Neurobiology of Stress 9 (2018) 74–83

Contents lists available at ScienceDirect

Neurobiology of Stress

journal homepage: www.elsevier.com/locate/ynstr

Neuroendocrine and neuroimmune adaptation to Chronic Escalating Distress (CED): A novel model of chronic stress

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ARTICLE INFO

Keywords:
Corticosterone
Habituation
Interleukin-1
Escalating stress
Sequential stress
Neuroimmune
Cytokine

ABSTRACT

Acute and chronic stress challenges have a profound influence on the development and expression of subsequent affective disorders, alcohol use disorders, and natural aging processes. These experiments examined adaptation in neuroimmune and neuroendocrine responses that occurred as a result of exposure to a novel model of chronic stress, termed chronic escalating distress (CED). This model involves exposure to highly predictable daily stress challenges involving a systematic escalation in both the intensity and length of daily stress challenges, and has recently been shown to profoundly alter alcohol sensitivity. Male Sprague-Dawley rats were exposed to an 11 day procedure where days 1–5 consisted of 60 min of restraint, days 6–10 consisted of 60 min of restraint immediately followed by 30 min of forced swim, and on day 11 subjects were exposed to a 2 h session of intermittent footshock. Experiment 1 examined adaptation in the corticosterone (CORT) response at key points in the 11 day procedure, and found that the escalation in stressors disrupted habituation to restraint, whereas the CORT response to daily forced swim exposure increased across days. Experiment 2 investigated the impact of this stress paradigm on the expression of several cytokine (IL-1β, IL-6, TNF-α) and cellular activation marker (c-Fos, CD14, CD200R) genes in key brain regions (PVN, HPC, & PFC) known to be influenced by stress. Interestingly, a history of CED had no effect on footshock-induced neuroimmune changes (increased IL-1 in the PVN; increased IL-6 in the HPC and PFC). In addition, acute footshock and CED produced similar c-fos induction within the PVN whereas CED led to enhanced c-fos induction in both the HPC and PFC. These findings support recent work indicating that neuroimmune responses to acute stress challenges persisted in rats with a recent history of repeated stress exposure, and that these effects occurred contemporaneously with ongoing changes in HPA axis reactivity. Overall, this CED model may serve as a highly tractable model for studying adaptation to chronic stress, and may have implications for understanding stress-induced alterations in alcohol sensitivity and natural aging processes.

1. Introduction

The incidence of adults reporting adverse effects from daily stress continues to grow in the United States, with two thirds of individuals reporting greater distress than a year ago (APA, 2017). It is therefore critical to develop highly tractable models of chronic stress in which the underlying physiological mechanisms of adaptation and ultimately, adverse health consequences of stress can be studied effectively. Toward this end, a wide variety of acute stress models are commonly utilized to study the underlying neural and hormonal consequences of stress, and may have important implications for understanding sensitivity to alcohol (Doremus-Fitzwater et al., 2018) and natural aging processes (Bale and Epperson, 2015). To model chronic stress experienced by humans, investigators often assemble daily stress challenges to specifically manipulate psychological features of the stressful experience, including the controllability (Maier et al., 1986), predictability (Weiss, 1970), duration (Martí et al., 1994), and general intensity (Natelson et al., 1988) of the stressful circumstances.

Indeed, repeated daily stress challenges that are consistent across exposures (often termed homotypic stressors) typically lead to habituation of stress responsive systems including, notably, habituation within the hypothalamic-pituitary-adrenal (HPA) axis. In contrast, chronic stress procedures that vary day by day (often termed heterotypic or variable stressors), typically lead to enhanced activity within stress responsive systems, an outcome that may portend stress-related maladies (Aguilera, 1998; Weinberg et al., 2009; Herman, 2013).

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https://doi.org/10.1016/j.ynstr.2018.08.007
Received 8 June 2018; Received in revised form 4 August 2018; Accepted 18 August 2018
Available online 18 August 2018

Importantly, habituation and sensitization effects are not unique to the HPA axis, but instead have been observed in the sympathetic-adrenal medullary response as evidenced by increased plasma catecholamine levels (Konarska et al., 1989), demonstrating generalization across multiple stress responsive systems. Thus, careful consideration of individual elements that are assembled into a chronic stress model require careful consideration, and will have a profound effect on study outcomes.

Studies utilizing chronic stress procedures tend to be on one extreme end or the other with respect to their general level of predictability. For instance, chronic variable stress (CVS) models utilize a range of challenges distributed intermittently across days to weeks, and are by design highly unpredictable. In contrast, repeated homotypic stress challenges such as chronic social defeat or repeated daily restraint are highly predictable and typically induce rapid habituation (e.g., Barnum et al., 2007). Further, a novel stress challenge in rats prehabituated to daily stress enhanced HPA axis activity and c-fos induction in the forebrain (Weinberg et al., 2009), yet no studies have examined sensitivity of neuroimmune genes to shifts in stress modality. Thus, one goal of the following studies was to establish a novel model of chronic stress that included a blend of predictable challenges, with discrete "escalations" in stress procedures that could be used to provide targeted assessments of how expectancies (or violations therein) regarding impending threat might influence physiological outcomes of stress (Martí et al., 1994; Pitman et al., 1995).

Stressful events are intrinsically salient and can rapidly induce the formation of associations, and learning occurs even more readily when stressors are able to be predicted. Thus, one would predict that in a repeated homotypic stress paradigm, the cessation of that stressor each day would become temporally predictable and would signal the organism to experience a sense of safety and "relief". If that expectation is violated and an additional stressor is applied, it would follow that a sensitized stress response might occur. Indeed, a recent study that looked at temporal stress expectation found that when rats were exposed to 10 min of restraint stress for 4 days then on the fifth day the length of stressor exposure was extended (violating the rats' expectation), corticosterone (CORT) responses were greater and struggling behavior was increased (Kearns and Spencer, 2013). Our lab recently utilized a "sequential stress" design where rats were exposed to 60 min of restraint followed immediately by 30 min of forced swim, and found that the CORT response was identical between subjects that had been exposed to restraint for 5 days prior and those that experienced the sequential stress without any history of stress whereas the PVN IL-1β response was attenuated in the group with stress history (Lovelock and Deak, 2017). Thus, different stress-responsive systems may respond with opposing patterns to unexpected stressors.

