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Chronic Stress-induced Behaviors Correlate with Exacerbated Acute Stress-induced Cingulate Cortex and Ventral Hippocampus Activation

Corey Fee, [a,b] **Thomas Prevot,** [a,c] **Keith Misquitta,** [a,b] **Mounira Banasr** [a,b,c] **and Etienne Sibille** [a,b,c]*

[a] *Campbell Family Mental Health Research Institute, Centre for Addiction and Mental Health, Toronto, ON, Canada*

[b] *Department of Pharmacology and Toxicology, University of Toronto, Toronto, ON, Canada*

[c] *Department of Psychiatry, University of Toronto, Toronto, ON, Canada*

Abstract—Altered activity of corticolimbic brain regions is a hallmark of stress-related illnesses, including mood disorders, neurodegenerative diseases, and substance abuse disorders. Acute stress adaptively recruits brain region-specific functions for coping, while sustained activation under chronic stress may overwhelm feedback mechanisms and lead to pathological cellular and behavioral responses. The neural mechanisms underlying dysregulated stress responses and how they contribute to behavioral deficits are poorly characterized. Here, we tested whether prior exposure to chronic restraint stress (CRS) or unpredictable chronic mild stress (UCMS) in mice could alter functional response to acute stress and whether these changes are associated with chronic stress-induced behavioral deficits. More specifically, we assessed acute stress-induced functional activation indexed by c-Fos+ cell counts in 24 stress- and mood-related brain regions, and determined if changes in functional activation were linked to chronic stress-induced behavioral impairments, summarized across dimensions through principal component analysis (PCA). Results indicated that CRS and UCMS led to convergent physiological and anxiety-like deficits, whereas working and short-term memory were impaired only in UCMS mice. CRS and UCMS exposure exacerbated functional activation by acute stress in anterior cingulate cortex (ACC) area 24b and ventral hippocampal (vHPC) CA1, CA3, and subiculum. In dysregulated brain regions, levels of functional activation were positively correlated with principal components reflecting variance across behavioral deficits relevant to stress-related disorders. Our data supports an association between a dysregulated stress response, altered functional corticolimbic excitation/inhibition balance, and the expression of maladaptive behaviors.

Key words: c-Fos, chronic stress, anterior cingulate cortex, ventral hippocampus, excitation, inhibition.

INTRODUCTION

The acute stress response is an adaptive mechanism that maintains homeostatic function by recruiting coordinated neuroendocrine, autonomic, and immune system activities (McEwen, 2003). In the brain, acute stress activates the hypothalamic-pituitary-adrenocortical (HPA) axis, releasing glucocorticoids (GCs) to mobilize energy and direct brain systems for effective coping (Ulrich-Lai and Herman, 2009). These mediators modulate both excitatory glutamatergic pyramidal neurons and inhibitory γ-aminobutyric acid (GABA)ergic interneurons to drive changes in the functional activity of corticolimbic brain regions (Moghaddam, 1993; Lowy et al., 1995; Duvarci and Paré, 2007; Di et al., 2009; Yuen et al., 2009; Popoli et al., 2012; Wang et al., 2016). Initially adaptive, sustained activation of this response during chronic stress can overwhelm feedback mechanisms and induce neuroplastic structural and functional changes that may underlie disrupted mood and cognitive functions (Sousa and Almeida, 2012; Shirazi et al., 2015). Indeed, exposure

*Correspondence to: E. Sibille, CAMH, 250 College Street, Room 134, Toronto, ON M5T 1R8, Canada.
E-mail address: Etienne.Sibille@camh.ca (E. Sibille).

Abbreviations: ACC, anterior cingulate cortex; AUC, area under the curve; BLA/BMA/CeA, basolateral/basomedial/central amygdala; BNST, bed nucleus of stria terminalis; CPu, caudate putamen; CRS, chronic restraint stress; DG, dentate gyrus; Ent, entorhinal cortex; EPM, elevated plus-maze; FST, forced-swim test; GABA, γ-aminobutyric acid; GCs, glucocorticoids; HPA axis, hypothalamic-pituitary-adrenocortical axis; IEG, immediate early gene; IHC, immunohistochemistry; Ins, insular cortex; LA, locomotor activity; LH, lateral hypothalamus; LSd, lateral septum; M1/2, motor cortex; MDD, major depressive disorder; mPFC, prefrontal cortex–medial; NAc, nucleus accumbens; NIH, novelty-induced hypophagia; NORT, novel object recognition test; NSF, novelty-suppressed feeding; OFT, open field test; PAG, periaqueductal gray; PCA, principal components analysis; PRh, perirhinal cortex; PT, phenotyper test; PVN, paraventricular nucleus; SCT, sucrose consumption test; UCMS, unpredictable chronic mild stress; v/dHPC, hippocampus – ventral/dorsal; VMN, ventromedial nucleus; vSub, ventral subiculum; VTA, ventral tegmental area; WM, white matter; YM, Y-maze.

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to prolonged stressors is a major precipitating factor for psychiatric disorders, including major depressive disorder (MDD) and anxiety disorders (McEwen, 2004, 2005), neurodegenerative disorders such as Alzheimer's and Parkinson's diseases (Vyas et al., 2016), and substance abuse disorders (Schwabe et al., 2011). Further, regional activity alterations are associated with specific symptoms of stress-related disorders, including disturbed executive function in MDD (Girotti et al., 2018) and enhanced fear responses in anxiety disorders (Shin and Liberzon, 2010). However, it remains poorly understood how dysregulation of the adaptive stress response translates to altered functional activity and subsequently, how regional changes in functional activation contribute to the development of maladaptive behaviors.

Preclinical models using exposure to chronic stress are useful tools for investigating pathological mechanisms relevant to human disorders. Among these, chronic restraint stress (CRS) and unpredictable chronic mild stress (UCMS) models are most common due to their construct, predictive, and face validity for modeling cell- and circuit-level pathological changes together with behavioral alterations related to human symptoms (Mineur et al., 2006; Chiba et al., 2012; Hill et al., 2012; Nollet et al., 2013), that are reversed by pharmacological treatments. For example, rodents exposed to chronic stress display neuronal atrophy in the prefrontal cortex (PFC) and hippocampus (HPC), but hypertrophy in the amygdala, concurrent with anxiety- and depressive-like behavioral deficits (Magarin ̧os and McEwen, 1995; Wellman, 2001; Vyas et al., 2002, 2003; Cook and Wellman, 2004; Radley et al., 2004; Banasr and Duman, 2008; Zhang et al., 2015), and closely resembling findings in humans with mood disorders (Sheline et al., 1996; Khundakar et al., 2009; Kang et al., 2012). That synaptic changes are altered in a brain region-specific manner implies a putative imbalance in neuronal activity and function. Indeed, chronic stress negatively impacts both glutamatergic and GABAergic neurons (reviewed in Fee et al., 2017), supporting theories that sustained activation leads to altered excitation/inhibition balance in cell circuits. These changes are also bi-directional. For instance, PFC and HPC feedback mechanisms regulate initiation and termination of the HPA stress response (Sapolsky, 1985; Sullivan and Gratton, 2002). Therefore, functional deficits emerging from altered activity of corticolimbic brain regions likely represent both a cause and consequence of sustained challenge on the adaptive stress response (McEwen and Gianaros, 2011).

Neuroimaging studies validate structural and functional changes in corticolimbic brain regions of patients with stress-related disorders (Drevets et al., 2008a; Pitman et al., 2012). However, deriving insights about chronic stress is less straightforward due to variability introduced by patient characteristics (e.g., medication history, comorbid disorders, timing and severity of stress exposure) and experimental conditions (e.g., resting state vs. task-related activity). Thus, rodent studies using systematic activity mapping after acute and chronic stress have been useful to understand functional consequences of stress-related pathology. For example, functional magnetic resonance imaging studies in CRS mice identified dendritic remodeling of the PFC, HPC, and amygdala with altered functional connectivity across regions of the default mode network (Henckens et al., 2015), in which activity changes are linked to symptom emergence in MDD (Jacob et al., 2020), post-traumatic stress disorder (Nathan et al., 2017), Parkinson's disease (Lin et al., 2020), and substance abuse disorders (Zhang and Volkow, 2019). Immediate early gene (IEG) markers, such as c-Fos, similarly yield high spatiotemporal resolution for characterizing functional alterations induced by chronic stress. Stress-induced recruitment of brain region activity initiates c-Fos expression, which varies depending on the type of stress, severity, and duration of exposure (Mohammad et al., 2000; Figueiredo et al., 2003). Additionally, patterns of acute activation can become modulated by prior exposure to chronic stress, potentially reflecting functional disturbances. However, findings are mixed as some studies report sensitization of the acute stress response by prior chronic stress exposure, reflected by enhanced c-Fos levels in the anterior cingulate cortex (ACC), HPC, hypothalamus, and amygdala (Matsuda et al., 1996; Chung et al., 1999), while others report response habituation and c-Fos attenuation following acute stress in distinct and overlapping regions (Melia et al., 1994; Umemoto et al., 1994; Stamp and Herbert, 1999).

