USING STRESS-BASED ANIMAL MODELS TO UNDERSTAND THE MECHANISMS UNDERLYING PSYCHIATRIC AND SOMATIC DISORDERS

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USING STRESS-BASED ANIMAL MODELS TO UNDERSTAND THE MECHANISMS UNDERLYING PSYCHIATRIC AND SOMATIC DISORDERS

Topic Editors:
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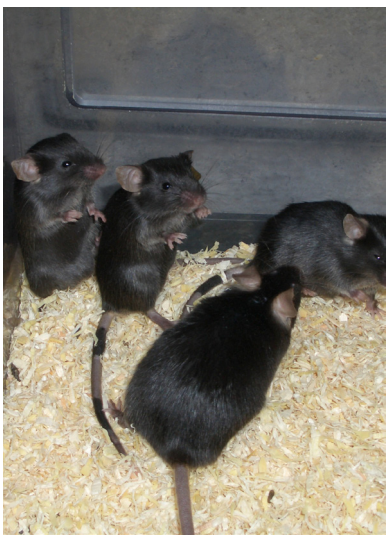

An example of a stress-based animal model, namely chronic subordinate colony housing (CSC), used to study the mechanisms underlying psychiatric and somatic disorders. Here the resident male mouse is shown defeating, and thus, subordinating, the intruder mice.

Cover photo provided by Stefan Reber.

Chronic or repeated stress, particularly psychosocial stress, is an acknowledged risk factor for numerous affective and somatic disorders in modern societies. Thus, there is substantial evidence showing that chronic stress can increase the likelihood of major depressive disorder and anxiety disorders, as well as cardiovascular diseases, irritable bowel syndrome and pain syndromes, to name but a few, in vulnerable individuals. Although a number of pharmacological agents are available to treat such stress-related disorders, many patients do not respond to them, and those who do often report a number of side effects. Therefore, a major emphasis in modern basic research is to uncover the underlying aetiology of these disorders, and to develop novel efficacious treatment strategies. This has led to a resurgence in developing, and using, appropriate animal models to study a wide variety of stress-related disorders.

Thus, the aim of this research topic "Using stress-based animal models to understand the mechanisms underlying psychiatric and somatic disorders" was to bring together novel research articles and comprehensive review articles from prominent stress researchers. In addition to describing the insights such models have provided relating to the aetiology

of psychiatric and somatic disorders, these articles also encompass mechanisms that are believed to underlie stress resilience and stress-protection. Finally, given the current prominence on the role of the brain-gut axis in health and disease, the research topic covers the emerging evidence showing how the gut, particularly the microbiota, influences affective behaviour and physiology.

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EDITORIAL
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Editorial: Using Stress-Based Animal Models to Understand the Mechanisms Underlying Psychiatric and Somatic Disorders

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Keywords: stress, microbiome, animal models, glucocorticoids, prefrontal cortex, behavior, irritable bowel syndrome

The Editorial on the Research Topic

Using Stress-Based Animal Models to Understand the Mechanisms Underlying Psychiatric and Somatic Disorders

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Chronic or repeated stress, particularly psychosocial stress, is an acknowledged risk factor for numerous affective and somatic disorders in modern societies. Thus, there is substantial evidence showing that chronic stress can increase the likelihood of major depressive disorder and anxiety disorders, as well as cardiovascular diseases, irritable bowel syndrome and pain syndromes, to name but a few, in vulnerable individuals. Although a number of pharmacological agents are available to treat such stress-related disorders, many patients do not respond to them, and those who do often report a number of side effects.

Consequently, a major emphasis in modern basic research is to uncover the underlying etiology of these disorders and to develop novel efficacious treatment strategies. Animal models can be utilized to unravel the biological mechanisms underlying specific disorders and test the efficacy of novel drugs. While animal models cannot mimic psychiatric and somatic disorders in their entirety, assessing the impact of certain risk factors for the disorders will facilitate the understanding of their etiology and treatment options. Consistent with this knowledge, and with clinical evidence purporting chronic stress to be a risk factor for such diseases, recent attempts have focused on the development of adequate stress paradigms. It is believed that psychosocial stress paradigms are more relevant to the human situation than non-social stress paradigms, because the vast majority of stressors reported by patients suffering from psychiatric or somatic disorders are social in nature. Therefore, there is a growing consensus that social stress paradigms are better placed to reveal the behavioral, neuroendocrine, or immunological mechanisms underlying chronic stress-induced pathology. However, in order to maximize our understanding of the mechanisms underlying stress-related disorders, various different models are necessary, including models employing physical (i.e., footshock) or pharmacological (e.g., corticosterone) stressors. Moreover, there is accumulating evidence suggesting that stress-induced gut dysbiosis and systemic low-grade inflammation play a causal role in the development of stress-associated somatic and affective disorders. Therefore, chronic/repeated stress models that result in reduced gut microbial diversity and/or promote chronic immune activation are of special relevance and importance.

In this research topic, we have collated novel research articles (four) and comprehensive review articles (five) from various leaders in the field of stress-based research. The overarching goal of the topic is to reveal how chronic/repeated stress models can help us to better understand the etiology of psychiatric and somatic disorders.

The first article describes a novel chronic unpredictable stress (CUS) protocol, which was developed for C57BL/6 mice. Given the unreliability of commonly utilized 4-week-long CUS protocols in C57BL/6, Monteiro et al. developed an 8-week-long CUS protocol that results in classical stress-related outcomes, such as increased adrenal weight and increased anxiety- and depressive-like behavior. Their results suggest that this 8-week protocol can be used to assess the underlying mechanisms of stress-related alterations in C57BL/6 mice.

Chronic stressor exposure can also be mimicked by treating rodents chronically with corticosterone. Here, Kinlein et al. demonstrate that non-invasive corticosterone administration *via* the drinking water for 28 days (25 μg/ml) results in a dysregulated response to a subsequent acute stressor. While corticosterone-treated mice did not show a plasma corticosterone response to swim exposure, their central *c-fos* response to this acute stressor was actually increased in numerous brain regions. Taken together, these findings suggest that this model can be used to determine how altered HPA axis function contributes to central dysregulation to an acute stressor.

Another system disrupted by chronic/repeated stressor exposure is the sleep/wake cycle. In their study, Greenwood et al. assess the effect of repeated (22 days) contextual fear conditioning on behavior and the sleep/wake cycle to a subsequent acute foot shock. Their findings demonstrate that the increased anxiety-related behavior and disrupted sleep/wake behavior evoked by acute stress is enhanced by prior fear conditioning. Taken together, this study suggests that repeated stress increases the vulnerability to novel subsequent stressors.

The extent to which chronic stress interacts with HIV-1 viral proteins to impact behavior and neuroinflammatory processes is largely unknown, but of growing research interest. Addressing this question, Rowson et al. expose male and female HIV-1 transgenic rats to chronic stress in adolescence (or non-stressed controls) and reveal that the presence of the transgene affects behavior and microglial structure, particularly in females. However, the addition of stressor exposure did not further affect these alterations, suggesting that there is little interaction between stress and HIV-1 viral proteins.

In the second section of the research topic, complementing the original research articles, are five review articles covering a range of topics related to stress-based research. The first review article describes the use of basic rat models to gain a better understanding of the effect of chronic stress on myocardial sensitivity to ischemic injury. Eisenmann et al. suggest that acute stress decreases the sensitivity to myocardial ischemia–reperfusion injury, while chronic stress even increases myocardial sensitivity to such an injury.

Stress has been well documented to affect synaptic plasticity and cause dramatic alteration of the glutamatergic system. In their review article, Musazzi et al. summarize our current understanding of the biphasic adaptations of the glutamatergic system in the prefrontal cortex during stressor exposure. The implications of these adaptations for our understanding of the etiology, and possible treatment, of stress-related disorders are then discussed.

There has been a growing reliance on chronic psychosocial stress models in basic research, due to the belief that such models more accurately reflect the clinical situation. Langgartner et al. discuss the knowledge that such models have provided in relation to psychiatric and somatic disorders. They focus particularly on one such model, the chronic subordinate colony housing (CSC) paradigm, which causes numerous stress consequences, including increased anxiety-related behavior, spontaneous colitis, and HPA axis changes.

Finally, given the growing interest in the role of the brain–gut axis on affective behavior and somatic disorders, there are two reviews focusing on this topic. Gur et al. discuss the evidence revealing that although the gut microbiota is relatively resistant to change, at particular time points, such as early life, stress can affect the microbiota and lead to detrimental outcomes for parturition and infant neurodevelopment. Moloney et al. review current animal models of stress-induced visceral pain, which aim to reflect irritable bowel syndrome in patients. Their wide-ranging review describes the influence of a number of factors, such as stress, gender, gut microbiota and epigenetic changes, in the etiology and potential treatment of visceral pain.

Taken together, the articles collated in this research topic provide a broad overview of the role that stress-based animal models have in deepening our understanding of stress-induced disorders. Although not comprehensive, the articles cover models relevant for anxiety disorder, major depressive disorder, alcohol abuse, cardiovascular diseases, ulcerative colitis, and irritable bowel syndrome. Importantly, given the large comorbidity between such disorders, placing these articles together in one research topic ensures an overview about how multiple systems interact during chronic stressor exposure. Such multimodal research is becoming increasingly necessary to enhance our understanding of psychiatric and somatic disorders.

AUTHOR CONTRIBUTIONS

Both the authors contributed to the writing of the editorial and agreed with the final version.

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ORIGINAL RESEARCH ARTICLE
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An efficient chronic unpredictable stress protocol to induce stress-related responses in C57BL/6 mice

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Exposure to chronic stress can have broad effects on health ranging from increased predisposition for neuropsychiatric disorders to deregulation of immune responses. The chronic unpredictable stress (CUS) protocol has been widely used to study the impact of stress exposure in several animal models and consists in the random, intermittent, and unpredictable exposure to a variety of stressors during several weeks. CUS has consistently been shown to induce behavioral and immunological alterations typical of the chronic stress-response. Unfortunately C57BL/6 mice, one of the most widely used mouse strains, due to the great variety of genetically modified lines, seem to be resistant to the commonly used 4-week-long CUS protocol. The definition of an alternative CUS protocol allowing the use of C57BL/6 mice in chronic stress experiments is a need. Here, we show that by extending the CUS protocol to 8 weeks is possible to induce a chronic stress-response in C57BL/6 mice, as revealed by abrogated body weight gain, increased adrenals weight, and an overactive hypothalamic–pituitary–adrenal axis with increased levels of serum corticosterone. Moreover, we also observed stress-associated behavioral alterations, including the potentiation of anxious-like and depressive-like behaviors and a reduction of exploratory behavior, as well as subtle stress-related changes in the cell population of the thymus and of the spleen. The present protocol for C57BL/6 mice consistently triggers the spectrum of CUS-induced changes observed in rats and, thus, will be highly useful to researchers that need to use this particular mouse strain as an animal model of neuropsychiatric disorders and/or immune deregulation related to CUS.

Keywords: chronic stress, CUS, neuropsychiatric disorders, immune dysfunction, anxiety, depressive-like behavior, social defeat

INTRODUCTION

Stressful life events can be triggering factors of numerous neuropsychiatric disorders namely anxiety, depression, and dementia (1), and many of these are accompanied by immune dysfunction (2). Moreover, prolonged-stress-induced immune dysfunction itself is regarded as a contributing factor for the effects of stress on health (3). In contrast with chronic stress, the acute stress-response is a beneficial event since it is an alarm reaction that prepares the body to a possible threat. This response is characterized by the secretion of stress mediators, such as glucocorticoids and epinephrine, which allows the stability of body function by adaptation to the stressor (4). However, when this response persists in time, it might render the system unable to cope with the stressor, ultimately leading to chronic-stress-associated illness.

Neuropsychiatric alterations are the most widely described effects of chronic stress exposure and include anxious-like behavior (5–8), depressive-like behavior (9, 10), and cognitive deficits (5, 11–14). However, the effects of chronic stress are not only limited to behavioral changes. Immune cells express receptors for glucocorticoids and catecholamines (15, 16), which can lead to alterations in gene transcription in response to stress (17). In fact, it is generally accepted that chronic-stress-associated changes in the immune system alter the vulnerability to infectious disease and auto-immunity (18).

Stress exposure variables, such as duration, intensity, and predictability, explain the spectrum of differential responses to stress but ultimately, the threshold in which the stress-response switches from physiological to deleterious is also dependent on neuroendocrine, neurochemical, and genetic factors that are responsible for individual differences in stress perception and response (19). Having this in mind, it seems logical that for the use of animal models, the chronic stress protocol needs to be adjusted to the animal species and even the strain used.