Thus, the goal of the following studies was to perform an initial characterization of a novel chronic stress model, which we will refer to as Chronic Escalating Distress (CED; see Fig. 1A). This 11-day procedure contains 3 distinct phases that capitalize on several well-established models/findings in the literature and has previously been shown to alter behavioral and neuroimmune sensitivity to a later alcohol challenge, effects that were categorically distinct from what was observed after exposure to footshock alone (Doremus-Fitzwater et al., 2018). Experiment 1 assessed body weight and CORT dynamics at several points during the CED procedure in peripheral blood as a first step toward understanding the impact of CED on general physiological manifestations of stress. Experiment 2 examined how recent stress history (Phases 1–2 of the CED model) impacted cytokine changes evoked by footshock exposure (Phase 3). Real time RT-PCR was utilized to assess pro-inflammatory cytokines (IL-1β, IL-6, and TNFα) known to be influenced by footshock (Hueston et al., 2011), as well as several cell surface markers indicative of glial activation (CD14, CD200, CD200R, GFAP, CHI313), and c-fos expression as a marker of neuronal activation. These target genes were examined in 3 structures (PVN, Hippocampus, PFC) critical for HPA axis regulation and that have also been reported to express neuroimmune genes in response to stress exposure (Hueston et al., 2011). Finally, to test whether the influence of CED on responses evoked by footshock were the result of the escalating nature of Phases 1–2, a separate control was included in which rats were exposed to an equivalent amount of restraint and forced swim as the CED group, but at random, unpredictable intervals (termed the "scrambled group").

2. Methods

2.1. Subjects

Adult male Sprague-Dawley rats purchased from Harlan Laboratories (Indianapolis, IN) were pair housed with access to food and water *ad libitum* and provided wooden chew sticks for enrichment. Colony conditions were maintained at 22 ± 1 °C with 12:12 light–dark cycle (lights on 06:30 h). Rats were given a minimum of 2 weeks to acclimate prior to experimentation and were handled for 3–5 min on each of two days before experimentation. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Binghamton University and animals were treated in accordance with PHS policy.

2.2. Restraint

Rats were restrained in a Plexiglas tube (length = 21.6 cm, inner diameter (ID) = 6.4 cm) with ample holes for ventilation for 60 min. The restraint stressor was devoid of any active immobilization, limb/tail tethering, or compression, and allowed sufficient movement so that animals could turn around within the tube (Barnum et al., 2007; Lovelock and Deak, 2017).

2.3. Forced swim stressor

Rats were transported to a dedicated procedural room and immediately placed in a cylinder (45 cm high, 20 cm diameter) filled 30 cm high with water that was carefully maintained at 25 °C, as previously described (Deak et al., 2003). Rats were forced to swim for 30 min, after which they were towel-dried and returned to their home cages.

2.4. Footshock procedure

Rats were exposed to 80 inescapable footshocks (1.0 mA, 5 s each, 90 s variable ITI) as previously described (Hueston et al., 2014).

2.5. Tail blood collection

Rats were briefly restrained in Plexiglas tubes (length = 21.6 cm, ID = 6.4 cm) and the tip of the tail (~1 mm) was transected with a razor blade. Blood (50–100 μL) was collected with gentle massaging of the tail into 0.5 ml microcentrifuge tubes and samples were immediately placed on ice. All blood samples were collected within 2 min to ensure serum measures of CORT reflected ambient levels untainted by the stress of the blood sampling procedure itself. Rats were returned to their home cages immediately afterwards or remained in restraint as dictated by group assignment. Serum was separated for 15 min at 3220 g in a refrigerated centrifuge and frozen at −20 °C until time of assay.

2.6. Measurement of CORT

Total serum CORT levels were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Enzo Life Sciences; Farmingdale, NY) according to manufacturer's instructions. Samples were heat-inactivated to denature endogenous corticosteroid

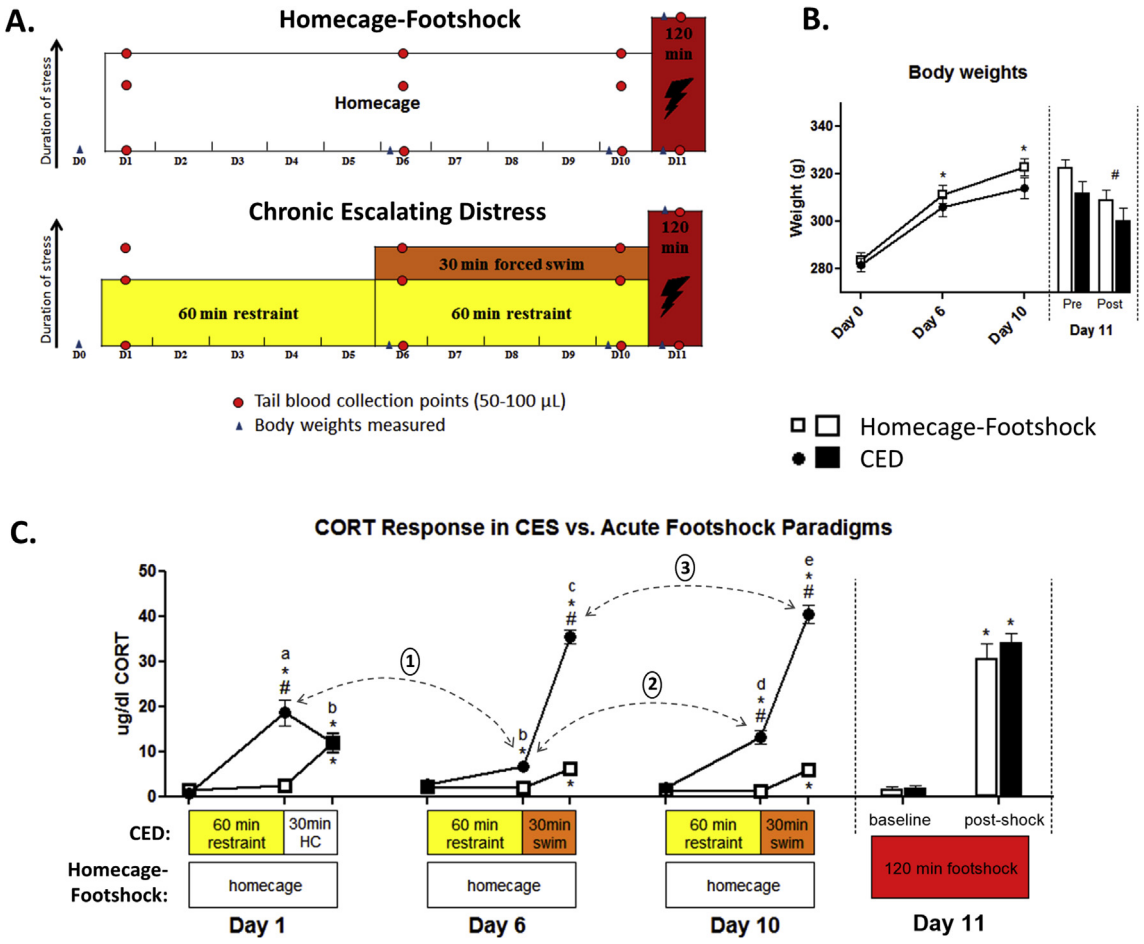

Fig. 1. A. Rats (n = 8 per group) were exposed to 10 total days of stress (5 days of restraint then 5 days of restraint followed immediately by forced swim) or were kept in their homecages for 10 days. All rats were then exposed to 2 h of footshock on the 11th day. Red dots depict the points at which tail blood samples were collected and blue triangles indicate points when body weights were measured. **B.** Body weight data. For days 0, 6, and 10, * indicate main effect of day. On day 11, # indicates a main effect of stress. **C.** CORT levels across the experiment. * represents a significant difference from that day's baseline within group and day, the # represents a difference between the two conditions at that specific day and timepoint, and different letters indicate significant differences in the CED condition at the 60 and 90 min timepoints. (1) highlights the expected habituation of the CORT response to restraint, (2) identifies an increase in the CORT response possibly due to the anticipation of forced swim following restraint, and (3) highlights a possible sensitization effect of forced swim. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

