1.5(0.4)	1.8(0.4) ^	2.9(0.6)	1.8(0.4)
Orbitofrontal ^{1,2,3}	2.0(0.5)	6.9(1.0) *	4.6(1.0)	5.1(0.8)	2.8(0.5)	3.4(0.7) ^	4.6(0.7)	2.3(0.5)
Prelimbic ^{1,2,3}	1.7(0.6)	19.8(2.6) *	4.5(1.4)	16.8(2.6) *	4.6(0.8)	3.5(0.9) ^	5.6(1.3)	4.3(1.0)
S1BF ^{1,2,3}	6.2(1.1)	85.3(7.7) *	32.0(8.4)	75.9(10.8) *	14.4(2.5)	33.8(4.5) ^	31.5(5.6)	31.8(7.9)
Hypothalamus:								
Dorsomedial ^{2,3}	1.8(0.5)	15.3(1.9) *	13.1(1.6) *	13.9(2.1) *	5.4(0.9)	5.6(1.0) ^	13.0(0.8) *	13.1(1.6) *
Medial preoptic area ²	0.1(0.05)	2.9(0.6)	4.2(1.5) *	4.7(1.2) *	0.4(0.1)	0.8(0.3)	3.3(0.5)	2.3(0.7)
PVN ^{1,2,3}	1.4(0.7)	22.0(2.6) *	9.7(2.7)	17.8(2.5) *	1.5(0.4)	2.0(0.4) ^	6.0(1.5)	5.6(1.6)
Thalamus:								
MGNdv ²	12.5(2.6)	23.2(3.4)	52.0(7.9) *	63.6(9.2) *	34.9(6.7)	25.7(5.0)	42.1(6.4)	43.3(8.7)
Ventral posterior ²	2.8(0.8)	10.4(2.0) *	10.8(2.6)	8.1(1.8)	5.2(0.9)	6.5(1.1)	8.5(1.6)	6.5(1.1)
Brainstem:								
Cochlear nucleus ²	4.4(1.4)	2.5(0.4)	21.8(4.0) *	9.3(2.9) *	12.2(2.0)	2.8(0.7)	20.8(3.5) *	23.5(4.2) *
Inferior colliculus ²	9.6(2.2)	31.1(8.3)	62.6(6.9) *	89.6(20.3) *	22.5(3.8)	33.9(5.3)	62.7(7.6) *	77.6(8.1) *

Mean integrated densities (divided by 100 $\pm$ SEM) measured in the CON-CON (control conditions days 1–15; n=7), CON-REST (control conditions days 1–14 and restrained on test day; n=8), CON-NOISE (control conditions days 1–14 and noise on test day; n=8), CON-N+R (control conditions days 1–14 and restraint + noise on test day; n=8), REST-CON (restraint days 1–14 and control conditions

on test day; n=8), REST-REST (restraint days 1–15; n=9), REST-NOISE (restraint days 1–14 and noise on test day; n=9), and REST-N+R (restraint days 1–14 and restraint + noise on test day; n=9) of *c-fos* mRNA expression on test day.

- ¹ indicates a significant main effect of prior treatment (ANOVA, $p < 0.01$).
- ² indicates a significant main effect of test day condition (ANOVA, $p < 0.01$).
- ³ indicates a significant interaction between main effects (ANOVA, $p < 0.01$).
- ^{*} indicates a significant difference from CON-CON group (Tukey's HSD, $p < 0.05$).
- [^] indicates a significant difference from CON-REST group (Tukey's HSD, $p < 0.05$).