The most commonly used unpredictable chronic stress paradigms are the unpredictable chronic mild stress (uCMS) and the chronic unpredictable stress (CUS). Although both terms, uCMS and CUS, tend to be used indiscriminately nowadays and that both protocols are widely used to study depression, the original purpose for which they were generated was quite distinct. uCMS paradigm have been long used to model depression, and consists in the continuous exposure of animals to stressful situations, usually for at least 4 weeks, including some stressors that

involve water and/or food deprivation. In contrast, CUS was originally used to study mechanisms underlying the stress-response and involves the intermittent exposure to a daily stressful stimulus, lasting at least 4 weeks, being one of the main advantages of this protocol the absence of stressors that interfere with water and/or food deprivation, which might better mimic everyday life stress.

Although rats are widely used as animal models of depression and other stress-related disorders, mice present advantages such as the availability of numerous genetically modified strains like transgenic and KO mice and the lower maintenance costs when compared to rats. Unfortunately, the most widely used inbreed strain of genetically modified mice, the C57BL/6, seems to be less vulnerable to stress than other mouse strains (20–25).

Our aim was to develop an improved CUS protocol to be used in C57BL/6 mice. In order to do so we modified the standard CUS protocol by including social defeat stress as one of the stressors and extending its duration to 8 weeks. By comparing the neuroendocrine, behavioral, and immune changes induced by the unmodified 4-week long CUS exposure and the optimized 8-week long CUS protocol we, herein, show the advantages of later for C57BL/6 mice.

MATERIALS AND METHODS

ANIMALS

Male C57BL/6 mice (C57BL/6J JAX™ mice strain) were purchased from Charles River (Charles River Laboratories, Barcelona, Spain) and housed (five animals per cage) under standard laboratory conditions (12 h light/12 h night cycles (8 h/20 h), 22–24°C, relative humidity of 55% and *ad libitum* access to water and food. All procedures were carried out in accordance to EU directive 2010/63/EU and Portuguese national authority for animal experimentation, Direção Geral de Veterinária (ID:DGV9457) guidelines on animal care and experimentation.

CHRONIC UNPREDICTABLE STRESS PARADIGM

One group of C57BL/6 animals was exposed to 4 weeks of CUS and compared to a control group that was subjected to gentle handling, twice a week, for the same period. Another group was exposed to 8 weeks of CUS and compared to other control group that was subjected to gentle handling, twice a week, for the same period. Mice were 8-week old when the CUS protocol was initiated. Each group consisted of 10–15 male C57BL/6 mice. We run two independent experiments to confirm our findings: data from the first, representative of our findings, are presented in the main paper, whereas data from the second experiment are shown as supplementary data (Figure S1 and Table S1 in Supplementary Material).

Briefly, the CUS paradigm consisted in exposure, once daily, to one of the following aversive stressors: **restraint** – mice were placed in a 50 ml plastic tube (Falcon) with openings in both sides for breathing, for 1 h; **shaking** – groups of five mice were placed in a plastic box container and placed in an orbital shaker for 1 h at 150 rpm; **social defeat** – mice were introduced in a cage of an aggressive mice and after being defeated, they were placed in a transparent and perforated plastic container, to avoid further physical contact, inside the resident homecage for 30 min (26); **hot air stream** – mice were exposed to a hot air stream from a hairdryer for 10 min; **overnight illumination** – mice were exposed to regular room light during the night period; **inverted light cycle** – regular room light was off during daytime and on during nighttime for 2 days; **tilted cage** – homecages were tilted in a 45° angle during 1 h. Stressors were presented in a random order in an unpredictable fashion (see **Table 1**). The stressors distribution for the group submitted to 4 weeks of CUS is a truncated version of **Table 1**. Body weight was monitored once a week and *post-mortem* thymus and adrenal weight were recorded.

CORTICOSTERONE QUANTIFICATION

Blood was collected through the tail by venopuncture within a maximum 120 s period since removal of each mouse from its homecage to the end of blood collection. Sera were separated by centrifugation at 13000 rpm, during 5 min and stored at −80°C. Serum corticosterone levels were measured on sera collected at *nadir* phase (9:00 a.m.) and at *zenith* phase (8:00 p.m.) using a commercial radioactive immunoassay kit (MP Biomedicals, CA, USA).

BEHAVIORAL ASSESSMENT

Mice were transported and left for habituation to the testing room for 1 h prior to the behavioral test. The order of the behavioral tests was: elevated-plus maze (EPM) and open field (OF) (Day 1), forced swimming test (FST) and tail-suspension test (TST) (Day 2), and Morris water maze (MWM) (Day 3–7).

Elevated-plus maze

Anxious-like behavior was assessed using the EPM test (27). Briefly, this test consists on placing each mouse in the hub of a plus-like apparatus elevated 72.4 cm from the floor, with two opposing open arms (50.8 cm × 10.2 cm) and two opposing closed arms (50.8 cm × 10.2 cm × 40.6 cm) (ENV560; Med Associates, Inc., Vermont, USA) and letting the animal freely explore it for 5 min. Time in the open arms and in the closed arms was used as a behavioral parameter of anxious-like behavior. EPM data from one animal from each group were not included in the analysis due to failure of the video recording system.

Open field

Locomotor and exploratory activities were assessed using the OF. Each mouse was left in the center of a squared arena (43.2 cm × 43.2 cm), which the mouse was free to explore for 5 min. This arena is equipped with infrared beams for activity detection (Med Associates, Inc., Vermont, USA). Data were collected using the activity monitor software (Med Associates, Inc., Vermont, USA). Distance traveled was used as a measure of locomotor activity and the number of vertical counts as a measure of exploratory activity.

Forced swimming test and tail-suspension test

Depressive-like behavior was assessed through the FST as described by Ref. (28) and through the TST (29). Briefly, in the

Table 1 | Example of stressors distribution.

	Mon	Tue	Wed	Thu	Fri	Sat	Sun
Week 1	BW hot drier	Shaking	Restraint	Social defeat	Restraint	Restraint	Tilted cage
Week 2	BW restraint	Shaking	Social defeat	Restraint	Shaking	Social defeat	Restraint
Week 3	BW restraint	Social defeat	Restraint	BC-*zenith*	Social defeat	Restraint	Shaking
Week 4	BW BC – *nadir* Social defeat	EPM OF Restraint	FST TST Social defeat	Shaking	Restraint	Shaking	Hot drier
Week 5	BW restraint	Sacrifice 4 weeks tilted cage	Cytometry hot drier	Restraint	Social defeat	Inverted light	Inverted light
Week 6	BW hot drier	Shaking	Restraint	Inverted light	Overnight illumination	Restraint	Hot drier
Week 7	BW restraint	Social defeat	Restraint	BC-*zenith*	Shaking	Restraint	Overnight illumination
Week 8	BWBC-*nadir*	EPM OF Restraint	FST TST Social defeat	MWM shaking	MWM restraint	MWM restraint	MWM shaking
Week 9	BW	Sacrifice 8 weeks	Cytometry				

BW, body weight measurement; BC, blood collection; EPM, elevated-plus maze; OF, open field; FST, forced swimming test; TST, tail-suspension test; MWM, Morris water maze test.

FST each mouse was placed in an inescapable transparent cylindrical tank filled with water (±24°C), for 6 min. In the TST, each animal was suspended by the tip of its tail for 6 min. The activity of each mouse, in both tests, was recorded using a video camera. Latency (time to the first stop), mobility and immobility times were scored manually by an investigator blind to the experimental conditions, using Etholog 2.2 software (30), and used as a measure of behavioral despair. TST data from one animal were not included in the analysis due to failure of the video recording system.

Morris water maze

In order to assess spatial reference memory, mice were tested in a white circular pool (170 cm diameter) filled with water (24–25°C) placed in a dimly lit room. Spatial cues were placed in the walls around the pool (square, stripes, triangle, and a cross). The pool was divided in four imaginary quadrants and a hidden transparent platform was placed in one of the quadrants. Data were collected by a fixed camera placed in the ceiling and connected to a video-tracking system (Viewpoint, Champagne-au-Mont-d'Or, France).

Mice had to learn the position of a hidden platform over a period of 4 days. In each day, mice were placed facing the wall of the pool at different quadrants (north, west, south, and east) as a starting point for each trial. Each trial was completed whenever the mouse reached the platform or when 120 s elapsed. Latency to reach the platform (escape latency) was recorded for each trial during the 4 days.

In the fifth day, the platform was removed and a single trial of 60 s was performed (probe trial). The percentage of time that each mouse swam in each quadrant was recorded to confirm the acquisition of platform location through reference memory.

FLOW CYTOMETRY

Thymus and spleen (8–10 animals per group) were dissected and homogenized in supplemented Dulbecco's modified eagle medium (DMEM) with 10% heat inactivated FCS, 10 mM HEPES buffer, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 μg/mL streptomycin, and 50 U/mL penicillin (all from Invitrogen, CA, USA) in order to obtain single-cell suspensions. Splenic erythrocytes were depleted by incubating for 5 min with a hemolytic solution (155 mM NH_4Cl, 10 mM $KHCO_3$, pH 7.2). To analyze the main cell populations in the thymus, the cells (1×10^6 cell) were stained with APC anti-mouse CD3 (clone 145-2C11, Biolegend, San Diego, CA, USA), V450 anti-mouse CD4 (clone RM4-5, BD Pharmingen, San Jose, CA, USA), and V500 anti-mouse CD8 (clone 53-6.7, BD Pharmingen, San Jose, CA, USA). Splenocytes (1×10^6 cell) were stained with APC anti-mouse CD3 (clone 145-2C11, Biolegend, San Diego, CA, USA) for T-lymphocytes, PE.Cy5.5 anti-mouse CD19 (clone 6D5, Biolegend, San Diego, CA, USA) for B-lymphocytes, V450 anti-mouse CD4 (clone RM4-5, BD Pharmingen, San Jose, CA, USA) for T helper cells, V500 anti-mouse CD8 (clone 53-6.7, BD Pharmingen, San Jose, CA, USA) for T cytotoxic cells, and FITC anti-mouse NK1.1 (clone PK136, Biolegend, San Diego, CA, USA) for natural killer cells. To analyze myeloid cell populations, splenocytes (1×10^6 cell) were stained with PE anti-mouse CD11b (clone M1/70, Biolegend, San Diego, CA, USA), and PE.Cy7 anti-mouse Gr1 (clone RB6-8C5, Biolegend, San Diego, CA, USA). Cells were first gated for singlets (FSC-H vs. FSC-A) and viable cells (FSC-H vs. SSC-H). Myeloid cells were selected using the gating strategy described previously (31). Briefly, myeloid cells were gated as $CD11b^+$ cells excluding the $NK1.1^+$ cells. Macrophages/dendritic cells were selected as the population $Gr1^+SSC^{low}$, neutrophils were selected as $Gr1^+SSC^{high}$ and eosinophils as $Gr1^-SSC^{high}$

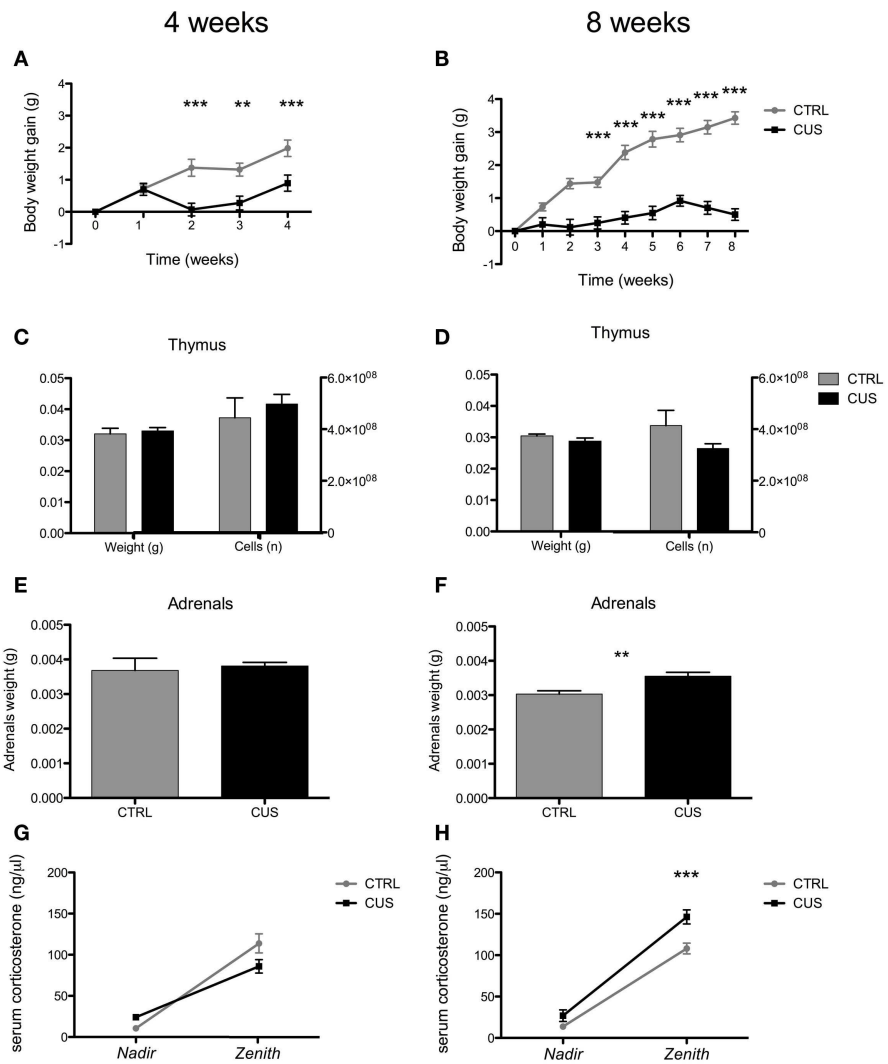

FIGURE 1 | Effect of 4 weeks vs. 8 weeks of CUS on biometric parameters. Body weight gain for animals submitted to 4 **(A)** and 8 **(B)** weeks of CUS. Thymus weight and cellularity after exposure to 4 **(C)** and 8 **(D)** weeks of CUS. Adrenals weight after 4 **(E)** and 8 weeks of CUS **(F)**. Corticosterone levels in the serum of animals from the group submitted to 4 **(G)** and 8 **(H)** weeks of CUS. Each bar/point represents the mean ± SEM from 10 animals per group. $^{**}p < 0.01$, $^{***}p < 0.001$.