binding globulin (CBG) via immersion in a 75 °C water bath for 60 min (Buck et al., 2011; Spencer and Deak, 2017). Prior assays found this procedure produced superior denaturation of CBG than the enzyme cleavage step provided in the kit (unpublished observations). Assay sensitivity was 27.0 pg/ml with an inter-assay coefficient of 8.11%.

2.7. Tissue collection

Tissue was harvested after rapid decapitation and trunk blood was collected in EDTA-coated vacutainers. Plasma was separated in a refrigerated centrifuge and frozen at −20 °C until time of assay. Brains were removed immediately after decapitation and whole brains were flash frozen in 2-methylbutane and stored at −80 °C. Key structures were identified with a brain atlas (Paxinos and Watson, 2005). For brain punches, brains were coronally sliced at 60 µm per slice on a cryostat at −20 °C and bilateral tissue punches (1.2 × 1 mm or 2 × 1.5 mm) were taken from structures of interest (see Lovelock and Deak, 2017 for schematic) and immediately stored at −80 °C.Each tissue sample was placed in a 2.0 ml Eppendorf tube with 500 µL Trizol® RNA reagent and a 5 mm stainless steel bead. Tissue was then homogenized using a Qiagen TissueLyser II™ (Qiagen, Valencia, CA) for 2–4 min at 20 Hz to ensure thorough homogenization of samples. Total cellular RNA was extracted from tissue using Qiagen RNeasy Mini kits according to the manufacturer's instructions. RNA was separated from the supernatant through chloroform extraction performed at 12,000 g for 15 min at 4 °C. Equal volume of 70% ethanol was added to the collected RNA and purified through RNeasy mini columns. Columns were washed and eluted with 30 µL of RNase-free water (65 °C). RNA yield and purity was determined using the NanoDrop 2000 spectrophotometer (NanoDrop, Wilmington, DE). RNA was stored at −80 °C prior to cDNA synthesis.

2.8. Real-time RT-PCR

Synthesis of cDNA was performed on 0.3–1.0 µg of normalized total RNA from each sample using the QuantiTect Reverse Transcription kit (Cat No. 205,313, Qiagen, Valencia, CA) which included a DNase treatment step. All cDNA was stored at −20 °C until further processing. Probed cDNA amplification was performed in a 20 µL reaction consisting of 10 µL IQ SYBR Green supermix (Bio-Rad Laboratories), 0.1 µL forward and reverse primer, 2 µL cDNA template, and 8.8 µL ribonuclease-free water run in triplicate in a 384-well plate (BioRad Laboratories) using the BioRad CFX 384 Real Time System C1000 Thermal Cycler (BioRad Laboratories). Relative gene expression was quantified using the delta-delta (2-ΔΔCT) method relative to the stable housekeeping gene β-actin (Livak and Schmittgen, 2001).

Housekeeping genes were analyzed separately to ensure stability across treatment groups prior to use as a reference. Primer sequences were recently published in Lovelock and Deak (2017) except for CHI3L3 (fwd: 5′-TGA ACG GGG CAG ATC CAA AC-3'; rev: 5′-GTT TGG ATC TGC CCC GTT CA-3).

2.9. Statistical analysis

Data were analyzed with Statistica software using either ANOVA designs as described below. Post-hoc testing was done using Tukey's test for all observed main effects. An α-level of 0.05 was used as the criterion for determining statistical significance.

2.10. Experiment 1: habituation of the CORT response to sequential stressor exposure

The goal of this experiment was to provide a detailed characterization of HPA axis adaptation at key time points (e.g. phase transitions) during the CED procedure, which consisted of an initial 5-day phase of once-daily restraint in Plexiglas tubes (30 min total), a second phase (days 6–10), in which the daily restraint continued but was followed immediately by placement into a forced swim challenge (60 min total), and on day 11 (phase 3), rats were exposed to a 2 h session of intermittent footshock (see Fig. 1A for design). Rats (n = 8/group, N = 16) were exposed to the first 10 days of CED and a separate group of non-stressed controls were kept in their homecages for 10 days (Homecage-Footshock). On the 11th day, all rats were exposed to 2 h of intermittent footshock (1.0 mA, 5 s each, 90 s variable inter-trial interval). Blood samples were collected on days 1, 5, 10, and 11 before restraint as a baseline measure, immediately post-restraint (immediately before swim), and immediately post-swim. Body weights were measured at on days 0, 6, 10, and 11, and also after footshock on day 11, as indirect measures of the impact of stress. We expected to replicate standard indices of CORT habituation during phase 1, and predicted that the addition of forced swim in phase 2 would lead to dishabituation. Although we made no *a priori* predictions about the directionality of CED effects on the CORT response to subsequent footshock, we expected that CED would produce signs of HPA axis dysregulation, which can manifest as either increased or decreased CORT.

2.11. Experiment 2: neuroimmune changes in response to repeated sequential stressor exposure

The goal of this experiment was to examine expression of cytokine and cellular activation marker genes in the paraventricular nucleus (PVN), prefrontal cortex (PFC), and hippocampus (HPC) after exposure to the CED model via real-time RT-PCR. A 2 × 2 design (stress history vs. footshock challenge) was used with an additional mixed stressor group (termed scrambled stress history), yielding a 5 group experiment designed to examine the effects of CED on pro-inflammatory cytokine expression and to determine the impact of elements that make up the procedure (see Fig. 2A for design). Adult male Sprague-Dawley rats (n = 8/group, N = 40) were randomly assigned to one of five conditions: Non-Stressed Controls, who remained in their home cages throughout the experiment, Stress History Only, who received CED without an acute footshock challenge, a Footshock Only group, CED (who received the full CED paradigm), and a Scrambled Stress History group in which subjects received equivalent exposure to restraint and swim as experienced in CED but the daily order and temporal contiguity was randomized across the 10 day chronic stress period prior to the footshock challenge on the final day. The order of stressor exposure for the scrambled condition was pseudo-randomly assigned using a random number generator followed by minor modification to ensure there was no predictability or escalation of stressor exposure across the ten day period. Body weights were measured the day before experimentation started and on the mornings of days 6, 10, and 11 of stressor exposure. Brains and plasma were collected immediately after stress termination under no-stress conditions immediately after footshock or at an equivalent time for controls. The day of stress initiation was varied so that all groups were equally represented on each day of tissue harvest. We predicted that CED would sensitize the IL-1β expression relative to acute footshock, and that the mixed group would show greater inflammatory cytokine expression in the PFC as compared to the CED group due to the difference in stressor predictability between the groups.