Here, we employed two mouse models of chronic stress exposure, longitudinal and end-point behavioral phenotyping, and comprehensive mapping of regional functional activation via c-Fos protein immunohistochemistry (IHC) following an acute stressor subsequent to chronic stress exposure. We sought to determine (a) whether chronic stress induced behavioral alterations along dimensions including physiological deficits, anxiety- and anhedonia-like behaviors, antidepressant-predictive behaviors, and memory impairments (b) whether region-specific responses to acute stress were dysregulated by prior chronic stress exposure and (c) whether regional activation changes were associated with behavioral deficits relevant to stress-related disorders. Control, CRS, and UCMS mice were used to avoid model-specific conclusions about the effects of chronic stress on behavioral and functional outcomes (Mohammad et al., 2000; Figueiredo et al., 2003; Willner, 2017a). Behavioral phenotyping assessed deficits along symptom-related dimensions, including physiological alterations, anxiety- and anhedonia-like behaviors, antidepressant-predictive behaviors, and memory impairments. Semi-quantitative c-Fos mapping was performed via IHC in 24 stress- and mood-related brain regions (plus motor cortex and white matter control regions) identified via *a priori* literature review (Drevets et al., 2008a; Tynan et al., 2010; Russo and Nestler, 2013; Bagot et al., 2015; Kim et al., 2016). Contributing relationships were investigated via correlational analyses between regional functional activation and unbiased parameters that captured variance across behavioral deficit dimensions. We hypothesized that prior chronic stress exposure alters acute stress-induced functional activation in corticolimbic brain regions, and that these changes

correlate with behavioral deficits. Our findings revealed that CRS and UCMS converged to exacerbate levels of functional activation by acute stress in ACC and vHPC subregions. These changes were positively correlated with stress-related behavioral dimensions, revealing a pathway by which the acute stress response becomes dysregulated and contributes to the expression of behavioral deficits.

EXPERIMENTAL PROCEDURES

Animals

Eight-week C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were kept under a 12/12 h light–dark cycle with food and water *ad libitum*. Group-housed mice underwent 2-week facility habituation before random assignment to control (daily handling), CRS, or UCMS groups (n = 12–14/group; 50% male/female). CRS/UCMS mice were single-housed at chronic stress initiation, whereas control mice were single-housed prior to end-point testing. All tests were performed in accordance with Canadian Council on Animal Care (CCAC) guidelines.

Chronic stress procedures

UCMS mice received 5 weeks of randomized mild stressors (2–4/day), and 3 weeks maintenance (1–2/day) during testing, based on validated methods (Nikolova et al., 2018). Stressors included: forced bath, wet bedding, predator odor, light cycle reversal/disruption, cage switching, tilted cage, reduced space, acute restraint, and enrichment removal. CRS mice received 5 weeks acute restraint (1 h; 2×/day), and 3 weeks maintenance (1×/day) during testing, based on validated methods (Prevot et al., 2019a).

Animal testing

Mice were tested in randomized order by experimenters blind to group assignments. Tests were performed during the light cycle, except for the overnight PhenoTyper Test (PT), on non-consecutive days and measured *physiological changes* i.e., coat state, weight gain, *anxiety-like behavior* i.e., PT, Elevated Plus-maze (EPM), Open Field Test (OFT), and Novelty-Suppressed Feeding (NSF), *anhedonia-like behavior* i.e., Sucrose Consumption Test (SCT), or *both* i.e., Novelty-Induced Hypophagia test (NIH) (Supplementary Fig. 1). Mice were also assessed on the *antidepressant-predictive* Forced Swim Test (FST), and for *memory impairment* i.e., *working memory* via Y-maze (YM) and *short-term memory* via Novel Object Recognition Test (NORT). To characterize the induction of stress-related deficits, *Longitudinal tests* (i.e., repeatable assessments not relying on novelty (Prevot et al., 2019b)) for weight, coat state, PT, and SCT were administered weekly, 6× from week 0 (before stress) to week 5 (5th week of chronic stress). To characterize the full extent of chronic stress-induced deficits, *End-point* tests included all tests mentioned previously, performed on non-consecutive days from weeks 6–8 (after 5+ weeks chronic stress).

Physiological tests

Animals' coat state quality was scored across 7 anatomical areas 0, 0.5, or 1, from maintained to unkempt, and a sum was calculated (Isingrini et al., 2010). Weight was tracked weekly and expressed as percent change (%) to measure deviation from normal development. LA was assessed for 25 min in a home cage-like environment with an overhead camera and tracked offline using ANY-maze software (Stoelting Co., Wood Dale, IL).

Anxiety-like behavior tests

Tests included the PT, a test developed by our lab to assess anxiogenic response to a light challenge in a home cage-like environment (Prevot et al., 2019b). Time spent in a PhenoTyper apparatus (Noldus, Leesburg, VA), with designated food (6.5 × 15 cm) and shelter (10 × 10 cm) zones was tracked during the dark cycle (7pm–7am). At 11 pm, an aversive spotlight was applied over the food zone for 1 h. All mice spend more time hiding in the shelter during this challenge, however, chronic stress-exposed mice show a protracted return to normal exploration after the challenge ends, reflected by increased shelter zone time and decreased food zone time for up to 10 h (Prevot et al., 2019b). Anxiogenic shelter/food zone response was represented by an area under the curve (AUC): the summed average of 1 h responses from the start of light challenge to end of the test (∼8 h). In the EPM, mice were recorded for 10 min exploring an elevated (55 cm) cross-shaped maze with two open arms (27 × 5 cm) and two partially-enclosed arms (27 × 5 × 15 cm), in a dimly lit room. ANY-maze software was used to measure time spent (s) and percentage of distance travelled (%, cm) in open and closed arms, excluding the neutral middle zone. In the OFT, mice were recorded for 10 min exploring an open arena (70 × 70 × 33 cm) in a dimly lit room. ANY-maze software delineated 3 concentric squares of equal area and tracked time spent (s) in the innermost center zone (40 × 40 cm) and outermost (70 × 70cm perimeter zone. In the NSF, food-deprived (16 h) mice were timed for latency to approach and feed on a food pellet (s) in a brightly lit arena (62 × 31 × 48 cm). Afterwards, home cage approach and feed latency was tracked to assess potential bias due to appetitive drive (Samuels and Hen, 2011).

Anhedonia-like behavior tests

Tests included NIH, involving two days reward habituation (1 mL of sweetened condensed milk), followed by measuring home cage reward approach latency on the third day as a measure of reward drive. On the fourth day, reward approach latency was measured in a novel brightly-lit cage, by an experimenter. The novel environment contributes to NIH being considered as an index of both anxiety- and anhedonia-like behaviors (Dulawa and Hen, 2005). In the SCT, home cage water bottles were replaced with sucrose solution (1%; Sigma, St. Louis, MO) for 48 h. Mice were then fluid-deprived overnight (∼14 h), and sucrose intake measured for 1 h. 2 days later, this process was repeated for water. Results

were interpreted as a sucrose preference percentage (sucrose consumed/sucrose + water consumed, %), to control for potential bias due to fluid intake.

Antidepressant-predictive behavior tests

Tests included the FST, a swim test that assesses chronic stress-induced increased immobility that is reversed by antidepressant treatment (Slattery and Cryan, 2012). Mice underwent a 6 min swim trial in a glass beaker (25 × 16 cm) of room temperature water and were then dried and warmed by a heat lamp. Trials were recorded and assessed offline for total immobility time, scored manually by an experimenter, blind to group assignments.

Memory tests

Tests included the YM, a black plastic Y-maze with 3 arms (26 × 8 × 13 cm) separated by 120° and gated by sliding doors. YM leverages the exploratory nature of rodents to alternate between 2 goal arms, across 7 successive trials (Olton, 1979). Mice were habituated to the apparatus and distal cues for 10 min sessions on 2 consecutive days. On day 3, mice underwent 7-trials to assess goal arm alternation with a non-challenging 30 s inter-trial interval (ITI). On day 4, alternation was assessed across 7-trials with a 90 s ITI, simulating increased cognitive load. An 8th trial with 5 s ITI was added to assess loss of motivation; mice failing this were excluded. Correct alternation percentage reflected an index of working memory performance (50% = random). The NORT leverages rodent's preference for novelty to assess short-term memory (recognition/recall) (Antunes and Biala, 2012). Mice were habituated to an empty open field (70 × 70 × 33 cm) and then to the field with 2 identical plastic objects ("familiar") over 10 min trials on consecutive days. On day 3, mice underwent a familiar pre-trial: tracking time spent with 2 identical familiar objects and then 3 h later were tested for recall in the trial: switching one familiar object for a novel object. A discrimination ratio measured time with left familiar object/time with both (pre-trial) or time with novel object/time with both (trial).

Immunohistochemistry

Forty-eight hours after end-point testing, mice received an acute stressor (5 min 18 °C cold swim) to stimulate c-Fos expression. Ninety minutes later, mice were deeply anesthetized with avertin (125 mg/kg, i.p.) and transcardially perfused with 50 mL 0.1 M phosphate-buffered saline (PBS, pH 7.4), followed by 75 mL 4% paraformaldehyde (PFA) in PBS. Extracted brains were post-fixed in PFA for 24 h at 4 °C, cryoprotected in 30% sucrose in PBS for 2 days, then frozen on dry ice and cryosectioned at 40 μm. Ten sets of full brain sections were collected yielding ~15 sections/set/mouse processed for IHC.