(**Figure 7C**). After staining cells were fixed in 2% paraformaldehyde for 20 min. Cell surface staining was acquired (100,000 events) in an eight-color LSRII flow cytometer (BD, Pharmingen, San Jose, CA, USA) and analyzed with FlowJo software version 7.6.4.

STATISTICAL ANALYSIS

All values were calculated as means ± SEM. Kolmogorov–Smirnov normality test was used to analyze if values departed from an approximate Gaussian distribution. Body weight, serum corticosterone levels, and reference memory task data were compared between groups using ANOVA repeated-measures on the average results of each week/phase/day, respectively. When the main effect was significant, *post hoc* Bonferroni test was performed in order to assess whether means differed significantly from each other. For all the other data, the differences among groups were analyzed using Student's *t*-test. Differences were considered significant if $p < 0.05$. Statistical analysis was performed with Graphpad Prism version 5.0b (La Jolla, San Diego, USA).

RESULTS

BIOMETRIC PARAMETERS AND CORTICOSTERONE MEASUREMENTS

Body weight gain, *post-mortem* thymus, and *post-mortem* adrenal weight and serum levels of corticosterone were monitored to control for stressors efficacy (**Figure 1**; Figure S1 in Supplementary Material). In the group submitted to the 4-week protocol of CUS, both time $[F_{(4,72)} = 23.85;\ p < 0.0001]$ and exposure to CUS $[F_{(1,18)} = 11.94;\ p = 0.003]$ had a significant impact on body weight (**Figure 1A**). Moreover, there was a significant

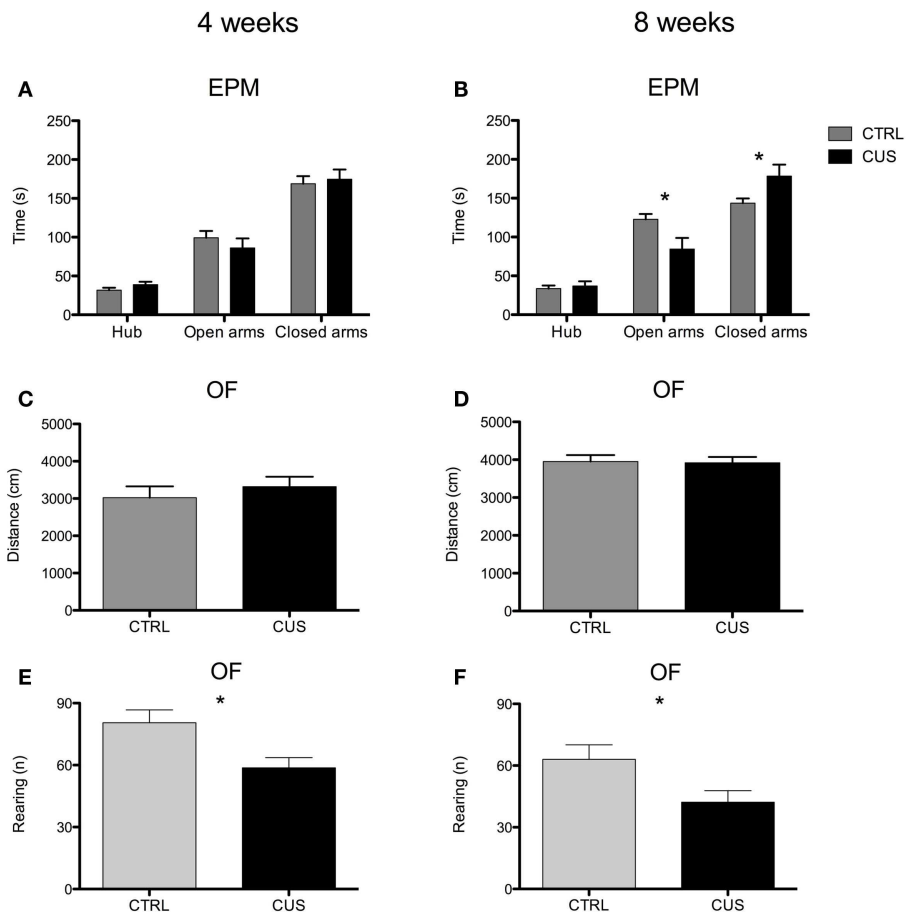

FIGURE 2 | Impact of 4 and 8 weeks of CUS on anxious-like and locomotor behavior and exploratory activity. Behavioral performance of mice exposed to 4 **(A)** and to 8 weeks of CUS **(B)** in the EPM. Locomotor function of mice submitted to 4 **(C)** and 8 weeks **(D)** of CUS measured in the OF. Exploratory activity of mice submitted to 4 **(E)** and 8 weeks **(F)** of CUS measured in the OF. Each bar represents the mean ± SEM from 8 to 10 animals per group. $^*p < 0.05$.

interaction between these factors $[F_{(4,72)} = 23.85;\ p < 0.0001]$ with stressed animals gaining significantly less weight over time (**Figure 1A**). In the group submitted to the 8-week protocol, repeated-measures ANOVA has shown again a significant effect of both time $[F_{(8,144)} = 80.13;\ p < 0.0001]$ and exposure to CUS $[F_{(1,18)} = 63.43;\ p < 0.0001]$ on body weight (**Figure 1B**). There was also a significant interaction between these factors $[F_{(8,144)} = 34.17; p < 0.0001]$ with stressed animals gaining significantly less weight over time (**Figure 1B**). CUS had no significant effect on thymus weight nor on thymic cell number, both in the group exposed to the 4- and the 8-week protocol of CUS (**Figures 1C,D**). CUS exposure during 4 weeks had no effect on adrenals weight, while exposure to CUS for 8 weeks led to a significant increase on adrenals weight $[t_{(18)=}3.449;\ p = 0.003]$ (**Figures 1E,F**). There were no statistically significant changes on corticosterone levels in the group submitted to 4 weeks of CUS, both at *nadir* and *zenith*. Repeated-measures ANOVA has shown a significant effect of exposure to 8 weeks of CUS on corticosterone levels $[F_{(1,18)} = 21.99;\ p = 0.0002]$. *Post hoc* test has shown a statistically significant increase of corticosterone levels in the *zenith* phase of the day, in the group submitted to 8 weeks of CUS $[t_{(18)} = 4.113;\ p < 0.001]$ (**Figures 1G,H**).

EXPOSURE TO 8 WEEKS OF CUS LEADS TO ALTERED EMOTIONAL BEHAVIOR BUT NOT TO MEMORY IMPAIRMENTS

There was a significant effect of exposure to 8 but not 4 weeks of CUS on anxious-like behavior, measured by a decreased time spent on the open-arms of the EPM $[t_{(16)} = 2.401;\ p = 0.029]$ and an increased time spent in the closed arms $[t_{(16)} = 2.176;\ p = 0.045]$ (**Figures 2A,B**; Figures S1I,J in Supplementary Material) by the 8-week CUS group when compared to controls. Exposure to CUS did not alter locomotor activity, assessed by the OF, both on the group exposed to 4 and 8 weeks of CUS (**Figures 2C,D**; Figures S1K,L in Supplementary Material), therefore validating behavioral tests that are dependent on an intact locomotor function. CUS had an impact on the exploratory activity, measured by a decrease on the number of rearings in the OF test, both in the group submitted to 4 $[t_{(18)} = 2.743;\ p = 0.013]$ (**Figure 2E**; Figure S1M in Supplementary Material) and 8 weeks of CUS $[t_{(18)} = 2.308;\ p = 0.033]$ (**Figure 2F**; Figure S1N in Supplementary Material).

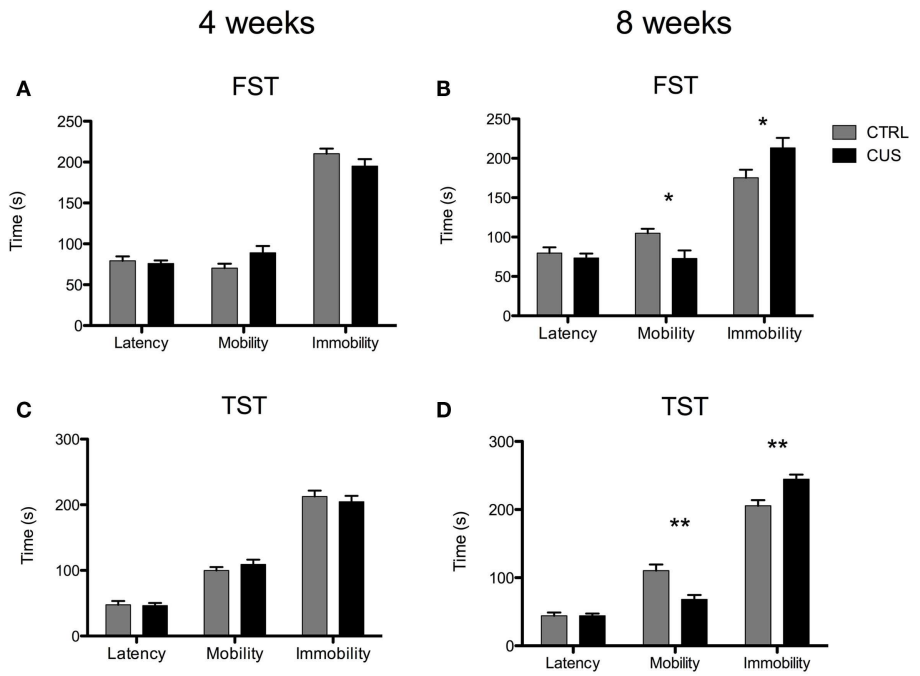

FIGURE 3 | Impact of 4 weeks vs. 8 weeks of CUS on depressive-like behavior. Behavioral performance of mice submitted to 4 **(A)** and 8 weeks of CUS **(B)** in the FST. Behavioral performance of mice submitted to 4 **(C)** and 8 weeks of CUS in the TST **(D)**. Each bar represents the mean ± SEM from 9 to 10 animals per group. $*p < 0.05$, $**p < 0.01$.

Animals exposed to 4 weeks of stress did not show any major differences in the FST when compared to controls (**Figure 3A**). The group of animals exposed to 8 weeks of CUS exhibited decreased mobility time $[t_{(18)} = 2.741;\ p = 0.013]$ and increased immobility time $[t_{(18)} = 2.310;\ p = 0.033]$ in the FST when compared to controls (**Figure 3B**). In the TST, the group submitted to 4 weeks of CUS did not show any major differences when compared to controls (**Figure 3C**), while the group submitted to 8 weeks of CUS exhibited a increased immobility time $[t_{(17)} = 3.710;\ p = 0.002]$ and an decreased mobility time $[t_{(17)} = 3.873;\ p = 0.001]$ (**Figure 3D**; Figure S1O in Supplementary Material); a typical phenotype of depressive-like behavior. No differences on latency time were found at any time point, both in the FST and TST (**Figure 3**).