3. Results

3.1. Experiment 1 results

3.1.1. Body weights

Raw body weights were analyzed with two separate analyses corresponding to the two phases of the experiment (days 0–10 and day 11). For the first analysis, a 2 × 3 repeated measures ANOVA was utilized to assess body weight gain across the chronic stress period of 10 days. An expected significant main effect of day [$F(2, 28) = 276.2$, $p < 0.0001$] was observed, indicating body weight gain throughout the experiment. In order to assess the impact of footshock on body weight, a separate 2 × 2 repeated measures ANOVA was used to examine body weight differences on day 11 before and after exposure to footshock. As expected, footshock significantly reduced body weight regardless of prior stress history [$F(1, 14) = 85.98$, $p < 0.00001$]. Body weights from both phases of the experiment are presented in Fig. 1B.

3.1.2. CORT

Similar to the body weight data, two separate analyses were performed corresponding to the two phases of the experiment. The CORT response over the 10 days of stressor exposure was analyzed with a 2 × 3 × 3 nested-design repeated measures ANOVA that found significant main effects of stress condition [$F(1,13) = 148.2$, $p < 0.0001$], day [$F(2,26) = 5.06$, $p < 0.0001$], and time [$F(2,26) = 181.9$, $p < 0.0001$]. Interactions between day and group [$F(2,26) = 18.8$, $p < 0.0001$], time and group [$F(2,26) = 66.8$, $p < 0.0001$], day and time [$F(4, 52) = 32.8$, $p < 0.0001$] and all three variables [$F(4, 52) = 62.1$, $p < 0.0001$] were significant. Post-hoc analysis revealed a mild but significant increase in CORT at the third sampling timepoint in the homecage group on each test day (all $p < 0.01$), likely a result of repeated sampling. On day 1 (60 min restraint), rats showed a post-restraint increase in CORT ($p < 0.0001$) and 30 min later a return to the level of the homecage control group. On day 6, restraint led to a modest increase in CORT ($p < 0.05$) relative to the baseline sample of the day, but there was no difference between stressed and homecage subjects at 60 min ($p > 0.05$), supporting the view that repeated daily exposure led to habituation. When restrained rats were then immediately exposed to swim, serum CORT increased substantially relative to the homecage group ($p < 0.0001$). On day 10, restraint induced a CORT response that was still habituated relative to day 1 ($p < 0.001$) but was higher relative to day 6 ($p < 0.0001$). Forced swim resulted in a greater CORT response on day 10 versus day 6 ($p < 0.001$), indicating sensitization of the CORT response.

When plasma CORT concentrations were analyzed from day 11 with a separate 2 × 2 ANOVA, footshock led to a substantial increase in CORT as evidenced by a main effect [$F(1, 14) = 240.0$, $p > 0.0001$]. Importantly, prior stress history had no significant effect on basal [$F(1, 14) = 1.06$, $p > 0.05$] or shock-induced CORT [$F(1, 14) = 0.63$, $p > 0.05$]. See Fig. 1C for CORT results.

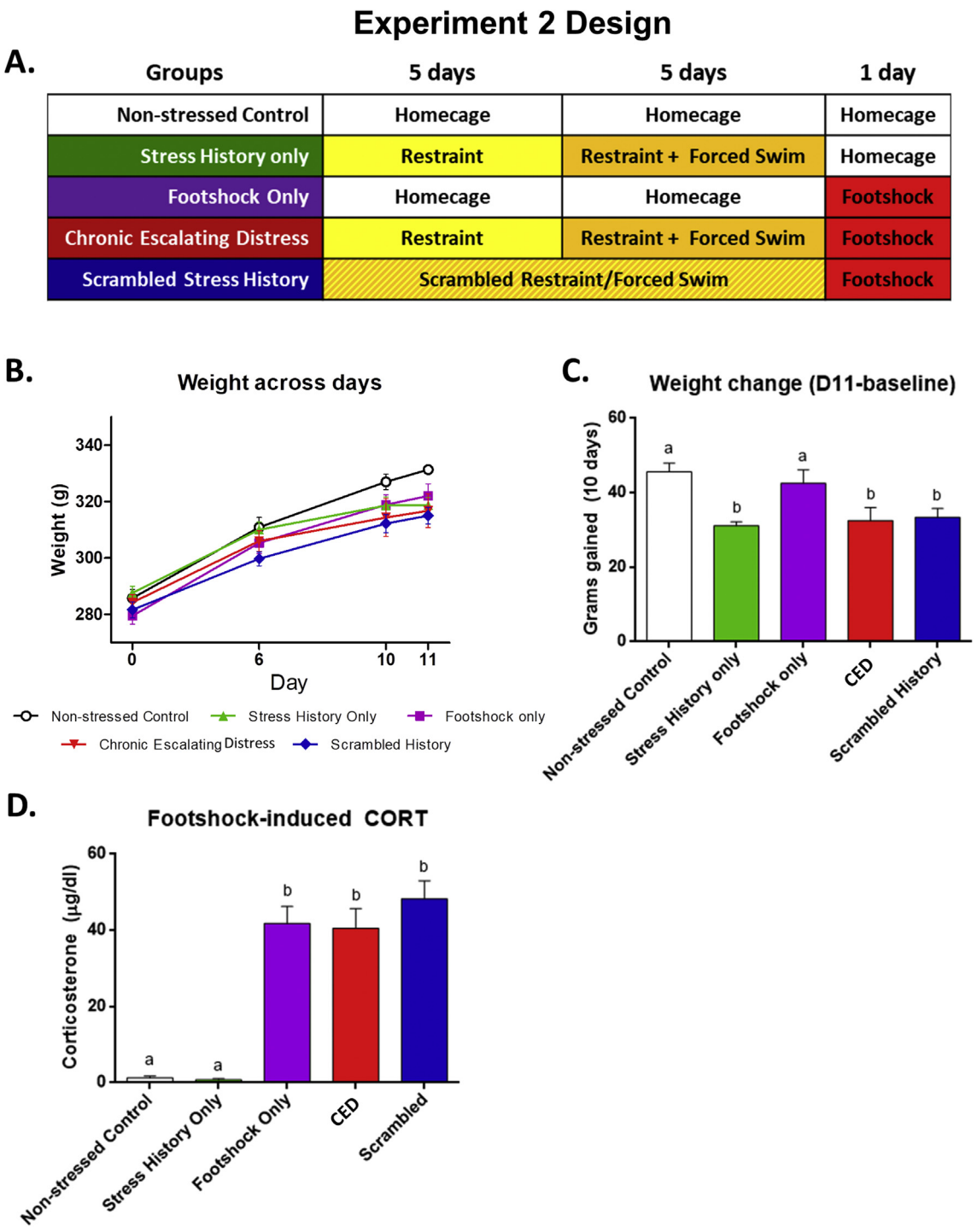

Fig. 2. Experiment 2 design, body weight data, and CORT data (n = 8/group). **A.** Rats were exposed to 10 days of stress (restraint for 5 days then 5 days of restraint immediately followed by forced swim) or remained in the homecage, then on the 11th day were exposed to 2 h footshock or were kept in the homecage. **B.** Body weight gain across days. **C.** Body weight gain from baseline to the final day of Experiment 2. **D.** Peripheral CORT measures after footshock on the 11th day of testing. Different letters indicate significant differences.