Functional activation was quantified via c-Fos protein immunolabeling (Kovács, 1998). Free-floating sections were rinsed in PBS, pretreated with 3% H_2O_2 for 20 min, and permeabilized in 0.3% Triton X-100 PBS for 20 min. Sections were again rinsed in 0.3% Triton-PBS before applying 10% normal goat serum (NGS) in 0.3% Triton-PBS as blocking agent for 30 min and incubated in primary antibody (rabbit anti-c-Fos 226003, 1:200 in 3% NGS/0.3% PBS-Triton; Synaptic Systems, Germany) for 72 h at 4 °C. Sections were rinsed with PBS and incubated with secondary antibody (biotinylated goat anti-rabbit IgG BA-1000, 1:500 in 0.3% Triton-PBS; Vector Laboratories, Burlingame, CA) for 2 h at room temperature. Reactions were enhanced with Vectastain ABC/HRP kit (Vector Laboratories) for 2 h, then rinsed and detected with 0.025% 3,3′-diaminobenzidine (DAB) and 0.025% of H_2O_2 in 0.5 M Tris-buffered saline (pH 7.4). After rinsing, sections were mounted, dehydrated, and coverslipped using DEPEX medium.

Microscopy

Quantitative analysis of c-Fos+ cell counts was performed using ZEN 2 software on scans acquired with a Zeiss AxioScanZ1 slide scanner (ZEISS Microscopy, Oberkochen, Germany). Regions were delineated at low magnification (2×) according to the Franklin and Paxinos Mouse Brain Atlas (Franklin and Paxinos, 2008). Cell counts were acquired in a semi-automated manner across full resolution 20× images using ZEN 2 with consistent thresholds for nuclei size, roundness, signal intensity, and segmentation. Cell counts for each region were obtained bilaterally across 2–3 evenly spaced (~400 μm) sections and expressed as mean cell density count (per 10,000 μm^2) per mouse per region, based on previous studies (Wheeler et al., 2013). As a summary measure, regional cell density counts were produced by averaging across subregions, where > 2 subregions were quantified (i.e., PFC, amygdala, dHPC and vHPC, and brainwide).

Statistical analysis

Data are visualized using box-and-whisker plots showing the median, lower and upper quartile ranges. Data were analyzed using SPSS (IBM, Armonk, NY). Behavioral parameters and cell counts were analyzed using one-way analysis of covariance (ANCOVA) with group as independent variable and sex as covariate, followed by Scheffe *post hoc* analysis. Time-course parameters from the coat state, weight gain, SCT, PT, and LA assessments were assessed by repeated measures ANCOVA for within- and between-subject comparisons using Greenhouse-Geisser correction for parameters failing sphericity assumptions. To address variability associated with classical rodent behavior tests (Carola et al., 2002; Guilloux et al., 2011), we employed principal components analysis (PCA) to generate summary scores reflecting overall depressive-like behavioral deficits, based on previous methods (Nikolova et al., 2018; Prevot et al., 2019b). Twenty-six parameters measuring baseline (i.e., week 0 or week 6–9 pre-trial readouts) and end-point (i.e., weeks 6–8 trial readouts) behaviors were Z-normalized (Guilloux et al., 2011) and loaded into PCA with varimax rotation, regarding mice as one experimental unit. For interpretation, parameter loadings onto principal components (PCs) were examined, wherein

those > 0.3 (absolute value) were considered significant based-on critical *r* values corresponding to $p < .05$ for the current sample size. Pearson's *r* was used to assess relationships between cell density and behavioral PCs.

RESULTS

Repeatable tests revealed progressive physiological and behavioral deficits during 5 weeks of chronic stress

Mice were assessed weekly for weight gain, fur coat deterioration, SCT, and PT from weeks 0 to 5, including 1-week baseline and 5-weeks chronic stress (Fig. 1). Repeated measures ANCOVA of weight gain revealed a significant main effect of group ($F_{2,36} = 19.92$; $p < .001$), time ($F_{3.8,134.19} = 3.99$; $p < .01$), and group*time interaction ($F_{7.6,332.73} = 4.95$; $p < .001$) (Fig. 1A). *Post hoc* analysis revealed that CRS altered weight gain relative to controls ($p < .01$), with significantly decreased change in weight gain observed the 1st, 4th, and 5th week of stress. ANCOVA revealed significant sex differences ($p < .01$) wherein females gained less weight across groups (Supplementary Table 1). Analysis of coat state scores showed a significant main effect of group ($F_{2,36} = 16.34$; $p < .001$), time ($F_{2.94,105.83} = 78.96$; $p < .001$), and a group*time interaction ($F_{5.88,105.83} = 9.49$; $p < .001$) (Fig. 1B). *Post hoc* analysis showed that CRS and UCMS exposure increased coat state deterioration from weeks 1 to 5 (*ps* < 0.01). ANCOVA revealed significant sex differences, wherein deterioration was more extensive in males ($p < .001$; Supplementary Table 1).

Analyses of week 0 to 5 sucrose preference ratio in the SCT revealed a significant main effect of group ($F_{2,34} = 10.02$; $p < .001$), time ($F_{5,175} = 4.98$; $p < .001$), and group*time interaction ($F_{10,175} = 2.34$; $p < .05$) (Fig. 1C). *Post hoc* comparisons revealed trend-level differences for CRS and UCMS mice vs. control mice (*ps* = 0.08) and a significant decrease in CRS vs. UCMS mice ($p < .001$). However, only trend-level differences were found at individual time points, including decreased sucrose preference for UCMS vs. CRS and control mice week 2 (*ps* = 0.6–0.7), and increased sucrose preference for CRS vs. control mice week 3 ($p = .052$). No main effect or interaction with sex was found.

PT was used to assess shelter zone activity before, during, and after a 1 h light challenge from week 0 to 5 (Fig. 1D–F). In week 0 (prior to stress), repeated measures ANCOVA revealed a significant effect of time ($F_{4.81,173} = 48.09$; $p < .001$) on shelter zone time, but no effect of group or their interaction (*ps* > 0.4) (Fig. 1D). In week 1, main effects for time ($F_{6.63,228.86} = 59.7$; $p < .001$), group ($F_{2,36} = 22.49$ $p < .001$), and group*time interaction ($F_{12.71,228.86} = 3.92$; $p < .001$) were found for shelter zone time (Fig. 1E). *Post hoc* analysis revealed significantly increased shelter zone time for CRS and UCMS mice relative to controls for each of the 4 hours following the light challenge (*ps* < 0.05). Shelter Zone AUCs were calculated to summarize anxiogenic light responses from week 0 to 5 PTs (Fig. 1F). Repeated measures ANCOVA of AUC data revealed significant main effects of time ($F_{2.65,95.39} = 57.09$; $p < .001$), group ($F_{2,36} = 43.11$; $p < .001$), and group*time interaction ($F_{5.3,228.86} = 7.65$; $p < .001$). Relative to control mice, CRS and UCMS mice spent more time in the shelter zone following the light challenge from weeks 1 to 5 (*ps* < 0.01). No main effect or interaction with sex was found.

End-point physiological measurements included final assessments of coat state and weight gain, plus LA (Fig. 2A–C). One-way ANCOVA revealed significant group effects on coat state ($F_{2,33} = 61.67$; $p < .001$) and weight gain ($F_{2,33} = 6.07$; $p < .01$). CRS and UCMS significantly induced coat state deterioration ($p < .001$) (Fig. 2A) in males and females. A sex ($F_{1,33} = 6.07$; $p < .001$) and sex*group interaction ($F_{2,33} = 2.95$; $p < .05$) was found wherein females overall and CRS/UCMS-exposed females relative to males had less deterioration (Supplementary Table 1). For % weight gain, significant effects of group ($F_{2,33} = 28.8$; $p < .001$), sex ($F_{1,33} = 103.53$; $p < .001$), and group*sex ($F_{2,33} = 4.36$; $p < .05$) were found showing that, relative to controls, weight gain was less extensive in CRS males and females ($p < .001$), but only UCMS males ($p < .001$) (Fig. 2B; sex effects Supplementary Table 1). Total distance travelled in the LA test was not significantly affected by group, sex, or their interaction (Fig. 2C).