The impact of different exposures to CUS was also tested in the MWM task in order to investigate whether the cognitive dimension was also affected. Although there was a slight tendency for a faster learning curve of the control group, especially on day 2 and 3, in comparison to CUS exposed animals, the ANOVA repeated-measures test revealed that there were no significant differences between groups, meaning that, at the end of the learning task, both CUS and control groups were able to successfully learn the task therefore exhibiting an intact spatial learning ability (**Figure 4**; Figure S1P in Supplementary Material).

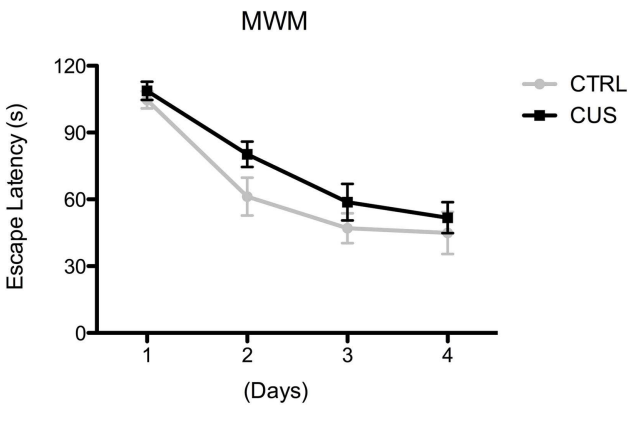

FIGURE 4 | Impact of 8 weeks of CUS on cognition. Behavioral performance of mice exposed to 8 weeks of CUS in the MWM. Each point represents the mean ± SEM from 10 animals per group.

THYMIC AND SPLENIC CELL POPULATION CHANGES BY EXPOSURE TO 8 WEEKS OF CUS

It is known that thymocytes are sensitive to stress hormones, such as glucocorticoids, which modulate several processes along their differentiation within the thymus (32). Due to this well-known susceptibility to stress hormones, the thymus weight, and cellularity have been widely used as indirect measures of stress. Thymocytes might be divided in four main differentiation populations depending on the expression of the CD4 and CD8 co-receptors ($CD4^-CD8^-$ double-negative – DN; $CD4^+CD8^+$ double-positive – DP; $CD4^+CD8^-$ single-positive CD4 – SPCD4; and $CD4^-CD8^+$ single-positive CD8 – SPCD8 cells). We therefore studied the major thymic subsets to determine if our CUS

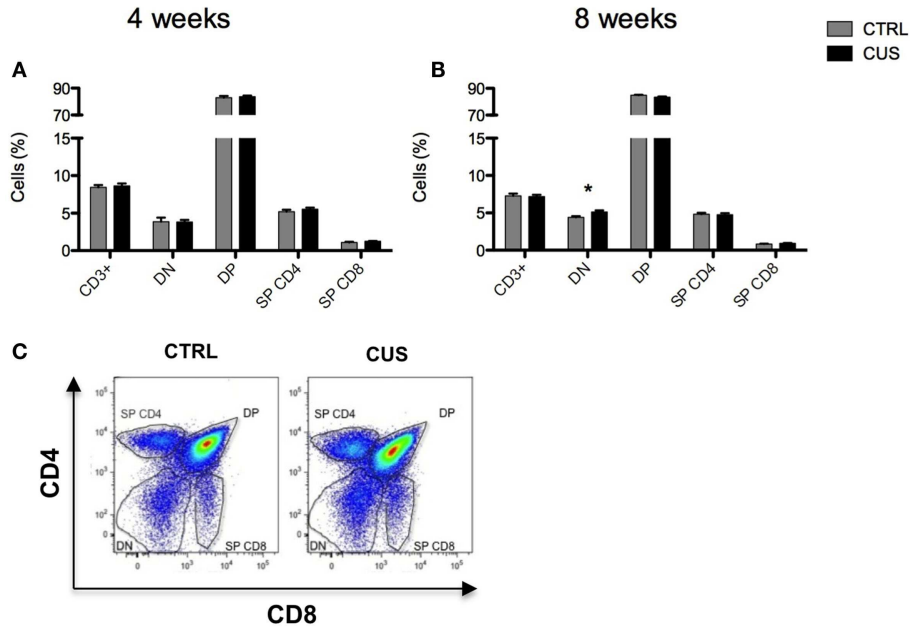

FIGURE 5 | Impact of 4 weeks vs. 8 weeks of CUS on thymocyte subsets. Percentage of main cell populations in thymus after exposure to 4 **(A)** and 8 **(B)** weeks of CUS. Flow cytometry plot showing the gating strategy for thymocyte subsets (gate) **(C)**. DN, double-negative thymocytes; DP, double-positive thymocytes; and SPCD4 and SPCD8, single-positive CD4 and CD8 thymocytes, respectively. Each bar represents the mean ± SEM from 10 animals per group. $*p < 0.05$.

protocols had a differential impact on them. We observed that 4 weeks of CUS did not alter the proportion of the four main thymocyte subsets (**Figure 5A**) while 8 weeks of stress led to an increase of the DN thymocytes proportion [$t_{(18)} = 2.681$; $p = 0.020$] (**Figures 5B,C**).

Since prolonged stress is known to influence the peripheral immune system we consider of relevance to investigate potential alterations caused by CUS on major lymphoid cell populations in the spleen, one of the most important lymphoid organs of the immune system. Animals exposed to 4 or 8 weeks of CUS did not show any differences on the percentage of splenic T and B cells (**Figures 6A,B** and **E**) nor in the $CD4^+$ and $CD8^+$ subsets among the T cells (**Figures 6C–E**). On the contrary, while animals exposed to 4 weeks of CUS did not show any major differences on the percentage of splenic eosinophils, neutrophils, and macrophages/dendritic cells (**Figure 7A**), the 8-week long CUS protocol led to an increased percentage of macrophages/dendritic cells [$t_{(14)} = 2.188$; $p = 0.046$] and neutrophils [$t_{(14)} = 3.327$; $p = 0.005$] in the spleen (**Figures 7B,C**).

DISCUSSION

In the present work, we have optimized a CUS protocol that results in a consistent stress-response in C57BL/6 mice. Published protocols on how to induce chronic stress on rodents are diverse and generate inconsistencies in their behavioral and immunological outcomes (33). Among the main reasons for such inconsistencies are strain inherent differences of stress susceptibility/resistance to distinct protocols. In mice, specifically, the C57BL/6 strain seems to be more resistant to CUS than other strains and/or other species (20–25). Yet, it is by far the most used mouse strain for genetic manipulations. This, and the fact that unpredictable chronic stress exposure is often used as a model of neuropsychiatric disorders, renders an effective CUS protocol in C57BL/6 mice, such as the one herein described, an important addition to the field.

Besides strain considerations, the type, diversity of stressors applied, and stress exposure length are also critical determinants of the impact of chronic stress. Some protocols use a single stressor, e.g., 6 h of daily restraint stress for a 4-week period (34, 35), which, despite being simpler to apply, have several limitations due to lack of unpredictability or the prolonged removal of animals from their homecages with no access to food or water for half of their resting period. On the other hand, reducing restraint stress to 1 h per day in order to overcome this difficulty results in a mild stress protocol.

Other widely used chronic stress protocol consists in the exposure to repeated bouts of social defeat stress, which have shown to induce a stressed phenotype in some C57BL/6 mice. However, both restraint and social defeat stress paradigms are characterized by repeated exposure to a single stressor, which lacks the variability of psychological and physical stressors generally encountered in life. Taking the aforementioned into account, we designed a CUS protocol, based on the appliance of a variety of stressors, presented randomly once per day, in an intermittent and unpredictable fashion, mimicking the variability of stressors encountered on everyday life (construct validity). Although not often used in mice, CUS protocols are widely used in rats and were shown to be highly effective in inducing a stress-related phenotype (6, 11, 12, 36). In addition, by extending this protocol to 8 weeks, instead of the usual

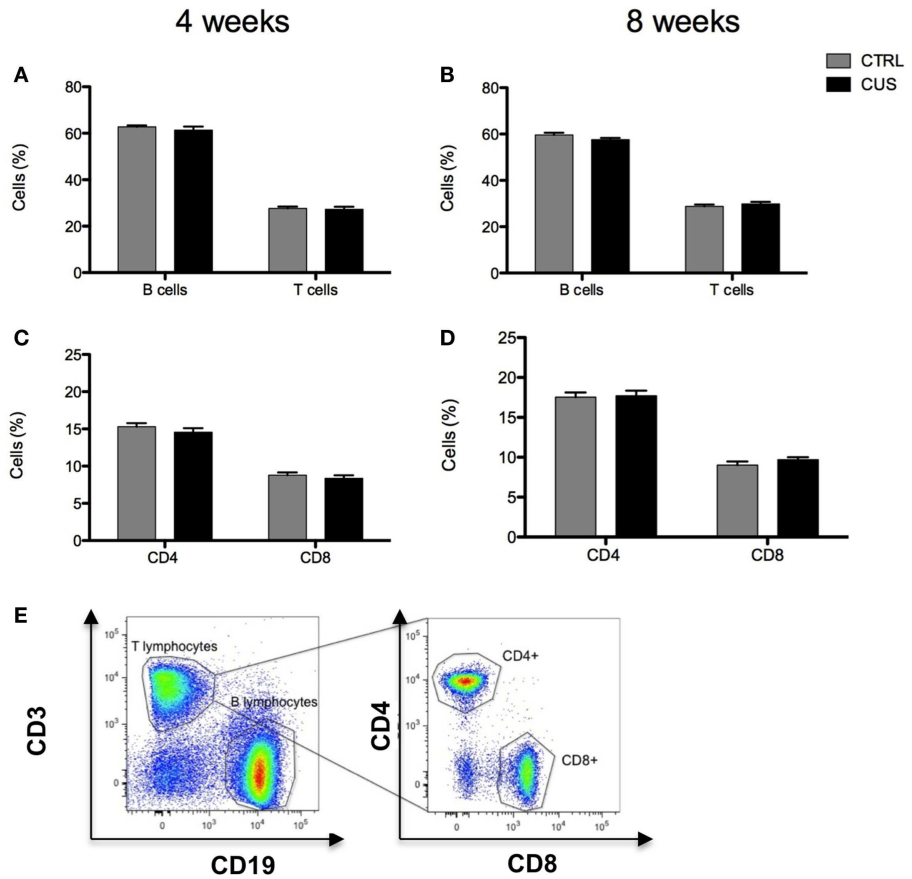

FIGURE 6 | Impact of 4 vs. 8 weeks of CUS on lymphoid cellular populations in the spleen. Percentage of T and B cells in spleen after exposure to 4 **(A)** and 8 **(B)** weeks of CUS. Percentage of $CD4^+$ and $CD8^+$ T cells in spleen after exposure to 4 **(C)** and 8 **(D)** weeks of CUS. Flow cytometry plot showing the gating strategy for T and B-lymphocytes (gates) **(E)**. Each bar represents the mean ± SEM from eight animals per group.

4 weeks, we were able to reach the point where this particular strain of mice clearly and consistently exhibits a maladaptive response to chronic stress with behavioral and immunological alterations (face validity).

One of the main advantages of this 8-week long CUS protocol is that there is no stressor that implies the disturbance of food and/or water consumption, which is of particular importance for metabolism studies, for example. Moreover, in this protocol FST or TST are not used as stressors, as used in some published protocols (9), which means that in our protocol these tests can still be used as behavioral measures.

Reduction on body weight gain, thymic involution (37–39), and increased adrenals weight (40) are typically used as markers of stressors efficacy. We have observed that although behavioral and immunological alterations were only evident after exposing mice to 8 weeks of stress, suppression of body weight gain was observed as early as after 2 weeks of exposure and was maintained throughout the duration of CUS. These findings suggest that, as a read-out of the maladaptive response to stress, body weight gain has a lower threshold than other changes and is not a good marker of the stress-impact in behavior and/or immunity. Moreover, we did not observe a consistent reduction on thymus weight; although we cannot discard the possibility of being unable to detect small differences of thymus weight, specially given that mice were previously transcardially perfused with 0.9% saline. Nevertheless, the concomitant lack of differences in thymic cellularity favors our observation that, in C57BL/6 mice, our CUS protocol does not impact thymus weight significantly. This observation strengths the idea that C57BL/6 are more resistant to the effects of chronic stress than other mouse strains.