3.2. Experiment 2 results

3.2.1. Body weights

When raw body weights were analyzed with a mixed 4 × 5 (day vs. stress condition) ANOVA, an expected main effect of day [$F(3, 105) = 496.19$, $p < 0.0001$] was observed. When average body weight gain across the experimental 10 days was tested with a one-way ANOVA, a significant effect of stress condition was observed [$F(4, 35) = 5.70$, $p < 0.01$]. Post-hoc analysis revealed that the three stress conditions (Stress History only, CED, and Scrambled) were significantly lower than the two homecage groups (non-stressed controls and footshock only; all $p < 0.05$). Results are summarized in Fig. 2B and C.

3.2.2. Corticosterone

Data were analyzed with a one-way ANOVA with 5 levels which revealed a significant effect of stress condition [$F(4, 35) = 52.44$, $p < 0.0001$] with all 3 groups that received footshock (footshock only, CED, and scrambled) having significantly elevated CORT as compared

Table 1
Real-time RT-PCR results from Experiment 2.

	Non-stressed Control	Stress History Only	Footshock Only	Chronic Escalating Distress	Scrambled	Statistical analyses
Paraventricular Nucleus of the Hypothalamus (PVN)						
β-actin	101.8 ± 7.8	114.9 ± 6.0	101.2 ± 10.2	87.75 ± 4.7	92.4 ± 7.1	F (4,34) = .2.04 p > 0.05
c-Fos	**101.4 ± 6.9**^{a}	**103.6 ± 9.7**^{a}	**625.2 ± 47.6**^{b}	**632.8 ± 25.1**^{b}	**595.5 ± 36.0**^{b}	F (4,34) = 86.30, p < 0.001
TNF-α	101.0 ± 5.7	82.0 ± 4.0	72.0 ± 6.0	78.6 ± 12.9	75.7 ± 7.7	F (4,34) = 1.87, p > 0.05
IL-1β	**106.1 ± 13.5**^{a}	**98.5 ± 10.4**^{a}	**253.0 ± 46.1**^{b}	**289.7 ± 68.5**^{b}	**300.2 ± 37.5**^{b}	F (4,34) = 5.47, p < 0.01
IL-6	102.8 ± 9.3	131.5 ± 7.2	135.22 ± 15.5	88.9 ± 13.7	120.0 ± 18.3	F (4,33) = 2.01, p > 0.05
CD14	**101.7 ± 7.7**^{a}	**109.1 ± 9.3**^{a}	**142.2 ± 7.2**^{b}	**168.5 ± 10.6**^{bc}	**170.4 ± 10.6**^{c}	F (4,34) = 11.72, p < 0.001
CD200	100.6 ± 4.8	103.0 ± 5.2	97.1 ± 3.6	117.7 ± 7.6	100.3 ± 4.1	F (4,34) = 2.39, p > 0.05
CD200R	105.0 ± 12.7	76.1 ± 8.9	87.1 ± 11.6	121.9 ± 14.8	88.3 ± 11.5	F (4,34) = 2.25, p > 0.05
GFAP	106.3 ± 14.0	84.8 ± 9.4	126.1 ± 11.6	119.6 ± 6.2	123.4 ± 12.5	F (4,34) = 2.48, p > 0.05
CHI3l3	122.3 ± 30.3	130.9 ± 16.2	160.8 ± 32.8	169.2 ± 24.8	188.6 ± 33.4	F (4,34) = 0.93, p > 0.05
Hippocampus (HPC)						
β-actin	126.6 ± 30.1	160.68 ± 22.3	155.9 ± 16.4	102.7 ± 20.3	153.8 ± 16.3	F (4,35) = 1.30, p > 0.05
c-Fos	**119.1 ± 30.7**^{a}	**94.2 ± 26.1**^{a}	**198.9 ± 24.0**^{b}	**283.0 ± 34.9**^{c}	**203.3 ± 10.3**^{b}	F (4,35) = 8.01, p < 0.001
TNF-α	**106.4 ± 14.5**^{a}	**105.4 ± 7.0**^{a}	**38.3 ± 5.0**^{b}	**52.0 ± 11.7**^{b}	**46.3 ± 3.8**^{b}	F (4,35) = 12.83, p > 0.001
IL-1β	**115.0 ± 23.3**^{a}	**84.6 ± 15.6**^{ab}	**43.2 ± 5.3**^{b}	**74.8 ± 13.0**^{ab}	**66.4 ± 8.9**^{b}	F (4,35) = 3.25 p > 0.05
IL-6	**105.8 ± 13.5**^{a}	**77.4 ± 7.6**^{a}	**185.8 ± 7.6**^{b}	**228.7 ± 42.3**^{b}	**170.7 ± 20.5**^{b}	F (4,35) = 7.45, p < 0.001
CD14	101.0 ± 5.2	88.9 ± 10.2	55.1 ± 6.4	105.5 ± 14.6	101.1 ± 24.5	F (4,35) = 1.19, p > 0.05
CD200	111.4 ± 18.0	82.0 ± 7.9	82.4 ± 6.7	105.0 ± 14.3	95.6 ± 9.8	F (4,35) = 1.87, p > 0.05
CD200R	108.4 ± 15.2	109.7 ± 11.0	90.2 ± 16.2	218.7 ± 78.1	137.7 ± 14.2	F (4,35) = 0.20, p > 0.05
GFAP	104.3 ± 11.3	89.5 ± 5.4	99.7 ± 19.0	93.3 ± 11.4	94.7 ± 13.0	F (4,35) = 0.44, p < 0.05
CHI3l3	104.0 ± 12.2	88.2 ± 9.5	86.9 ± 9.6	93.8 ± 11.1	97.9 ± 10.4	F (4,35) = 0.91 p > 0.05
Prefrontal Cortex (PFC)						
β-actin	103.4 ± 9.1	93.1 ± 14.0	108.2 ± 12.3	107.3 ± 7.9	115.7 ± 9.7	F (4,34) = 0.58, p > 0.05
c-Fos	**102.3 ± 8.4**^{a}	**91.33 ± 12.1**^{a}	**474.11 ± 17.4**^{b}	**616.8 ± 34.0**^{b}	**593.6 ± 36.5**^{b}	F (4,33) = 121.87, p < 0.001
TNF-α	**102.8 ± 9.3**^{a}	**91.4 ± 11.1**^{a}	**41.3 ± 5.6**^{b}	**24.1 ± 2.8**^{b}	**32.1 ± 3.7**^{b}	F (4,31) = 22.60, p < 0.001
IL-1β	114.1 ± 20.1	137.2 ± 31.3	141.7 ± 25.9	96.3 ± 27.1	88.0 ± 11.3	F (4,33) = 0.77, p > 0.05
IL-6	**116.5 ± 21.6**^{a}	**105.6 ± 26.7**^{a}	**189.4 ± 23.7**^{b}	**201.0 ± 31.7**^{b}	**154.5 ± 22.3**^{ab}	F (4,32) = 2.80, p < 0.05
CD14	108.1 ± 17.7	117.5 ± 8.3	96.8 ± 10.7	111.4 ± 27.1	107.2 ± 10.7	F (4,32) = 0.25, p > 0.05
CD200	226.4 ± 38.6	229.2 ± 29.8	200.8 ± 14.7	256.0 ± 34.9	214.8 ± 17.0	F (4,32) = 0.49, p > 0.05
CD200R	139.6 ± 30.9	143.8 ± 7.8	162.3 ± 22.8	182.9 ± 20.3	141.4 ± 20.2	F (4,32) = 0.61, p > 0.05
GFAP	108.6 ± 17.8	104.9 ± 13.3	111.6 ± 10.5	84.7 ± 12.1	82.3 ± 13.9	F (4,32) = 0.98, p > 0.05
CHI3l3	113.3 ± 18.7	85.1 ± 7.5	100.7 ± 18.7	89.0 ± 8.8	101.5 ± 20.4	F (4,32) = 0.50, p > 0.05