End-point anxiety-like behavior was assessed in the PT, EPM, OFT, and NSF. In the PT, group assignment had a significant effect on shelter ($F_{2,33} = 18.56$; $p < .001$) and food zone AUCs ($F_{2,33} = 11.51$; $p < .001$) (Fig. 2D, E). The light challenge significantly increased shelter zone time and decreased food zone time in CRS and UCMS mice relative to controls (*ps* < 0.001), suggesting persistent avoidance of the previously lit zone and reflecting a protracted anxiogenic response. A significant sex effect was found for shelter zone AUC ($F_{1,33} = 7.84$; $p < .01$), but *post hoc* analysis revealed only a trend-level increase for males ($p = .064$; not shown). In the EPM, group assignment significantly altered time spent in closed arms ($F_{2,35} = 7.1$; $p < .01$), without changes in time spent (not shown) or % of distance travelled in open arms (Fig. 2F, G). Closed arm time was significantly elevated in CRS mice relative to controls ($p < .01$). In the OFT, center zone and outermost zone time were not affected by chronic stress (Fig. 2H, I). In the NSF, we found a main effect of group on latency to approach ($F_{2,36} = 4.87$; $p < .05$) and feed ($F_{2,36} = 3.66$; $p < .05$) on a food pellet in the novel arena (Fig. 2J, K). UCMS increased latency to approach relative to CRS and control mice (*ps* < 0.05) and latency to feed relative to control mice only ($p < .05$). Home cage latencies were unchanged, reflecting no bias due to appetitive drive (L-M). Sex was not a significant covariate in the EPM, OFT, or NSF.

End-point anhedonia-like behavior were assessed using reward approach and consumption in NIH and SCT. In the NIH, chronic stress did not significantly

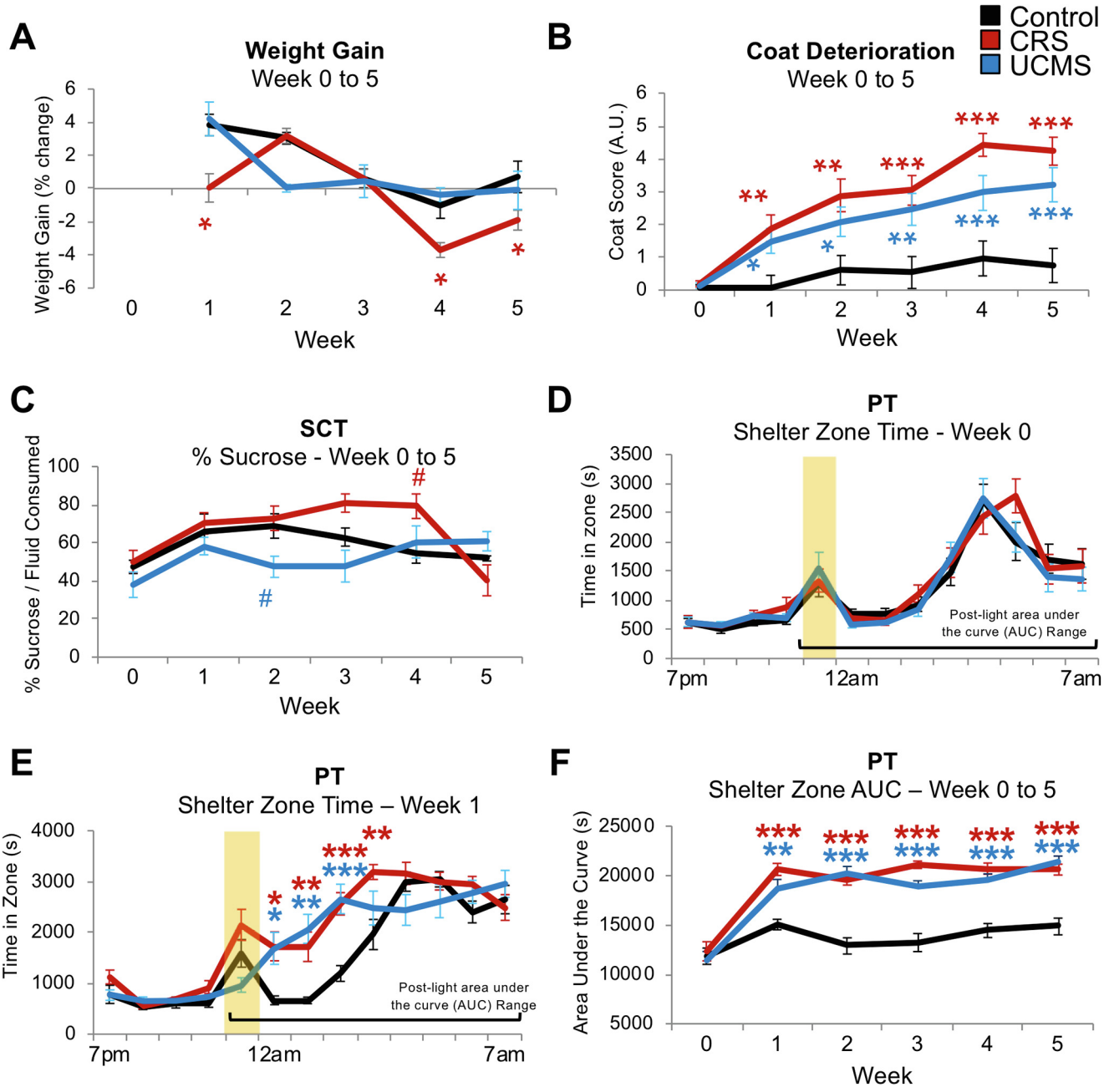


Fig 1. Longitudinal analysis using weekly repeatable physiological and behavior tests to confirm induction of stress-related deficits in mice assigned to control (daily handling), CRS, or UCMS conditions (n = 12–14/group; 50% male/female). **(A)** Percentage weight gain. **(B)** Fur coat state deterioration scores (AU = arbitrary units). **(C)** Percent sucrose of total volume consumed in the sucrose consumption test (SCT). **(D)** Time spent in the shelter zone of the PhenoTyper Test (PT) in week 0 prior to chronic stress. **(E)** Time spent in the shelter zone of the PT after 1 week of chronic stress. **(F)** Area under the curve (AUC) for shelter zone time in PT from the beginning of the light challenge (yellow vertical bars) until the end of the test. ***p < .001; **p < .01; *p < .05; $^{\#}p$ < .1 for CRS (gray symbols) or UCMS (black symbols) vs. controls. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

affect latency to drink milk in novel or home cage tests (Fig. 2N, O). Sex was a significant covariate ($F_{2,33}$ = 5.91; p < .05) as females had increased latency to drink in a novel environment relative to male mice (p < .05; Supplementary table 1). In the SCT, sucrose preference ratio was significantly affected by group ($F_{2,36}$ = 3.42; p < .05), but not sex. CRS mice had decreased consumption relative to UCMS mice (p < .05) (Fig. 2P).

End-point measures of working and short-term memory were assessed in the YM and NORT, respectively. In YM, one-way ANCOVA revealed significant effects of stress on alternation rate ($F_{2,36}$ = 4.48; p < .05) (Fig. 2R). Specifically, under cognitive load of 90 s ITI, UCMS mice alternated arm choice less than controls (p < .05), suggesting a working memory deficit. In the NORT, pre-trial discrimination ratio (measuring time spent with 1 of 2