An overactive hypothalamic–pituitary–adrenal (HPA) axis is also a feature of a maladaptative response to chronic stress (41). In fact, resistance to chronic stress can be associated with an effective negative feedback system that is able to shut down the excessive production of glucocorticoids occurring in response to stress (42). We observed that the 8-week long CUS protocol was the only one that led to a persistent increase on circulating corticosterone levels and increased adrenals weight, features consistent with a hyperactive HPA axis. Of note, based on corticosterone levels at zenith we identified a reduced number of resistant animals (2 out of 10 in one of the experiments and 2 out of 10 in the replicated experiment), a proportion of stress-resistance very similar to what already have been described in other models of chronic stress (26). Accumulating evidence shows that glucocorticoids modulate the

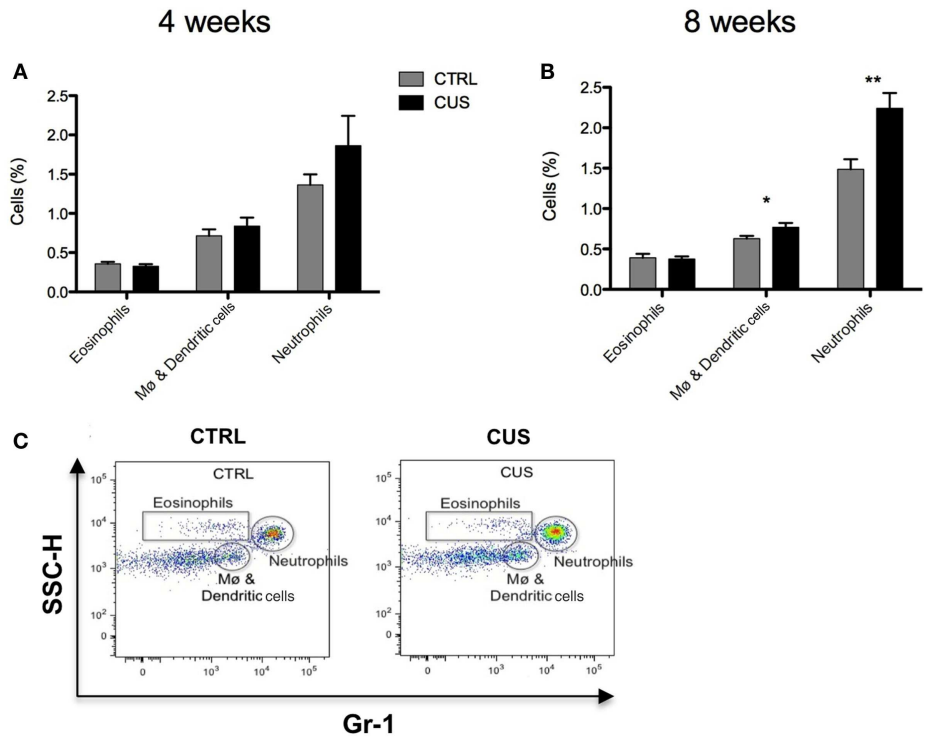

FIGURE 7 | Impact of 4 vs. 8 weeks of CUS on myeloid cellular populations in the spleen. Percentage of eosinophils, macrophages/dendritic cells, and neutrophils in spleen after exposure to 4 weeks **(A)** and 8 **(B)** weeks of CUS. Flow cytometry plot showing the gating strategy of myeloid splenocytes subsets (gates) **(C)**. Each bar represents the mean ± SEM from 8 animals per group. $*p < 0.05$, $**p < 0.01$.

behavioral effects of chronic stress (43, 44). In accordance, we observed that the 8-week long CUS protocol, the only that induced a hyperactive HPA axis, had a negative impact on emotional behavior. Specifically, we observed an enhanced anxious-like behavior, revealed by an increased time spent in the closed arms, and a decreased time in the open arms of the EPM. These animals also displayed behavioral despair, a symptom of depressive-like behavior, as they spent more time immobile in the FST. Of notice, this was further confirmed by performing the TST, another validated test for depressive-like behavior assessment.

Despite the emotional changes caused by 8 weeks of CUS exposure, cognitive functioning, namely spatial learning, seems to be intact, confirming data from other model of chronic stress (uCMS) (10). In fact, we observed that although stressed animals at the end of 4 days of MWM training were able to learn task at the same level as controls, there was a tendency for a slower learning progression on day 2 and 3. This type of learning pattern was previously shown using rats submitted to CUS (11), therefore emphasizing that the effects of CUS on spatial learning are more subtle than those on emotional behaviors. Contrary to the above mentioned effects, chronic stress triggers a decreased exploratory behavior of mice, both at 4 and 8 weeks of CUS, which might not be dependent on increased levels of corticosterone.

Although we cannot completely discard the possible confounder effect from performing two behavioral tests in the same day, we believe that data from the OF and TST was not significantly affected by acute stress caused by prior testing; indeed motor function (measured by OF) is not known as a target of acute stress, whereas data from TST were confirmed by the findings of the FST.

Glucocorticoids play a crucial role on thymopoiesis (32, 39), a process that occurs in the thymus in which immature precursor cells differentiate into mature T cells. In accordance, it was previously shown that rats exposed to chronic stress, with increased levels of circulating corticosterone levels, exhibit an increase in the percentage of DN thymocytes, while the percentage of SPCD4 was decreased (39). In our model, an increase in the percentage of DN thymocytes was observed. Still, contrary to the previously described (39), we did not observe any differences on the SPCD4 and SPCD8 populations of thymocytes, which may be due to the stress-resistance inherent to this particular strain of mice. T and B-lymphocytes in the spleen were not altered by exposure to chronic stress. However, we observed that exposure to 8 weeks of CUS (and not to 4 weeks) led to alterations in the cell composition of the spleen, characterized by an increased percentage of myeloid cells (macrophages/dendritic cells and neutrophils), in agreement with previous reports in both mice (45, 46) and humans (46). Glucocorticoids were shown to inhibit neutrophils' apoptosis, which may explain the persistent presence of these cells with short life span (47) in the spleen of chronically stressed animals. Moreover, it was shown that stress, through norepinephrine signaling from sympathetic nerve fibers, increased the proliferation of hematopoietic progenitors in the bone marrow giving rise to an increase on disease-promoting monocytes and neutrophils output (46). Stress was also shown to

increase monocyte recruitment to the brain by increased expression of cytokines and chemokines in specific brain regions. And more importantly, this monocyte recruitment to the brain was shown to be essential for the development of anxiety behavior induced by stress (45).

The absence of neuroendocrine, major behavioral and immunological alterations seen in the 4-week CUS exposed group could reflect the temporal dynamics of the stress-response rather than a failure to respond to stress. In fact, it should be noted that stress did impact the body weight gain and exploratory behavior on this group. This absence of major alterations resembles the Hans Selye's resistance phase of the so-called "syndrome of adaptation" (48) in which adaptative processes reinstall homeostasis during stress, including the normalization of glucocorticoid secretion. Therefore, the 4-week CUS protocol may be preferable to studies that target this specific stage of the stress-response like for example those that want to show a negative impact of a particular treatment on the stress-response, since the 8-week CUS alterations may approach a "ceiling effect." In contrast, the alterations observed in the 8-week version of CUS are consistent with phase 3 of this syndrome, where the system is no longer able to cope with stressors and is exhausted, which renders this version a robust model of the maladaptative response to chronic stress.

The establishment of a robust mouse model of stress-related disorders on C57BL/6 background represents a valuable research tool endowing the study of different genetic contributions to chronic stress-responses, which may enhance current knowledge on the neurobiology and immunology of complex neuropsychiatric and other stress-related disorders.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fpsyt.2015.00006/abstract

REFERENCES

1. McEwen BS. Protection and damage from acute and chronic stress: allostasis and allostatic overload and relevance to the pathophysiology of psychiatric disorders. *Ann N Y Acad Sci* (2004) **1032**:1–7. doi:10.1196/annals.1314.001
2. Glaser R, Kiecolt-Glaser JK. Stress-induced immune dysfunction: implications for health. *Nat Rev Immunol* (2005) **5**:243–51. doi:10.1038/nri1571
3. Haroon E, Raison CL, Miller AH. Psychoneuroimmunology meets neuropsychopharmacology: translational implications of the impact of inflammation on behavior. *Neuropsychopharmacology* (2012) **37**:137–62. doi:10.1038/npp.2011.205
4. McEwen BS. Stressed or stressed out: what is the difference? *J Psychiatry Neurosci* (2005) **30**:315–8.
5. Bondi CO, Rodriguez G, Gould GG, Frazer A, Morilak DA. Chronic unpredictable stress induces a cognitive deficit and anxiety-like behavior in rats that is prevented by chronic antidepressant drug treatment. *Neuropsychopharmacology* (2008) **33**:320–31. doi:10.1038/sj.npp.1301410
6. Pego JM, Morgado P, Pinto LG, Cerqueira JJ, Almeida OF, Sousa N. Dissociation of the morphological correlates of stress-induced anxiety and fear. *Eur J Neurosci* (2008) **27**:1503–16. doi:10.1111/j.1460-9568.2008.06112.x
7. McEwen BS, Eiland L, Hunter RG, Miller MM. Stress and anxiety: structural plasticity and epigenetic regulation as a consequence of stress. *Neuropharmacology* (2012) **62**:3–12. doi:10.1016/j.neuropharm.2011.07.014
8. Ventura-Silva AP, Pego JM, Sousa JC, Marques AR, Rodrigues AJ, Marques F, et al. Stress shifts the response of the bed nucleus of the stria terminalis to an anxiogenic mode. *Eur J Neurosci* (2012) **36**:3396–406. doi:10.1111/j.1460-9568.2012.08262.x
9. Strekalova T, Spanagel R, Bartsch D, Henn FA, Gass P. Stress-induced anhedonia in mice is associated with deficits in forced swimming and exploration. *Neuropsychopharmacology* (2004) **29**:2007–17. doi:10.1038/sj.npp.1300532
10. Bessa JM, Mesquita AR, Oliveira M, Pego JM, Cerqueira JJ, Palha JA, et al. A trans-dimensional approach to the behavioral aspects of depression. *Front Behav Neurosci* (2009) **3**:1. doi:10.3389/neuro.08.001.2009
11. Cerqueira JJ, Mailliet F, Almeida OF, Jay TM, Sousa N. The prefrontal cortex as a key target of the maladaptive response to stress. *J Neurosci* (2007) **27**:2781–7. doi:10.1523/JNEUROSCI.4372-06.2007
12. Dias-Ferreira E, Sousa JC, Melo I, Morgado P, Mesquita AR, Cerqueira JJ, et al. Chronic stress causes frontostriatal reorganization and affects decision-making. *Science* (2009) **325**:621–5. doi:10.1126/science.1171203
13. Conrad CD. A critical review of chronic stress effects on spatial learning and memory. *Prog Neuropsychopharmacol Biol Psychiatry* (2010) **34**:742–55. doi:10.1016/j.pnpbp.2009.11.003
14. Morgado P, Silva M, Sousa N, Cerqueira JJ. Stress transiently affects Pavlovian-to-instrumental transfer. *Front Neurosci* (2012) **6**:93. doi:10.3389/fnins.2012.00093
15. Dhabhar FS, McEwen BS. Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: a potential role for leukocyte trafficking. *Brain Behav Immun* (1997) **11**:286–306. doi:10.1006/brbi.1997.0508
16. Padgett DA, Glaser R. How stress influences the immune response. *Trends Immunol* (2003) **24**:444–8. doi:10.1016/S1471-4906(03)00173-X
17. Dhabhar FS, Malarkey WB, Neri E, McEwen BS. Stress-induced redistribution of immune cells – from barracks to boulevards to battlefields: a tale of three hormones – Curt Richter Award winner. *Psychoneuroendocrinology* (2012) **37**:1345–68. doi:10.1016/j.psyneuen.2012.05.008
18. Harpaz I, Abutbul S, Nemirovsky A, Gal R, Cohen H, Monsonego A. Chronic exposure to stress predisposes to higher autoimmune susceptibility in C57BL/6 mice: glucocorticoids as a double-edged sword. *Eur J Immunol* (2013) **43**:758–69. doi:10.1002/eji.201242613
19. Franklin TB, Saab BJ, Mansuy IM. Neural mechanisms of stress resilience and vulnerability. *Neuron* (2012) **75**:747–61. doi:10.1016/j.neuron.2012.08.016
20. Anisman H, Lacosta S, Kent P, McIntyre DC, Merali Z. Stressor-induced corticotropin-releasing hormone, bombesin, ACTH and corticosterone variations in strains of mice differentially responsive to stressors. *Stress* (1998) **2**:209–20. doi:10.3109/10253899809167284
21. Anisman H, Hayley S, Kelly O, Borowski T, Merali Z. Psychogenic, neurogenic, and systemic stressor effects on plasma corticosterone and behavior: mouse strain-dependent outcomes. *Behav Neurosci* (2001) **115**:443–54. doi:10.1037/0735-7044.115.2.443
22. Parfitt DB, Walton JR, Corriveau EA, Helmreich DL. Early life stress effects on adult stress-induced corticosterone secretion and anxiety-like behavior in the C57BL/6 mouse are not as robust as initially thought. *Horm Behav* (2007) **52**:417–26. doi:10.1016/j.yhbeh.2007.06.002
23. Razzoli M, Carboni L, Andreoli M, Ballottari A, Arban R. Different susceptibility to social defeat stress of BalbC and C57BL6/J mice. *Behav Brain Res* (2011) **216**:100–8. doi:10.1016/j.bbr.2010.07.014
24. Razzoli M, Carboni L, Andreoli M, Michielin F, Ballottari A, Arban R. Strain-specific outcomes of repeated social defeat and chronic fluoxetine treatment in the mouse. *Pharmacol Biochem Behav* (2011) **97**:566–76. doi:10.1016/j.pbb.2010.09.010
25. Savignac HM, Finger BC, Pizzo RC, O'Leary OF, Dinan TG, Cryan JF. Increased sensitivity to the effects of chronic social defeat stress in an innately anxious mouse strain. *Neuroscience* (2011) **192**:524–36. doi:10.1016/j.neuroscience.2011.04.054
26. Golden SA, Covington HE III, Berton O, Russo SJ. A standardized protocol for repeated social defeat stress in mice. *Nat Protoc* (2011) **6**:1183–91. doi:10.1038/nprot.2011.361
27. Walf AA, Frye CA. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nat Protoc* (2007) **2**:322–8. doi:10.1038/nprot.2007.44
28. Can A, Dao DT, Arad M, Terrillion CE, Piantadosi SC, Gould TD. The mouse forced swim test. *J Vis Exp* (2012) **59**:e3638. doi:10.3791/3638
29. Steru L, Chermat R, Thierry B, Simon P. The tail suspension test: a new method for screening antidepressants in mice. *Psychopharmacology (Berl)* (1985) **85**:367–70. doi:10.1007/BF00428203