Note. Means and SEM for Experiment 2. Gene expression data in the paraventricular nucleus of the hypothalamus (PVN), prefrontal cortex (PFC), and hippocampus (HPC) adjusted to β-actin as housekeeper and expressed as percent change from the control group (homecage). Bold text indicates a significant effect; data points marked with differing letters are significantly different from one another ($p < 0.05$).

to non-stressed controls ($p < 0.0001$ for all). A trend emerged for a greater CORT response in the scrambled condition than the CED group, but this effect did not achieve significance ($p = 0.099$). Results are illustrated in Fig. 2D.

3.2.3. Gene expression

As a comprehensive summary, the results for all brain structures and gene targets are summarized in Table 1, whereas our *a priori* targets of interest, c-Fos and key inflammatory cytokine results, are highlighted in Fig. 3. One-way ANOVAs showed no effect of stress manipulations on β-actin expression in any brain structure (PVN [$F(4,34) = 2.04$, $p > 0.05$]; HPC [$F(4,35) = 1.30$, $p > 0.05$]; PFC [$F(4,34) = 0.58$, $p > 0.05$]), so all gene expression data were adjusted to β-actin as a housekeeper.

3.2.4. Cytokines

One-way ANOVAs showed an effect of stress exposure on IL-1β in the PVN [$F(4, 34) = 5.47$, $p < 0.01$]. Post-hoc analysis revealed that expression levels were elevated in all of the groups that received footshock (footshock only, $p < 0.05$; CED, $p < 0.01$, scrambled, $p < 0.01$). An effect on IL-1β expression was also seen in the HPC [$F(4, 35) = 3.24$, $p < 0.05$], with the footshock only ($p < 0.01$) and scrambled ($p < 0.05$) conditions displaying a significant reduction in expression. A trend was seen for suppressed IL-1β expression in the CED group, but it did not reach the level of significance ($p = 0.059$). One-way ANOVA analysis of IL-6 expression found significant effects in the HPC [$F(4, 35) = 7.44$, $p < 0.001$] and PFC [$F(4, 32) = 2.80$, $p < 0.05$]. Post-hoc analysis in the HPC revealed significant elevations in expression in the three groups that experienced footshock (footshock only, $p < 0.05$; CED, $p < 0.001$; scrambled, $p < 0.05$). In the PFC, elevations were also seen in the footshock only ($p < 0.05$) and CED ($p < 0.05$) conditions but not the scrambled group ($p > 0.05$). One-way ANOVA analysis of TNF-α expression again revealed effects in the HPC [$F(4, 35) = 12.92$, $p < 0.0001$] and PFC [$F(4, 31) = 22.60$, $p < 0.0001$]. In the HPC, TNF-α expression was reduced in all three groups exposed to footshock (all $p < 0.001$) as compared to controls, and the same pattern was seen in the PFC with suppressed TNF-α expression in the same three groups (all $p < 0.0001$). Cytokine data are illustrated in Fig. 3A–I.

3.2.5. Cellular activation

In all three structures, stress manipulations significantly affected c-Fos induction (PVN [$F(4,34) = 86.30$, $p < 0.001$]; HPC [$F(4,35) = 8.01$, $p < 0.0001$]; PFC [$F(4,33) = 121.87$, $p < 0.0001$]). In the PVN, c-Fos expression was elevated in the three footshock-exposed conditions (all p's < 0.0001). In the HPC, c-Fos was also elevated in all three footshock-exposed conditions (footshock only, $p < 0.05$; CED, $p < 0.001$; scrambled, $p < 0.05$) and expression was higher in the CED group as compared to the footshock only ($p < 0.05$) and scrambled ($p < 0.05$) conditions. In the PFC, c-Fos expression was elevated in all three footshock-exposed conditions (all $p < 0.0001$), with higher expression seen in the CED group versus footshock only ($p < 0.001$) and also in scrambled versus footshock only ($p < 0.001$). See Fig. 3J–L for c-Fos results. One-way ANOVAs revealed a significant effect of stressor exposure on CD14 in the PVN [$F(4,34) = 11.72$, $p < 0.0001$]. The footshock only ($p < 0.01$), CED ($p < 0.0001$), and scrambled ($p < 0.0001$) groups were all elevated relative to non-stressed controls. The scrambled condition exhibited increased CD14 expression compared to the footshock only group ($p < 0.05$), and a trend was seen for increased expression in the CED group versus the