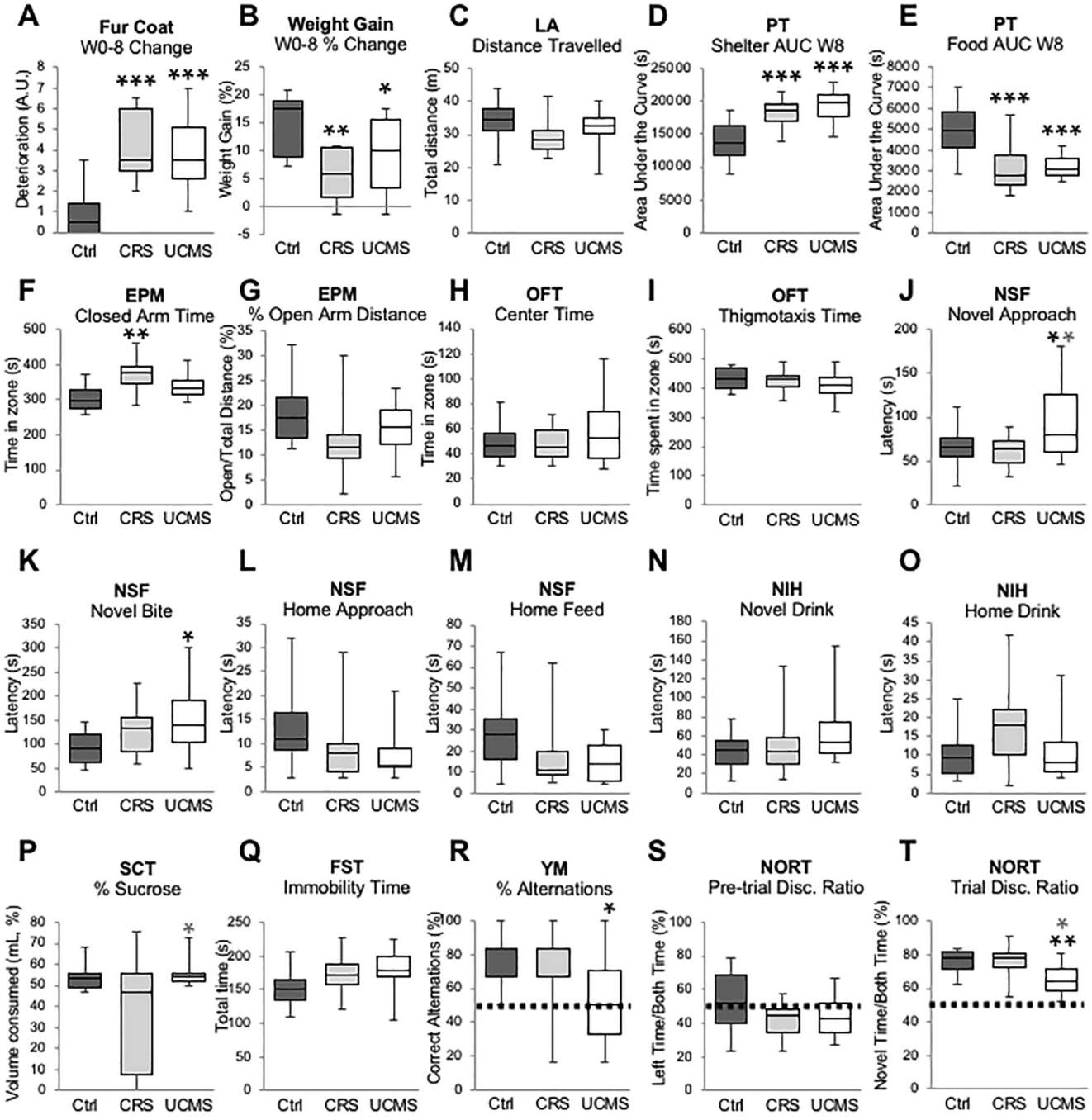


Fig 2. End-point analysis using multiple behavior tests to characterize induction of deficits along physiological **(A–C)**, anxiety-like **(D–M)**, anhedonia-like **(N–P)**, antidepressant-predictive **(Q)**, and memory impairment **(R–T)** dimensions in control, CRS, and UCMS mice (n = 12–14/group; 50% male/female). **(A)** Fur coat change from week 0 to 8. **(B)** Percent weight gain from week 0 to 8. **(C)** Distance travelled in 25 min locomotor activity (LA) test. **(D)** Area under the curve (AUC) of shelter zone time. **(E)** Shelter zone and **(F)** food zone time area under the curve (AUC) in PhenoTyper Test (PT) from the beginning of the light challenge until the end of the test. **(F)** Time spent in closed arms and **(G)** % of distance travelled in open arms in elevated-plus maze (EPM). **(H)** Time spent in the center and **(I)** thigmotaxis zones in open-field test (OFT). **(J)** Time to approach and **(K)** feed on a food pellet in novel environment, and **(L)** approach and **(K)** feed on a pellet in homecage, in the novelty-suppressed feeding test (NSF). **(N)** Latency to drink in a novel and **(O)** homecage environment in the novelty-induced hypophagia test (NIH). **(P)** Percent sucrose of total volume consumed in the sucrose consumption test (SCT). **(Q)** Total time immobile in the forced-swim test (FST). **(R)** Percent correct alternations from 7-trials in the Y-maze (YM). **(S)** Discrimination ratio for pre-trial with identical objects and **(T)** trial with familiar/novel objects in the novel object recognition test (NORT). ***p < .001; **p < .01; *p < .05 for indicated group vs. control (black) or CRS (gray).

identical objects) was not significantly affected by stress (p > .12; Fig. 2S), indicating that no arena side preference existed. Short-term recall was assessed 3 h later by trial discrimination ratio (measuring novel object/novel + familiar object time) indicating a significant group effect for object recall ($F_{2,36}$ = 7.13; p < .01). UCMS significantly decreased discrimination ratio compared to control and CRS mice (ps < 0.05) (Fig. 2T). Sex was not a significant covariate in the YM or NORT.

Principal components analysis reveals 2 components associated with stress-induced variance across behavioral tests

As an alternative approach to assessing individual behaviors, we used dimension reduction via PCA to generate parameters capturing behavioral variance across tests. PCA was conducted on 26 z-normalized parameters (including baseline and end-point readouts) revealing a 5-component solution accounting for 56.7% of the total variance (Supplementary Fig. 2). One-way ANCOVA revealed a significant main effect of group on the top two components: PC1 ($F_{2,36}$ = 15.2; p < .001, 16.43% of variance) and PC2 ($F_{2,36}$ = 3.29; p < .05, 14.27% of variance) (Fig. 3A, B), with no effect of sex or their interaction. Relative to control mice, CRS and UCMS mice had significantly higher PC1 scores (p < .001), whereas only CRS mice had significantly higher PC2 scores (p < .05). Strong loadings were found for parameters of physiological and anxiety-like deficits for both PC1 (Fig. 3C) and PC2 (Fig. 3D), indicating that these components captured emotional reactivity across behavioral dimensions and groups. However, qualitative inspection revealed that PC1 loaded stronger on physiological, antidepressant-predictive, and memory deficits, whereas PC2 loaded mainly on anxiety-like deficits and negatively loaded on memory deficits, consistent with reflecting variance solely from CRS mice (Fig. 3A, B). PC3-5 were independent of group and/or sex effects, reflecting variance from other unrelated sources.

Chronic stress exacerbates the effect of acute stress on c-Fos+ cell density in prefrontal cortex and ventral hippocampal subregions

We next assessed the impact of prior chronic stress exposure on levels of c-Fos+ cell density induced by acute stress. We first established the ability for acute stress to induce c-Fos activation via analysis of c-Fos+ cell density in 26 brain regions, including 24 stress- and mood-related brain regions, 2 control regions (motor cortex and white matter), plus 5 summary measures (total PFC, Amygdala, dHPC, vHPC, and brainwide) in a separate cohort of naïve adult C57BL/6J mice following an acute swim stress or normal home cage environment (n = 6/group; 50% male/female). Acute stress induced significant elevations in c-Fos+ cell density in 15/26 regions examined, including the PFC (areas 24a, 24b, and 32), basomedial amygdala, dorsal HPC (CA1), ventral HPC (CA1, CA3, subiculum), nucleus accumbens, lateral septum, bed nucleus of stria terminalis, lateral hypothalamus, periaqueductal gray, entorhinal cortex, and motor cortex (ps < 0.05), but not the basolateral amygdala, dorsal dentate gyrus, caudate putamen, ventromedial nucleus, ventral tegmental area, insular cortex, perirhinal cortex, or white matter (Supplementary Fig. 3). Acute stress induced trend-level elevations in c-Fos+ cell density in the central amygdala and the dorsal HPC (CA2, CA3) (p < .1).

To test whether chronic stress alters the induction of c-Fos activation, control, CRS, and UCMS mice from behavioral experiments were then subjected to the same acute swim stressor and brains were extracted and processed for IHC (Fig. 4A). Significant main effects of group on c-Fos+ cell density were observed only in area 24b of the PFC ($F_{2,36}$ = 4.11; p < .05), overall ventral hippocampus (vHPC; $F_{2,36}$ = 6.47; p < .01) and specifically vHPC CA1 ($F_{2,36}$ = 4.92; p < .01), CA3 ($F_{2,36}$ = 5.97; p < .01) or subiculum (vSub; $F_{2,36}$ = 3.75; p < .05) (Fig. 4B, I). *Post hoc* analyses revealed significant elevations in acute stress-induced functional activity among CRS and UCMS mice relative to controls in A24b (Fig. 4A), vCA1, and vCA3 (Fig. 4D), and in CRS mice relative to controls in vSub and overall vHPC (ps < 0.05). No main effect or interaction with sex was found.

These results demonstrate that functional activation induced by an acute swim stressor is exacerbated by prior chronic stress exposure in the PFC and vHPC, when compared to acute swim stress-induced functional activation in all other tested brain regions tested.

Levels of functional activation in prefrontal A24b and vHPC subregions positively correlate with stress-related behavioral components

We next sought to investigate associations between behavioral component and functional activation via correlational analyses of cross-dimensional principal components and z-normalized c-Fos+ cell densities in subregions affected by chronic stress (Fig. 5A, B). These analyses revealed significant positive correlations between PC1 and c-Fos levels in A24b (r = 0.329, p = .041) and vCA3 (r = 0.506, p = .007) (Fig. 5A), as well as PC2 and c-Fos levels in A24b (r = 0.361, p = .024), vCA1 (r = 0.588, p < .001), and vSub (r = 0.447, p = .032) (Fig. 5B). For all comparisons, mice with higher levels of regional c-Fos activation had greater PC1 or PC2 scores, reflecting increased behavioral emotionality. Sex was not a significant covariate in these analyses.