30. Ottoni EB. EthoLog 2.2: a tool for the transcription and timing of behavior observation sessions. *Behav Res Methods Instrum Comput* (2000) **32**:446–9. doi:10.3758/BF03200814
31. Rose S, Misharin A, Perlman H. A novel Ly6C/Ly6G-based strategy to analyze the mouse splenic myeloid compartment. *Cytometry A* (2012) **81**:343–50. doi:10.1002/cyto.a.22012
32. Berki T, Palinkas L, Boldizsar F, Nemeth P. Glucocorticoid (GC) sensitivity and GC receptor expression differ in thymocyte subpopulations. *Int Immunol* (2002) **14**:463–9. doi:10.1093/intimm/14.5.463
33. Nestler EJ, Hyman SE. Animal models of neuropsychiatric disorders. *Nat Neurosci* (2010) **13**:1161–9. doi:10.1038/nn.2647
34. Yun J, Koike H, Ibi D, Toth E, Mizoguchi H, Nitta A, et al. Chronic restraint stress impairs neurogenesis and hippocampus-dependent fear memory in mice: possible involvement of a brain-specific transcription factor Npas4. *J Neurochem* (2010) **114**:1840–51. doi:10.1111/j.1471-4159.2010.06893.x
35. Voorhees JL, Tarr AJ, Wohleb ES, Godbout JP, Mo X, Sheridan JF, et al. Prolonged restraint stress increases IL-6, reduces IL-10, and causes persistent depressive-like behavior that is reversed by recombinant IL-10. *PLoS One* (2013) **8**:e58488. doi:10.1371/journal.pone.0058488
36. Bessa JM, Ferreira D, Melo I, Marques F, Cerqueira JJ, Palha JA, et al. The mood-improving actions of antidepressants do not depend on neurogenesis but are associated with neuronal remodeling. *Mol Psychiatry* (2009) **14**(764–773):739. doi:10.1038/mp.2008.119
37. Dominguez-Gerpe L, Rey-Mendez M. Time-course of the murine lymphoid tissue involution during and following stressor exposure. *Life Sci* (1997) **61**:1019–27. doi:10.1016/S0024-3205(97)00606-1
38. Ashwell JD, Lu FW, Vacchio MS. Glucocorticoids in T cell development and function. *Annu Rev Immunol* (2000) **18**:309–45. doi:10.1146/annurev.immunol.18.1.309
39. Zivkovic IP, Rakin AK, Petrovic-Djergovic DM, Kosec DJ, Micic MV. Exposure to forced swim stress alters morphofunctional characteristics of the rat thymus. *J Neuroimmunol* (2005) **160**:77–86. doi:10.1016/j.jneuroim.2004.11.002
40. Ulrich-Lai YM, Figueiredo HF, Ostrander MM, Choi DC, Engeland WC, Herman JP. Chronic stress induces adrenal hyperplasia and hypertrophy in a subregion-specific manner. *Am J Physiol Endocrinol Metab* (2006) **291**:E965–73. doi:10.1152/ajpendo.00070.2006
41. Sousa N, Almeida OF. Disconnection and reconnection: the morphological basis of (mal)adaptation to stress. *Trends Neurosci* (2012) **35**:742–51. doi:10.1016/j.tins.2012.08.006
42. Taliaz D, Loya A, Gersner R, Haramati S, Chen A, Zangen A. Resilience to chronic stress is mediated by hippocampal brain-derived neurotrophic factor. *J Neurosci* (2011) **31**:4475–83. doi:10.1523/JNEUROSCI.5725-10.2011
43. Cerqueira JJ, Pego JM, Taipa R, Bessa JM, Almeida OF, Sousa N. Morphological correlates of corticosteroid-induced changes in prefrontal cortex-dependent behaviors. *J Neurosci* (2005) **25**:7792–800. doi:10.1523/JNEUROSCI.1598-05.2005
44. Sousa N, Cerqueira JJ, Almeida OF. Corticosteroid receptors and neuroplasticity. *Brain Res Rev* (2008) **57**:561–70. doi:10.1016/j.brainresrev.2007.06.007
45. Wohleb ES, Powell ND, Godbout JP, Sheridan JF. Stress-induced recruitment of bone marrow-derived monocytes to the brain promotes anxiety-like behavior. *J Neurosci* (2013) **33**:13820–33. doi:10.1523/JNEUROSCI.1671-13.2013
46. Heidt T, Sager HB, Courties G, Dutta P, Iwamoto Y, Zaltsman A, et al. Chronic variable stress activates hematopoietic stem cells. *Nat Med* (2014) **20**(7):754–8. doi:10.1038/nm.3589
47. Saffar AS, Ashdown H, Gounni AS. The molecular mechanisms of glucocorticoids-mediated neutrophil survival. *Curr Drug Targets* (2011) **12**:556–62. doi:10.2174/138945011794751555
48. Selye H. A syndrome produced by diverse nocuous agents. *Nature* (1936) **138**:32–32. doi:10.1038/138032a0

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Dysregulated hypothalamic–pituitary–adrenal axis function contributes to altered endocrine and neurobehavioral responses to acute stress

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Organisms react to environmental challenges by activating a coordinated set of brain–body responses known as the stress response. These physiological and behavioral countermeasures are, in large part, regulated by the neuroendocrine hypothalamic–pituitary–adrenal (HPA) axis. Normal functioning of the HPA axis ensures that an organism responds appropriately to altered environmental demands, representing an essential system to promote survival. Over the past several decades, increasing evidence supports the hypothesis that disruption of the HPA axis can lead to dysregulated stress response phenotypes, exacting a physiological cost on the organism commonly referred to as allostatic load. Furthermore, it has been recognized that high allostatic load can contribute to increased vulnerability of the organism to further challenges. This observation leads to the notion that disrupted HPA function and resulting inappropriate responses to stressors may underlie many neuropsychiatric disorders, including depression and anxiety. In the present set of studies, we investigate the role of both the normally functioning and disrupted HPA axis in the endocrine, neural, and behavioral responses to acute stress. Using a model of non-invasive chronic corticosterone treatment in mice, we show that dysregulating the normal function of the HPA leads to a mismatch between the hormonal and neural response to acute stress, resulting in abnormal behavioral coping strategies. We believe this model can be leveraged to tease apart the mechanisms by which altered HPA function contributes to neurobehavioral dysregulation in response to acute stress.

Keywords: corticosterone, allostatic load, prefrontal cortex, hippocampus, *c-Fos*

INTRODUCTION

The term stress carries with it negative connotations, but it describes an important neurobehavioral and physiological response that is essential for survival. In response to an environmental stressor, the body quickly orchestrates changes in brain activity followed shortly thereafter by secretion of "stress mediators," including cytokines, metabolic hormones, and corticosteroids (1–4). These circulating chemical messages then act at peripheral tissues and in the brain to modify physiological processes and behavioral outputs (1, 4). The primary neuroendocrine axis that regulates the stress response in mammals is the hypothalamic–pituitary–adrenal (HPA) axis. Activation of this system is initiated by projections from the brainstem and limbic system to the paraventricular nucleus (PVN) of the hypothalamus (2–4). Upon activation, corticotropin-releasing hormone (CRH) cells in the PVN secrete CRH onto proopiomelanocortin (POMC)-containing cells at the median eminence, triggering release of adrenocorticotropic hormone (ACTH) into the circulation via activation of CRH receptors (2–6). As circulating ACTH levels increase, melanocortin-2 receptors in the adrenal glands are activated, leading to secretion of glucocorticoid hormones (7), primarily cortisol in humans and corticosterone (CORT) in rodents. Circulating CORT causes increased metabolic activity in peripheral tissues and acts centrally at the brain via glucocorticoid receptors (GRs) in order to induce negative feedback in the circuit (1, 8). This negative feedback largely occurs via indirect connections to the hypothalamus [through the bed nucleus of the stria terminalis (BNST) as well as through changes in endocannabinoid signaling in limbic, cortical, and subcortical brain regions] (1, 4, 9, 10). It is well known that two main CORT responsive brain regions – the medial prefrontal cortex (mPFC) and hippocampal formation – act both directly and indirectly to inhibit the drive to CORT production at the level of the amygdala and the PVN (1–4), thus closing the loop and terminating the acute stress response.

The stress response orchestrated by the HPA axis is well-choreographed multi-system response, involving behavioral, physiological, and metabolic responses, with tightly regulated components that need to become active at the appropriate times and in the appropriate contexts (11, 12). Thus, it is not surprising that disruptions in the biological response to stress can lead to changes in neuronal function, behavior, and immune function (13–18). Disorders related to disrupted HPA function include depression, post-traumatic stress disorder (PTSD), metabolic dysfunction, and anxiety disorders (8, 19–22). These disorders affect millions worldwide, inflicting significant economic and personal

costs. Developing animal models to understand how dysfunctions in stress responses lead to altered behavioral outputs is an important goal for modern neuroscience, and could lead to direct translational benefits once mechanisms have been elucidated. Thus, determining common themes in stress-related disorders will be a useful approach in isolating relevant factors for further mechanistic investigation.

Since many neuropsychiatric disorders are associated with changes in basal HPA function, as well as with altered responses to stressors, identifying the hormonal and neurobehavioral systems that are affected could provide important insights as to potential mechanisms by which these changes occur. Using low-dose CORT administered in the drinking water of adult mice, our goal was to determine how this treatment affects endocrine, neural, and behavioral responses to acute stress. Our results show that this non-invasive disruption of the HPA can fundamentally alter both neural and behavioral responses to acute stress and demonstrate that this can be a viable model for dissecting the mechanisms by which HPA dysregulation results in inappropriate neurobehavioral responses.

MATERIALS AND METHODS

ANIMALS AND CORTICOSTERONE TREATMENT

Adult male C57/BL6 mice (aged 35–42 days) were obtained from Harlan Laboratories, Inc. Animals were allowed 1 week acclimatization time upon arrival prior to treatment. Animals were then treated with vehicle (1% ethanol) or CORT (Sigma Aldrich, St. Louis, MO, USA; 25 μg/ml in 1% EtOH) for 28 days prior to testing or tissue collection (23–25). Animal weights were measured weekly and solutions were replaced during that time. All animal experiments were conducted with approval of the Washington State Institutional Animal Care and Use Committee.

FORCED-SWIM TEST

Acute stress was accomplished using the forced-swim test (FST). Animals were subjected to 10-min FST (4 l glass beaker, water temperature of 21–23°C) at ZT6. Following FST, animals were allowed to recover for 10 min in their home cage prior to the open-field test (OFT), blood collection, or RT-PCR. Time spent struggling (defined as limb movements in excess of those needed to simply stay afloat) or time spent immobile was scored by an experimenter blinded to the conditions. Swim chambers were emptied, cleaned, and refilled between each trial.