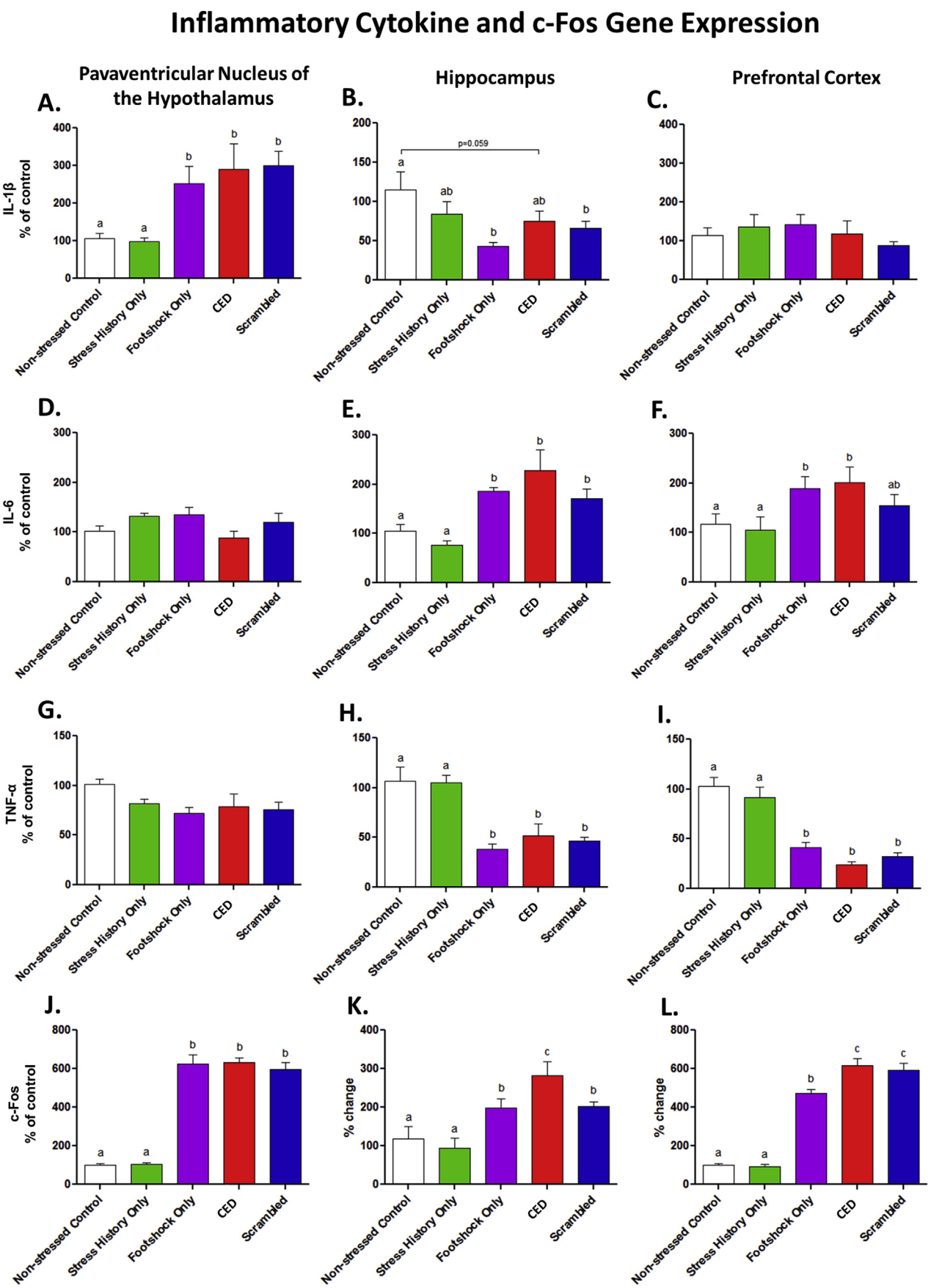

Fig. 3. Inflammatory cytokine and c-Fos real-time RTPCR results from Experiment 2. All data is expressed relative to the control group (homecage) and normalized to β-actin. Each group consisted of 6–8 subjects. Different letters indicate significant differences. A–C: IL-1β; D–F: IL-6; G–I: TNF-α; J–L: c-Fos.

footshock only group, though significance was not quite achieved ($p = 0.051$).

4. Discussion

The present studies were designed to perform an initial characterization of the CED paradigm, with the specific goals of: (i) measuring adaptation of the CORT response at key points during administration of CED and (ii) identifying adaptations of neuroimmune and cellular activation markers to escalating stress, with a focus on inflammatory cytokines that have been found to be highly responsive to stress in previous studies. Understanding the course of adaptation during the "ramping up" period of the CED paradigm can provide insight into how neuroimmune mechanisms might impact adaptation within other physiological systems after chronic stress. These studies are not the first to utilize exposure to sequential stress challenges to create a composite, prolonged experience of distress. For instance, the single prolonged stress model of PTSD involves sequential exposure to restraint, swim and ether, and produces enduring changes in behavior that resemble hallmark features of PTSD (Liberzon et al., 1997, 1999; Yamamoto et al., 2009). Thus, adaptation of these basic concepts into a chronic stress procedure that allows for targeted "phase transitions" in which features of stress adaptation and expectancies to occur with temporal precision is a strength of the model utilized here.

Consistent with this, CORT results from Experiment 1 highlighted a fundamental advantage of the CED experimental design, utilizing within-subject comparisons at key transitional timepoints. As expected, the CORT response to 60 min of restraint was habituated after 5 days (see ①, Fig. 1C), as the CORT response was reduced on day 6, which was the first day that 30 min of forced swim immediately succeeded the 60 min of restraint. On day 10, after 5 days of restraint + swim, the post-restraint CORT response showed an elevation relative to day 6 (see ②, Fig. 1C). This disruption of habituation may be indicative of an anticipatory CORT response as the initially unpaired restraint stressor transitioned into becoming predictive of the forced swim stressor that followed. The post-swim timepoint on day 10 was also elevated relative to that on day 6, revealing evidence of HPA axis dysfunction (see ③, Fig. 1C). Another recent study in our lab (Lovelock and Deak, 2017) found that 5 days of repeated restraint + swim did not lead to sensitization of the CORT response, and that extending the procedure to 10 days led to signs of habituation. Whether the current finding is reflective of sensitization, impaired feedback inhibition, or simply reflects an elevation due to the higher post-restraint CORT level remains to be determined. The homecage-footshock group showed a small but significant increase in CORT at the third sampling point (90 min) on each day, likely due to the repeated tail blood sampling procedure. Footshock on day 11 resulted in a robust CORT response that did not differ between groups. The same result was observed in Experiment 2, where CORT levels were high in all groups that received footshock but did not differ between animals with different stress histories. However, it may be important to consider that footshock evokes a strong CORT response that may obfuscate potential differences due to a ceiling effect, and only a single time point was examined. Thus, future studies examining HPA reactivity after the CED paradigm using pharmacological procedures (CRH, ACTH challenge, Dexamethasone suppression, etc) to probe axis function further.