DISCUSSION

In this report, we characterized the effect of prior chronic stress exposure on brain region-specific functional activation induced by acute stress and investigated associations with behavioral deficits relevant to symptoms of human stress-related disorders. We first demonstrated that two prominent chronic stress models, CRS and UCMS, lead to convergent progressive outcomes over 8 weeks of exposure in male and female mice. This included physiological and anxiety-like deficits in both groups, and memory impairment only in UCMS mice. To capture common elements underlying behavioral variance across tests, we employed dimension reduction via PCA of all baseline and end-point readouts. This analysis revealed that the top two principal components, explaining 30.7% of total variance, were strongly associated with stress-related outcomes, including physiological, anxiety-like, antidepressant-predictive, and memory deficits (based

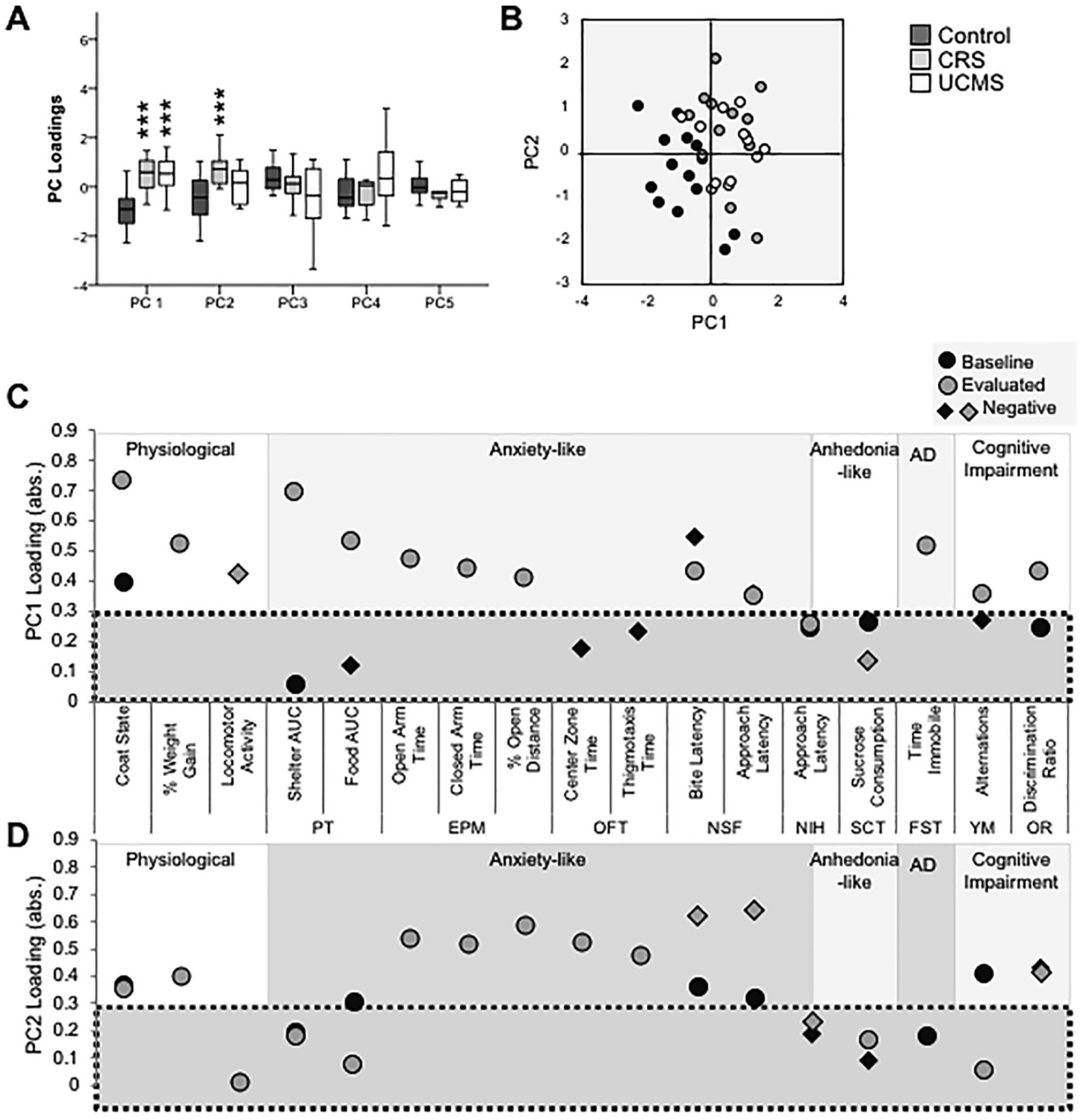
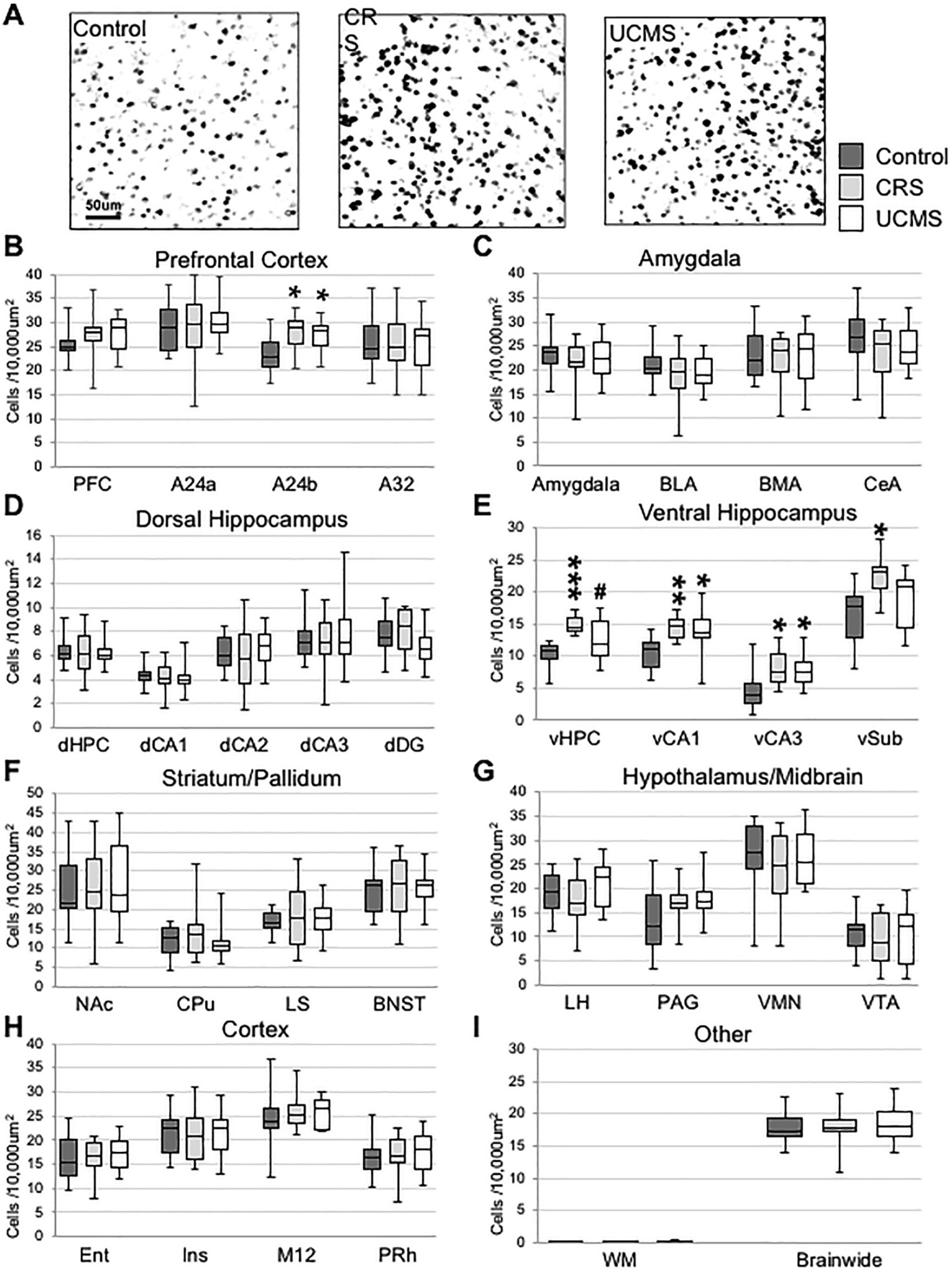


Fig 3. Dimension reduction via principal component (PC) analysis of behavioral data from mice at baseline (week 0 or pre-trial conditions) or evaluated (weeks 6–8 trial conditions) states. **(A)** PC loadings by group indicating captured behavioral variance related to chronic stress for CRS and UCMS mice for PC1 and CRS mice for PC2. Absolute values are reported; diamonds = negative loadings. **(C)** Visualization of clustering by groups and PC1/2. **(D)** PC1 and **(E)** PC2 loadings for individual tests demonstrating that both components captured variance across multiple symptom-like behavioral dimensions. ***$p < .001$; **$p < .01$; *$p < .05$ for indicated group vs. controls.