CORTICOSTERONE AND ADRENOCORTICOTROPIC HORMONE RIA

To determine changes in diurnal plasma CORT following vehicle or chronic CORT treatment, a group of mice ($N = 4$–5/group/time point) was killed by rapid decapitation at 6 h intervals at zeitgeber times 0, 6, 12, and 18 (with ZT0 being time of lights ON, and ZT12 being time of lights OFF). To determine endocrine stress responses, a separate group of mice was killed 10 min after the 10-min FST by rapid decapitation ($N = 4$–5/group/treatment). In both cases, trunk blood was collected in EDTA-coated tubes. Brains from the FST group were then used to detect stress-induced mRNA changes via RT-PCR (see below). Blood was spun at 1500 rcf for 15 min in a refrigerated centrifuge, and plasma removed, aliquoted, and stored at −80°C until assay. Plasma was assayed for total CORT using the Siemens Coat-a-Count kit for rat CORT according to the manufacturer's protocol. To further probe the endocrine response, plasma ACTH was measured in a previously unthawed plasma aliquot from a subset of stressed vehicle- or CORT-treated mice ($N = 5$/group), with an ACTH 125I Kit (DiaSorin, Inc., Stillwater, MN, USA) using the option A (overnight incubation) protocol. For both assays, tubes were counted on a Packard Cobra II gamma counter, and the intrassay coefficient of variability was less than 10%.

NOVEL OPEN-FIELD TEST

To test behavioral responses following FST, an OFT was conducted. The OFT consisted of a white plastic chamber (27.5 cm × 27.5 cm × 20 cm). Lighting was set at 13–18 lux at floor level. Ten minutes following the end of FST (or at ZT6 for unstressed controls, $N = 8$/group/treatment), each animal was placed in the corner of the chamber and allowed to roam freely for 5 min before being removed. The chamber was cleaned with 70% ethanol after each trial. Locomotor behavior was scored digitally using Noldus Ethovision XT software (Leesburg, VA, USA). Animal location was determined by the center point of the animal.

IN SITU HYBRIDIZATION FILM AUTORADIOGRAPHY

To detect CRH mRNA, *in situ* hybridization and film autoradiography was used as previously described (26), with a mouse CRH riboprobe generously provided by Dr. A. Jasnow, Kent State University, OH, USA (27). Briefly, a separate group of unstressed vehicle- or CORT-treated mice (25 μg/ml; $N = 4$–5/group) was killed via rapid decapitation at ZT6, and brains were removed and immediately frozen on dry ice. Brain sections were made on a cryostat (20 μm thick) and mounted on slides. Slide-mounted sections were fixed in 3.7% formaldehyde for 10 min, rinsed in a series of phosphate-buffered saline (PBS) washes, followed by rinses in triethanolamine-HCl (TEA) and TEA with acetic anhydride. Slides were then washed in 2× sodium chloride citrate (SCC) and dehydrated in a series of graded alcohols (70, 95, and 100%), delipidated in chloroform, and rinsed in 100% alcohol. For pre-hybridization, slides were exposed to hybridization solution (225 μl/slide), coverslipped, placed in a humidity chamber, and incubated at 55°C for 1 h. Slides were then washed in 2× SCC and again dehydrated in a series of graded alcohols (70 and 95%). For hybridization, slides were exposed to hybridization buffer (225 μl/slide) with the 35S-labeled antisense or sense ribonucleotide probes (approximately 1×10^6 cpm/slide), coverslipped, placed in a humidity chamber, and incubated at 55°C overnight. Following hybridization, slides were washed in 2× SCC buffer and incubated with RNase A (10 μg/ml) in digestion buffer at 37°C for 30 min and then digestion buffer alone for 10 min. Slides were then rinsed in series of 2× SCC and 0.2× SCC washes at 55°C and dehydrated in a series of graded alcohols. Slides were air dried for 24 h and then apposed to Kodak BioMax MR film (Sigma) for 2 days to generate autoradiograms.

QUANTIFICATION OF CRH mRNA

Relative optical densities (RODs) were measured from the autoradiograms using computerized image analysis software (MCID-M4, Imaging Research, Inc., St. Catharines, Canada). Background

measurements were made from areas adjacent to the PVN and subtracted from the ROD. The same size circular tool, smaller than the size of the brain region (PVN or amygdala) being measured, was used to ensure that the same size sample was measured from section to section, and mouse to mouse. At least two bilateral measurements were made for each mouse.

QUANTITATIVE RT-PCR

As above, brains from each group were collected 10 min after the end of a 10-min FST ($N = 4$–5/group/treatment), and frozen immediately on dry ice. Brain regions of interest were punched using 0.5 mm ID biopsy corer (Fine Science Tools, Foster City, CA, USA) and kept at −80°C until extraction. mRNA isolation was performed using Qiazol (Trizol-chloroform) extraction with RNeasy column clean-up (Qiagen, Valencia, CA, USA). Samples were stored at −80°C in 1 mM sodium citrate, pH 6.4 (Life Technologies, Grand Island, NY, USA) prior to cDNA synthesis. mRNA concentrations were measured using spectrophotometry and diluted to the same concentration for all samples. cDNA synthesis was preformed with MultiScribe™ MuLV reverse-transcriptase following the protocol for the high capacity cDNA synthesis kit (Life Technologies). cDNA was stored at −20°C. cDNA synthesis reaction was preformed in triplicate for vHC samples. Assays were performed using the TaqMan chemistry and off the shelf assays from Life Technologies. Assay IDs were: Gapdh-Mm99999915_g1 and cFos-Mm00487425_m1. Samples were run in triplicate on a Life Technologies 7900HT real-time PCR machine with a 20 µl reaction volume. Samples were compared using the $\Delta\Delta C_T$ method of relative quantification. GAPDH was used to normalize between biological replicates.

STATISTICS

All statistical analyses were accomplished using Prism 5 (GraphPad Software, La Jolla, CA, USA). Two-tailed *t*-tests and one-way or two-way ANOVAs were undertaken where appropriate, and Tukey *post hoc* tests were used to probe interactions. In all cases, results were considered statistically significant at the $P = 0.05$ level.

RESULTS

CORT TREATMENT DOES NOT AFFECT BODY WEIGHT

After 4 weeks of treatment, no statistically significant difference was observed in weight gained between chronic CORT-treated animals and vehicle-treated animals (data not shown; $F_{1,37} = 0.7057$, $P = 0.4063$), replicating results from our previous work in this model (23, 25).

CORT TREATMENT RESULTS IN ALTERED DIURNAL PATTERNS OF PLASMA CORT

After 4 weeks of treatment, mice ($N = 4$–5/group) were killed at one of four time points: lights ON (ZT0), mid-light (ZT6), lights OFF (ZT12), and mid-night (ZT18). A significant main effect was detected for both treatment (two-way ANOVA; $F_{1,25} = 44.29$, $P < 0.0001$) and time ($F_{3,25} = 13.39$, $P < 0.0001$), as well as a significant interaction ($F_{3,25} = 16.75$, $P < 0.0001$). Tukey *post hoc* analysis showed that chronic CORT resulted in higher plasma CORT levels at both ZT18 and ZT0 (both $P < 0.001$), but nadir levels were statistically indistinguishable from vehicle-treated mice at ZT6 and ZT12 (both $P > 0.05$; **Figure 1**).

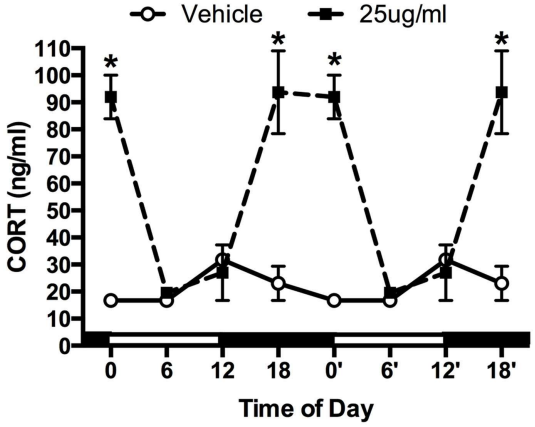

FIGURE 1 | Chronic low-dose CORT treatment in the drinking water maintains rhythmic, yet slightly higher, levels of plasma CORT. Treatment with CORT (25 µg/ml) in the drinking water resulted in rhythmic diurnal changes in plasma CORT that reached a peak during the dark phase (ZT12–ZT0), but reached the same nadir levels as vehicle-treated mice (ZT6). Data from 0′ to 18′ are re-plotted from 0 to 18 to aid in visualization of the rhythm. Asterisks indicate statistically significant effects at the $P < 0.05$ level.

CORT TREATMENT RESULTS IN A BLUNTING OF THE ENDOGENOUS STRESS RESPONSE

To determine how chronic CORT affects the peripheral (endocrine) stress response, we exposed CORT-treated mice to an acute FST stress. Given that ZT6 basal CORT levels were not different between vehicle- and chronic CORT-treated mice, we tested the endogenous hormonal stress response 10 min after a 10-min FST ($N = 4$–5/group). We found main effects of both CORT treatment (two-way ANOVA, $F_{1,15} = 161.6$, $P < 0.0001$) and stress ($F_{1,15} = 187.7$, $P < 0.0001$), and a significant interaction ($F_{1,15} = 106.0$, $P < 0.0001$). Tukey *post hoc* analysis showed that FST resulted in increased CORT only in vehicle-treated mice ($P < 0.05$), while stressed chronic CORT mice showed no increase in plasma CORT over basal levels ($P > 0.05$). There were no differences in non-stressed levels of plasma CORT between vehicle- and chronic CORT-treated mice (**Figure 2**). In a subset of only FST mice ($N = 4$–5/group), we also measured plasma ACTH at the same time point as CORT (Figure S1 in Supplementary Material), showing that CORT treatment blocked the normal increase in plasma ACTH following FST (*t*-test, $t = 5.257$, $P = 0.0012$). This suggests the endogenous hormonal stress response is impaired in CORT-treated mice.

CHRONIC CORT TREATMENT REDUCES CORTICOTROPIN-RELEASING HORMONE mRNA IN THE PARAVENTRICULAR NUCLEUS, BUT NOT THE AMYGDALA

To determine the effects of chronic CORT on central HPA function, using *in situ* hybridization, we assayed CRH mRNA levels following vehicle or CORT treatment ($N = 4$/group) within the PVN of the hypothalamus, a key central node in the HPA axis, and the amygdala (**Figure 3**). We found a main effect of CORT

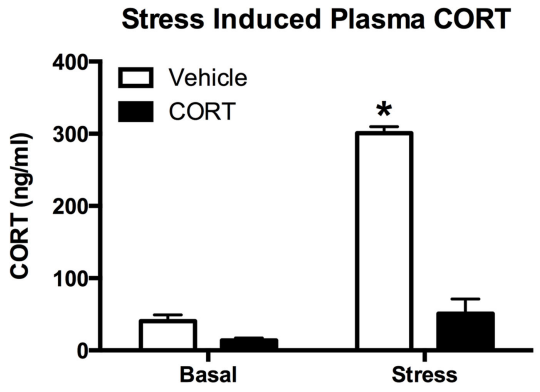

FIGURE 2 | Chronic low-dose CORT treatment blocks the normal endocrine response to stress. Treatment with CORT (25 μg/ml) in the drinking water for 4 weeks resulted in reduced CORT secretion following a 10-min forced-swim stress at ZT6, with no effect on basal plasma CORT levels. Asterisk indicates statistically significant difference at the $P < 0.05$ level.

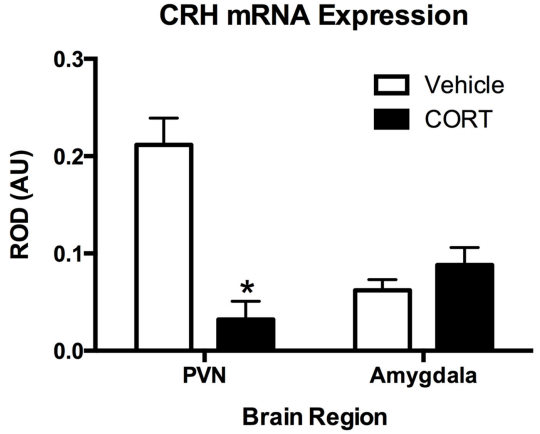

FIGURE 3 | Chronic low-dose CORT treatment reduced CRH mRNA expression in the PVN but does not affect expression in the amygdala. *In situ* hybridization showed that treatment with CORT (25 μg/ml) in the drinking water for 4 weeks resulted in a statistically significant decrease in CRH mRNA expression in the PVN, but not in the amygdala. Asterisk indicates statistically significant difference at the $P < 0.05$ level.