Experiment 2 focused on the impact of stress exposure on neuroimmune and cellular activation marker gene expression changes. IL-1β expression was increased in the PVN (Fig. 3A), replicating our previous findings after acute footshock (Blandino et al., 2013; Hueston et al., 2014), though stress history did not modify the response. Interestingly, IL-1β mRNA expression was suppressed in the HPC in the footshock only and scrambled conditions, and a trend for suppression was seen the CED group. Most studies have found either elevations of hippocampal IL-1β in response to various stressors, such as predator stress (Barnum et al., 2012), tail shock (Johnson et al., 2004), and immobilization (Suzuki et al., 1997) in rats, or no effects in the hippocampus as seen with chronic mild stress (Mormede et al., 2003) in rats, social defeat in mice (Gibb et al., 2011), and maternal separation in guinea pigs (Hennessy et al., 2004). However, IL-1β mRNA was suppressed in the HPC by chronic social defeat (Bartolomucci et al., 2003), demonstrating that the specific stressor, time of stressor exposure, and species/strain of subjects may be a critical component in determining the IL-1β response within the HPC (Deak et al., 2017). Regardless, the apparent site-specific induction of IL-1β in the PVN and reduced expression in the HPC may be explained by site-specific differences in glucocorticoid sensitivity, since glucocorticoids are known to suppress expression of IL-1β via action on glucocorticoid receptors (Laue et al., 1988). Further studies examining the expression of glucocorticoid receptors in the HPC may be able to shed some light on the interplay of these elements.

IL-6 was found to be elevated in the HPC in all groups that experienced footshock, which fits with prior data from our lab (Blandino et al., 2009). Interestingly, in another recent study our lab found that the typical elevation in IL-6 after intubation with ethanol (Doremus-Fitzwater et al., 2014, 2015) was attenuated in rats that had completed the CED procedure 24 h prior, suggesting that the experience of CED induced at least short-term changes in the HPC. Expression in the PFC was similar with the footshock only and CED groups showing an elevation and the scrambled group trending towards increased levels. TNF-α expression in the HPC and PFC was suppressed in all groups that received footshock, which again may be a result of high CORT levels acting on glucocorticoid receptors that are known to be highly concentrated in these regions, thereby inhibiting inflammation (Sapolsky et al., 1984). IL-6 was not increased in the PVN nor in the HPC, which fits with previous findings (Hueston and Deak, 2014) and highlights the cytokine- and site-specific effects of footshock.

The present work focused on three CNS sites involved in negative affect regulation that are known to evince neuroimmune changes as a result of stress exposure. In all three brain regions, c-Fos was elevated after footshock and baseline levels were not modified by the experience of the 10-day escalating portion of the CED design as indicated by the lack of effects in the stress history only group. In the PVN there were no differences in the level of c-Fos induction between the footshock only, CED, and scrambled groups, whereas in the PFC the CED and scrambled groups had higher expression than the acute footshock group. Dendritic remodeling in the PFC is known to occur 24 h after exposure to chronic restraint stress (Shansky and Morrison, 2009), which likely involves increased c-Fos immediately after the cessation of stress. In the HPC, c-Fos expression was greater in the CED condition as compared to both the footshock only and scrambled conditions, suggesting that specific adaptations to CED, but not those to a randomized but equal daily stress condition (scrambled group), resulted in a greater c-Fos response. Thus, the predictable nature of CED appears to be reflected by differential activational responses within the hippocampus and PFC, though overall we were surprised to see relatively few differences between the scrambled and CED groups. Of course, measuring c-Fos via RT-PCR represents a limitation of the studies presented here, since multiple cell types are present in focal punches such as those used here. This approach was adopted due to the advantages of RT-PCR for measuring multiple cytokines and neuroimmune factors within tissue punches, which could not likely be accomplished via standard IHC or *in situ* hybridization procedures. Further studies will be necessary to identify the specific cellular populations within these structures and their potential alteration by CED, and the extent to which scrambled and CED groups might differ when more refined approaches are used.

Other than c-fos, the only change seen in cell surface markers was in CD14 in the PVN, where all footshock conditions exhibited increased CD14 expression, replicating our prior findings (Hueston et al., 2014). No changes were observed in other microglial activational markers (CD200R, CHI3l3), neuronally-expressed microglial activation regulators (CD200), or astrocytic activational markers (GFAP) which is

consistent with prior data from our lab (Blandino et al., 2009; Catanzaro et al., 2014). It may be that the timepoint of tissue collection is not ideal for observing changes in other cell-surface markers as the timecourse of changes in glial markers has not been well-characterized, and glial changes often occur in a delayed fashion (24 h after stress termination) in other studies (Tynan et al., 2010; Wohleb et al., 2011). The finding that the only increase in microglial activity marker expression was found in the PVN, which also exhibited IL-1β induction, fits well with prior data showing that minocycline, a putative microglial inhibitor, blocked stress-responsive IL-1β after acute footshock (Blandino et al., 2006, 2009). Further, microglia have been shown to be the first IL-1β producing cells in response to insults such as nerve injury, CNS lesions, and neurodegenerative diseases, followed by astrocytic IL-1β production a few days later (Herx and Yong, 2001; Liu et al., 2011). It is not yet known whether astrocytes take on a similar role after repeated exposure to stress, though our data showing no changes in GFAP mRNA expression across the present study do not support that notion. Overall, prior stress history had no impact on any of the cytokine changes induced by footshock.

In summary, these experiments serve as a foundation for establishing the CED model as a tractable model for exploring neuroendocrine and neuroimmune adaptations to chronic stress. We found that the escalation of stressors in a systematic, predictable manner led to partial disruption of habituation of the CORT response (see Fig. 1C) whereas the peak CORT response was largely unaffected, though overall the effects of stress history were more prevalent than effects due to “escalating distress” per se (as evidenced by differences between the CED and scrambled groups in Exp 2). Nevertheless, the finding that hippocampal c-Fos was more strongly induced by footshock CED group raises many questions about how the HPC is modulated by the CED procedure, and highlights this region as highly plastic in responding to predictable stressors. Indeed, other work from our laboratory has recently demonstrated that acute footshock exposure led to enhanced IL-6 responses evoked by acute ethanol intoxication, whereas CED exposure led to a blunted increase in hippocampal IL-6 as well as altered behavioral sensitivity to ethanol-induced loss of righting reflex (Doremus-Fitzwater et al., 2018). Furthermore, the transition of the brain toward a more inflamed state during later stages of natural aging might be accelerated by life history of stressful events, as stress early in life is a known threat to natural aging (Bale and Epperson, 2015). Though the present studies were not designed to determine the mechanisms underlying stress adaptation in the CED model, it is possible that changes in overall expression and nuclear trafficking of glucocorticoid and mineralocorticoid receptors, or other factors such as glutamatergic plasticity and noradrenergic regulation that are known to be impacted by stress exposure (Popoli et al., 2012; Valentino et al., 1997), may be responsible. These and other studies are important steps towards a better understanding of how these vital stress responsive systems impact overall brain health and may have implications for natural aging processes.

Acknowledgments

Research reported in this publication was supported by the National Institute of Health Grants P50AA017823 and RO1AG043467 to T. Deak, and the Center for Development and Behavioral Neuroscience at Binghamton University. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the above stated funding agencies. The authors have no conflicts of interest to declare.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ynstr.2018.08.007.

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