on parameter loadings). We then performed c-Fos mapping to characterize the functional consequences of chronic stress on the acute stress-mediated recruitment of 26 brain regions previously associated with stress responses. This analysis revealed that functional activation by acute stress was similarly exacerbated by CRS or UCMS in 4 out of 24 stress- and mood-related brain subregions investigated: the PFC A24b (ACC) and the ventral hippocampus CA1, CA3, and subiculum. Moreover, in regions dysregulated by chronic stress, levels of functional activation were positively correlated with stress-related principal components, suggesting that chronic stress-induced functional alterations in the recruitment of ACC or vHPC subregions by acute stress co-occur with or contribute to stress-related maladaptive behaviors.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Ventral Hippocampus
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vCA1
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vSub
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CPu
LS
BNST
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Hypothalamus/Midbrain
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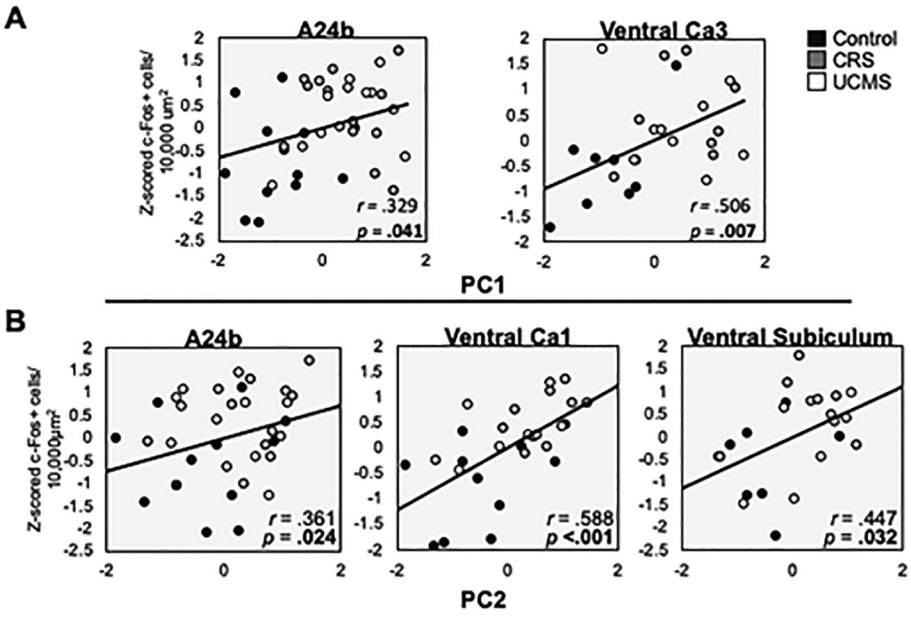

Fig 5. Correlational analysis of acute stress-mediated c-Fos+ cell density (*z*-scored) in regions dysregulated by chronic stress (A24b, vCA1, vCA3, vSub) and principal components (PC) of trans-dimensional stress-related behavioral alterations. **(A)** PC1 and **(B)** PC2 correlations with c-Fos+ cell density among control (black), CRS (gray), and UCMS (white) mice. Associations between PC1 and vCA1/vSub or PC2 and vCA3 were not significant (not shown).

As an advantage of our approach, we replicated findings of altered functional activation and maladaptive behaviors in parallel among two cohorts exposed to distinct stressor paradigms (i.e., CRS and UCMS), addressing key challenges in reliability and replicability associated with rodent chronic stress models and improving the likelihood that these results are meaningfully related to chronic stress-induced biological changes. Further, we combined a comprehensive phenotyping approach, using multiple tests assessing human symptom-like dimensions, with a dimension reduction technique to capture the full extent of behavioral variance attributable to chronic stress. This "bottom-up" PCA approach relies upon variance in behavioral data in an unbiased manner, and is well-suited for the current study to control for power bias introduced by an uneven number of tests loading onto individual parameters, which could skew correlational analyses, e.g., relative to "top-down" methods that rely on assigning behavioral outputs to pre-determined categories (e.g., "anxiety-like behavior"), such as with z-scoring (Guilloux et al., 2011). We also included males and females in the current study based on evidence for a strong moderating role of sex in chronic stress processes (Cahill, 2006; Garrett and Wellman, 2009). However, biological outcomes were consistent across sexes and behavioral outcomes did not vastly differ in covariate analyses as, e.g., weight gain alterations were more severe in females week-to-week, but in males overall from week 0 to 8. Thus, we did not find divergent effects of chronic stress reported by others, for instance where males and females show distinct depressive-like behaviors when tested during the light cycle (Huynh et al., 2011), but cannot preclude this possibility due to the moderate sample size employed (6–7 males/females per group).

In this study, we confirmed that acute stress increases functional activation measured via c-Fos+ cell density across 18/24 stress- and mood-related brain regions examined, using an acute swim stressor. The rapid induction and short half-life of c-Fos make it an ideal marker to characterize functional changes in the acute stress response (Matsuda et al., 1996; Kovács, 1998). Numerous reports demonstrate that acute stress recruits regional activation via c-Fos expression patterns that overlap with our findings (Senba et al., 1994; Senba and Ueyama, 1997; Bubser and Deutch, 1999; Silva et al., 2012). We found that prior chronic stress exposure exacerbated the effect of acute stress on functional activation of ACC and vHPC subregions. While this finding is interesting and relevant to reported dysregulations of these two brain regions in stress-related disorders (Phan et al., 2002; Quirk and Beer, 2006; Etkin et al., 2011; Tannenholz et al., 2014), past studies using prior exposure to chronic social or restraint stress reported contrasting attenuation of acute stress-induced c-Fos activation (Melia et al., 1994; Umemoto et al., 1994; Stamp and Herbert, 1999; Mohammad et al., 2000). This discrepancy may be attributable to experimental design, as past studies used chronic stress protocols that were shorter (5–10 days), known to produce heterogeneous or resilient subgroups (e.g. social

Fig 4. Functional activation quantified regionally via absolute density counts for c-Fos+ cells 90 min after exposure to an acute stressor in control, CRS, and UCMS mice (n = 12–14/group; 50% male/female). **(A)** Representative qualitative images of c-Fos staining in A24b (8-bit, thresholded). **(B)** prefrontal cortex (PFC) as an average index of A24a, A24b, and A32. **(C)** Amygdala as an average index of basolateral (BLA), basomedial (BMA), and central (CeA) parts. **(D)** Dorsal hippocampus (dHPC) as an average index of CA1, CA2, CA3, and dentate gyrus (DG). **(E)** Ventral hippocampus (vHPC) as an average index of CA1, CA3, and subiculum (sub). **(F)** Striatum/pallidum groups comprising nucleus accumbens (NAc), caudate putamen (Cpu), lateral septum (LSd), and bed nucleus of stria terminalis (BNST). **(G)** Hypothalamus/midbrain groups comprising lateral hypothalamus (LH), periaqueductal gray (PAG), ventromedial nucleus (VMN), and ventral tegmental area (VTA). **(H)** Other cortical regions including entorhinal (Ent), insular (Ins), motor (M1/2), and perirhinal (PRh) cortex. **(I)** Control and overall regions: white matter (WM) and brain-wide (average of all regions). ***p < .001; **p < .01; *p < .05 for indicated group vs. controls (black).

defeat) (Krishnan et al., 2007), or that lacked validation with behavioral tests. Further, compared to our approach using a distinct acute stressor (i.e., cold swim vs. CRS/UCMS), past studies investigated response to the same stressor for acute and chronic phases, likely contributing to habituation (Mohammad et al., 2000; Figueiredo et al., 2003). For example, one study employing prior chronic social defeat stress before acute restraint stress revealed enhanced central amygdala activation, together with increased anxiety-like behavior (Chung et al., 1999). However, we did not confirm this finding in the central amygdala, possibly due to lack of significant c-Fos induction by acute swim stress in this region, precluding insights about alterations in control vs. chronic stress-exposed mice. Consistent with our findings, another study found that c-Fos activation following chronic social defeat stress persisted at 24 hours in only 5/60 regions that were sensitive to activation by acute defeat, including the ACC and HPC (Matsuda et al., 1996).

Although we confirmed enhanced activation of the ACC and vHPC, we did not fully replicate findings from studies using long-lasting IEG markers that demonstrate sustained activation by chronic stress in HPA-sensitive regions, such as the amygdala, PAG, and BNST (Perrotti et al., 2004; Flak et al., 2012; Laine et al., 2017). These differences likely reflect our approach to measure functional response to acute stress vs. changes in baseline activity levels, but may have also been influenced by the type or intensity of stressors applied. Specifically, control vs. CRS/UCMS differences could have been masked by a ‘‘ceiling effect” on c-Fos activation due to the severity of stressor used, or else it may have been difficult to distinguish control mice due to repeated behavioral testing under stressful conditions. It is also important to mention that c-Fos expression is a proxy of neuronal activation and does not rule out the functional recruitment of brain regions through other mediators (Ericsson et al., 1994). Finally, regional activity levels do not fully capture the complexity of circuit-level changes, wherein for instance, others showed that mPFC-vHPC activity becomes more tightly coupled in anxiogenic environments (Adhikari et al., 2010).

Combining our findings in stress-naïve and chronic stress-exposed mice, we demonstrated that activation of the ACC and vHPC is recruited by acute stress, and that this effect is exacerbated by prior exposure to chronic stress. Parallel changes after chronic stress may reflect the fact that reciprocal connections between the ACC/vHPC (extending also to the amygdala, NAc, and BNST) exert important regulatory control of HPA-mediated stress responses (Bannerman et al., 2004). Indeed, lesion studies targeting the PFC/ACC (Diorio et al., 1993; Radley et al., 2006) or vHPC/vSub (Jankord and Herman, 2008) demonstrated enhanced endocrine response to stress associated with greater c-Fos activation in GC-secreting structures. These findings are consistent with neuroimaging studies in healthy controls showing that smaller ACC volume was associated with enhanced HPA responses (MacLullich et al., 2006). Similarly, HPC atrophy was associated with concomitant enhanced HPA responses and minor depressive symptoms in a study of AD patients (O’Brien et al., 1996). Taken together, it seems that whereas acute recruitment of these brain regions is necessary for HPA regulatory feedback, chronic activation may negatively impact regulatory processes, leading to runaway downstream activity that support the transition from adaptive to pathological stress reactivity. Taken together, these findings provide strong justification for future studies to investigate functional changes using multiple indicators of neuronal activity (e.g., functional magnetic resonance imaging, short- and long-lasting IEG mapping, electrophysiology), and considering the dynamic interplay between markers of functional activation and endocrine stress response mediators both within and across functional brain circuits.

Although we did not assess specific cellular mediators, activity alterations are a likely consequence of deficits in excitatory and inhibitory neurons that are frequently observed after chronic stress. Indeed, human and animal studies focusing on the PFC/ACC and HPC revealed reductions in glutamatergic principal neuron morphology and synaptic connectivity that are reversed by rapid-acting antidepressant drugs, such as ketamine (Li et al., 2011; Popoli et al., 2012; McEwen et al., 2015; Zhao et al., 2016). Similarly, altered structural and functional markers of inhibitory GABA neurons represent a pathological hallmark across disorders, are recapitulated by rodent chronic stress paradigms, and normalize following antidepressant treatment (Banasr et al., 2017; Fee et al., 2017; Ghosal et al., 2020). In contrast to our findings of enhanced excitation, multiple rodent studies reported that chronic stress increased GABAergic and decreased glutamatergic markers in the mPFC, potentially recapitulating prefrontal hypofunction in humans stress-related disorders (reviewed in McKlveen et al., 2019). For example, 1-week repeated restraint or unpredictable stress reduced glutamatergic neurotransmission in mPFC pyramidal neurons in juvenile male rats (Yuen et al., 2012). Another study showed that 2-week exogenous corticosterone administration reduced vmPFC glutamate receptor subunits, which correlated with impaired fear extinction (Gourley et al., 2009). Finally, 2- or 4-week UCMS exposure increased GABAergic PV cell markers in the mPFC, but reduced local activation by novel environment stimuli only in female mice (Shepard et al., 2016; Shepard and Coutellier, 2018). In light of these differences, it is important to distinguish that several past findings rely on stress paradigms shorter than the 2-week chronic stress minimum endorsed by a majority of researchers (Willner, 2017a). Further, a study using 9-week UCMS recapitulated human stress-related GABAergic deficits together with decreased inhibition of mPFC pyramidal neurons in susceptible rats (Czéh et al., 2018). Similarly, 3-weeks CRS exposure in rats exacerbated glutamatergic activation of HPC CA3 by an acute stressor (Yamamoto and Reagan, 2006), consistent with our results demonstrating that control mice clustered on low CA3 activation/behavioral emotionality PCs contrasting the high CA3 activation/behavioral emotionality PCs in chronic stress-exposed mice. Although these findings may seem discordant, they likely represent an imbalance attributable to the impact of chronic stress on both

excitatory and inhibitory components. Although the precise mechanisms underlying these changes are unclear, one candidate is glutamatergic excitotoxicity (Sousa and Almeida, 2012; Duman et al., 2016), supported by evidence that acute stress enhances extrasynaptic glutamate activity in the PFC and vHPC (Lowy et al., 1995; Venero and Borrell, 1999; Maggio and Segal, 2009; Yuen et al., 2011; Treccani et al., 2014), consistent with our c-Fos findings. This is supported by evidence that antidepressants and anxiolytics prevented the induction of stress-induced c-Fos activation in the ACC, HPC, CeA, PVN, and VTA (Beck and Fibiger, 1995; Morrow et al., 2000; Lino-de-Oliveira et al., 2001; Silva et al., 2012), supporting that dysregulation of the adaptive stress response precedes the structural and functional deficits that are targeted by pharmacological treatments.

In the current study, we identified altered ACC and vHPC activation together with physiological and anxiety-like deficits (CRS and UCMS) and impaired memory (UCMS only). These findings are consistent with demonstrated roles for the ACC and vHPC in the regulation of mood and cognition, plus other functions that are disrupted in chronic stress-related disorders, including fear conditioning, reward processing, and motivation (Phan et al., 2002; Quirk and Beer, 2006; Etkin et al., 2011; Tannenholz et al., 2014). Indeed, postmortem studies of patients with MDD and bipolar disorder demonstrate reduced gray matter volume and glial cell density in ACC A24b and adjacent subregions (Öngür et al., 1998; Cotter et al., 2001; Drevets et al., 2008b). Elevated task-induced ACC activation has been identified in neuroimaging studies of patients with post-traumatic stress disorder, anxiety disorders, and obsessive–compulsive disorder (Fitzgerald et al., 2005; Etkin and Wager, 2007; McClure et al., 2007; Nitschke et al., 2009). Although one meta-analysis revealed ACC hypoactivity in MDD (at baseline and during task-induction) (Fitzgerald et al., 2008), others show that this was reversed when correcting for volumetric atrophy, revealing increased activity (Drevets et al., 2008a). Reduced HPC volume, although moderate relative to ACC changes, was also consistently found in patients with MDD and post-traumatic stress disorder (Koolschijn et al., 2009; Stratmann et al., 2014; Henigsberg et al., 2019). In the HPC, findings are mixed. Postmortem studies in bipolar disorder and MDD subjects demonstrated reduced synapse and gray matter volume in vCA1 and the subiculum, whereas imaging studies in live MDD patients reported volumetric changes in the HPC, but not when stratified to the anterior part (homologous to the vHPC in rodents) (Neumeister et al., 2005; Bonne et al., 2008). Functional changes also parallel those found in the ACC, showing hyperactivity during emotive processing in both regions, and associations between familial MDD risk and HPC hyperactivity that was reversed by antidepressants (Mayberg et al., 2000; Kennedy et al., 2001; Mannie et al., 2014; Jaworska et al., 2015).

Our study design revealed behavioral alterations consistent with past rodent chronic stress studies showing that >5-week CRS or UCMS exposure in rodents induces physiological alterations in weight gain and fur coat quality, anxiety-like behavior in the EPM, NSF, and PT, and memory impairments in working and short-term memory in the YM and NORT, respectively (Mineur et al., 2006; Chiba et al., 2012; Willner, 2017b). Comparisons of CRS and UCMS models often focus on differences in face and construct validity that parallel human disease experiences (Willner, 2017b; Prevot et al., 2019b). However, in line with recent Research Domain Criteria (Anderzhanova et al., 2017), our approach aimed to focus on the convergent biological and behavioral alterations produced by both models to identify mechanisms connecting common disease risk factors (i.e., chronic stress) to maladaptive behaviors. Thus, we attribute the observed variability in behavioral alterations within and between models to well-documented challenges of using classical behavioral tests as measuring tools (see discussions on consistency, reliability, and variability in Ramos, 2008; Willner, 2017a; and Prevot et al., 2019b), rather than meaningful conclusions about the validity of UCMS vs. CRS. For example, we frequently observe YM deficits in CRS mice (Prevot et al., 2019a), but did not here. To overcome these challenges, we implemented multiple end-point tests assessing top-down behavioral dimensions (Ramos, 2008) and used repeatable tests to (a) track the induction of deficits over time, and (b) provide baseline and end-point readouts to measure the transition into maladaptive behavior (via PCA). Longitudinal tests revealed physiological and anxiety-like deficits persisting after 1–5 weeks of stress, supporting the utility of repeatable behavioral tests such as the PhenoTyper test (Maluach et al., 2017; Nikolova et al., 2018; Prevot et al., 2019b). However, it is important to note that repeated behavioral testing may in itself introduce variability in rodent behavioral outputs (Voikar et al., 2004). Thus, for correlational analyses, we partially controlled for this variability by summarizing behavioral alterations through dimension reduction via principal component analysis, based on past approaches (Nikolova et al., 2018; Prevot et al., 2019b). We found that baseline parameters (e.g., week 0 or pre-trial readouts) had weak or negative loadings vs. the strong positive loadings of evaluated parameters (e.g., week 6–8 or trial readouts), indicating that these variables captured variance associated with maladaptive behavioral states. These PCs were positively correlated with c-Fos activation in regions altered by chronic stress, suggesting that dysregulation of the stress response in the ACC and vHPC co-occurs with or contributes to stress-related behaviors.

In summary, our data contribute evidence supporting a potential association between chronic stress, altered ACC and vHPC regional activation by acute stress, and maladaptive behaviors relevant to stress-related disorders. Although, the correlational design of the current study does not support causal inferences, future studies should investigate the modulation of excitatory and inhibitory components within these target regions to improve understanding of how chronic stress leads to pathological changes and to identify opportunities for treatment development.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuroscience.2020.05.034.

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