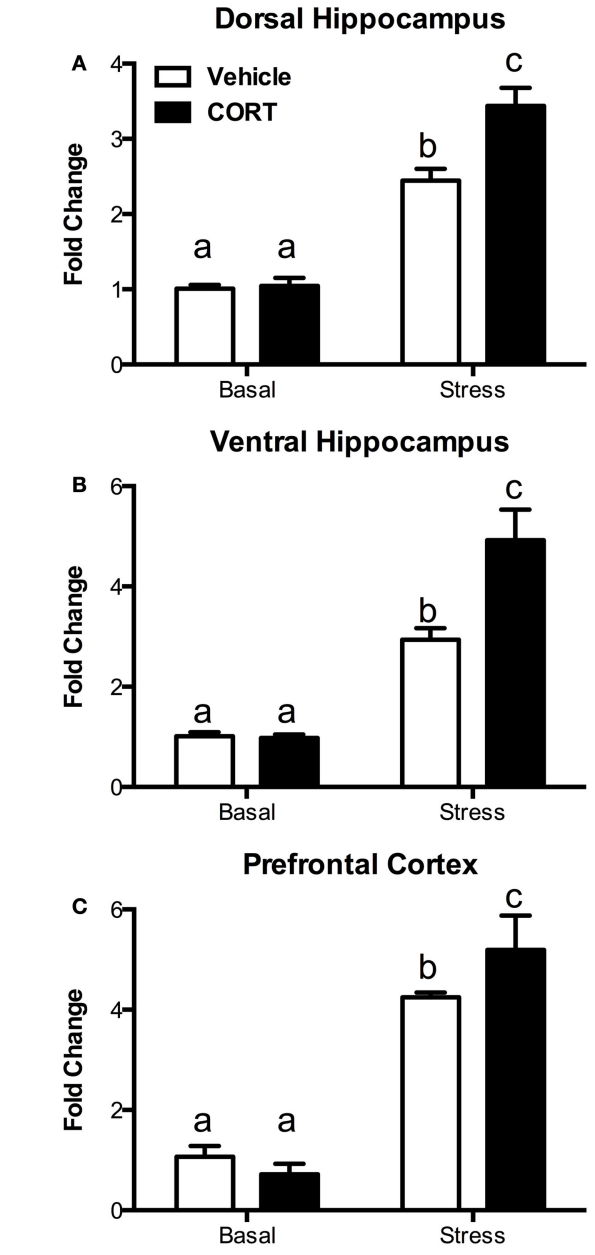

FIGURE 4 | Treatment with CORT in the drinking water results in exaggerated stress-induced *c-Fos* responses in the brain. Following a 10-min forced-swim stress and a 10-min recovery period, CORT (25 μg/ml) drinking water-treated mice showed an exaggerated *c-Fos* mRNA response as measured by RT-PCR in **(A)** the dorsal hippocampus, **(B)** the ventral hippocampus, and **(C)** the prefrontal cortex. CORT treatment did not affect basal non-stressed levels of *c-Fos*. Columns that share the same letter are not statistically different from each other at the $P < 0.05$ level.

treatment and brain region (two-way ANOVA, $F_{1,12} = 15.15.$, $P = 0.002$; $F_{1,12} = 5.602$, $P = 0.0356$), and a significant interaction ($F_{1,12} = 27.21$, $P = 0.0002$). Tukey *post hoc* analysis demonstrates that CRH mRNA levels are reduced in the PVN following 4 weeks of CORT treatment ($P < 0.05$), while CRH mRNA in the amygdala remained unaffected, compared to controls.

STRESS RESULTS IN EXAGGERATED *c*-Fos mRNA EXPRESSION IN CORT-TREATED MICE

To determine if the blunting of the hormonal stress response was accompanied by changes in neural activation following the stress, we investigated changes in *c-Fos* mRNA using quantitative RT-PCR in the dorsal and ventral hippocampus (dHipp and vHipp), as well as in the prefrontal cortex (PFC) 10 min after the termination of a 10-min FST (**Figure 4**, $N = 4$–5/group).

In the dHipp (**Figure 4A**), we found significant main effects of both stress (two-way ANOVA, $F_{1,15} = 149.4$, $P < 0.0001$) and CORT treatment ($F_{1,15} = 10.81$, $P = 0.005$), as well as a significant

interaction ($F_{1,15} = 9.366$, $P = 0.0079$). Tukey *post hoc* analysis showed that while stress increased dHipp *c-Fos* mRNA, the increase was greater in the chronic CORT-treated mice ($P < 0.05$), while there was no difference in non-stressed basal *c-Fos* expression.

Similarly, in the vHipp (**Figure 4B**), we found significant main effects of stress ($F_{1,14} = 64.81$, $P < 0.0001$) and chronic CORT treatment ($F_{1,14} = 7.150$, $P = 0.0182$), as well as a significant interaction ($F_{1,14} = 7.655$, $P = 0.0151$). *Post hoc* analysis indicated that, as in the dHipp, stress increased *c-Fos*, but this increase was greater in the chronic CORT mice ($P < 0.05$), without any differences in the non-stressed basal expression of *c-Fos*.

In the PFC (**Figure 4C**), while we found a significant main effect of stress ($F_{1,15} = 373.2$, $P < 0.0001$), we did not find a statistically significant main effect of CORT treatment ($F_{1,15} = 2.282$, $P = 0.1516$). However, there was a significant interaction ($F_{1,15} = 10.75$, $P = 0.0051$), with Tukey *post hoc* tests revealing a similar pattern where CORT-treated mice show enhanced *c-Fos* expression in the PFC following stress compared to vehicle-treated mice ($P < 0.05$), with no effect on non-stressed basal *c-Fos* expression.

Thus, in all instances, while chronic CORT does not affect basal non-stressed *c-Fos* expression, it does result in a statistically significant increase in *c-Fos* expression following FST in the dHipp, vHipp, and PFC.

CHRONIC CORT TREATMENT RESULTS IN ALTERED BEHAVIORAL RESPONSES FOLLOWING STRESS

Given the altered hormonal and gene expression responses following acute FST stress in CORT-treated mice, we next wanted to determine if prior CORT treatment causes changes in acute stress-induced behavior. To these ends, we investigated stress-induced changes in swim behaviors in the FST, as well as rearing and grooming behavior in a novel open-field environment in vehicle- or CORT-treated mice, with or without stress ($N = 8$/group/treatment; **Figure 5**).

Behavior in the FST

Struggling in the FST was scored to determine if there was any effect of chronic low-dose CORT treatment on activity in the FST that may contribute to altered responses in rearing or grooming. We found that 4 weeks of chronic CORT did not affect time struggling or time spent immobile in the FST (**Figure 5A**; two-tailed *t*-test, $t = 1.594$, $P = 0.13$). We also found no effect of low-dose CORT on latency to immobility (data not shown; vehicle 58.63 ± 4.769 s vs. CORT 51.25 ± 2.782 s; two-tailed *t*-test, $t = 1.336$, $P = 0.203$).

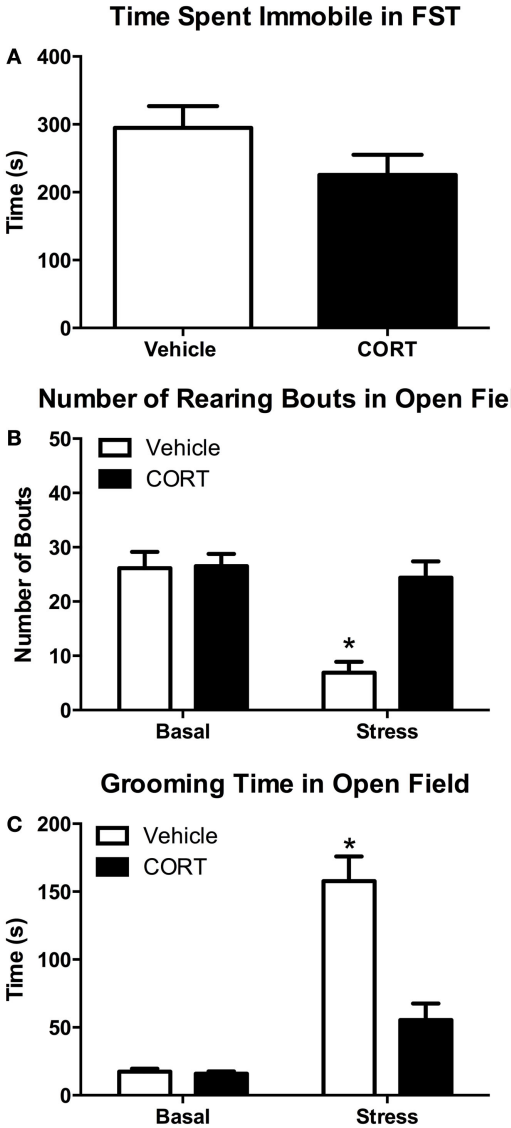

FIGURE 5 | Chronic low-dose CORT does not affect behavior in the FST, but blocks normal stress-induced anxiety behaviors in a novel environment after the FST. Four weeks of CORT (25 μg/ml) in the drinking water does not alter time spent immobile in the FST **(A)**. Following the FST, vehicle-treated mice show subsequent anxiety-like behavior in an open field as measured by decreased rearing **(B)** and increased grooming **(C)**, while CORT-treated mice are statistically indistinguishable from controls, suggesting they do not show normal stress-induced anxiety-like behavior. Asterisks indicate statistically significant differences at the $P < 0.05$ level.

Rearing behavior

One episode of forced-swim stress resulted in statistically significant differences in the frequency of rearing behavior in the OFT (**Figure 5B**). While there was a main effect of FST stress ($F_{1,28} = 16.64$, $P = 0.0003$) and CORT treatment ($F_{1,28} = 11.64$, $P = 0.0020$) on rearing frequency, there was also a statistically significant interaction between stress and CORT treatment ($F_{1,28} = 10.68$, $P = 0.0029$). *Post hoc* analyses revealed that VEH mice showed a significant decrease in grooming ($P < 0.0003$). However, no statistically significant difference in rearing frequency was observed between stress/CORT animals and non-stress/VEH animals ($P = 0.9645$) or non-stress/CORT animals ($P = 0.9392$), suggesting chronic CORT blocked the normal stress-induced decreases in rearing behavior.

Grooming behavior

Similar to the effects on rearing behavior, FST and CORT treatment resulted in main effects on grooming duration (**Figure 5C**;

$F_{1,27} = 62.36$, $P < 0.0001$ and $F_{1,27} = 20.83$, $P < 0.0001$, respectively). However, there was also a statistically significant interaction between stress and CORT treatment ($F_{1,27} = 19.60$, $P < 0.0001$). *Post hoc* analyses revealed that FST increased grooming duration in VEH-treated mice ($P < 0.0001$). However, no statistically significant difference in grooming duration was observed between stress/CORT mice and non-stress/VEH mice ($P = 0.1191$), or non-stress/CORT mice ($P = 0.0832$), further supporting the notion that CORT induced disruption of the HPA blocks normal behavioral responses to acute stress.

DISCUSSION

The stress response is an essential brain–body response that protects homeostasis and ensures survival in the face of threatening environmental stimuli (12, 28–30). The coordinated response following stress allows for mediators of allostasis to mobilize and allow an organism to adapt to a temporary environmental shift. As such, these responses can be considered protective, and in fact, healthy allostatic responses are a sign of resilience (31–33). However, if allostatic mediators are over active, improperly regulated, poorly terminated, or engaged inappropriately, allostatic load can increase. Eventually this results in allostatic overload, which, rather than protecting the organism, leads to a series of cascading failures in multiple physiological systems. Thus, the systems that usually impart resilience instead result in increased vulnerability (12, 31, 32, 34). Given the central role of the HPA axis stress response in allostatic responses, we probed how our model of disrupted HPA function might lead to altered neural and behavioral outputs, and perhaps contribute to negative mental and physical health outcomes.

Disruption of normal HPA function is a hallmark of a varied set of physical and neuropsychiatric diseases, such as obesity, depression, and anxiety (21, 22, 35). For example, PTSD is associated with dysregulated HPA function. Even though some reports suggest a hyperactive HPA is related to development of PTSD (36, 37), while others propose that HPA hypoactivity may be a key factor (38–40), the overarching theme remains: that in PTSD the HPA is disrupted and does not respond normally to stressors. Whether these changes in HPA activity are a cause or consequence of such disease states is unclear, though it is feasible that disrupting the normal responses to environmental stress could predispose individuals to negative health outcomes. In the present study, we explored how disruption of the HPA via a non-invasive drinking water treatment could change the normal neuroendocrine stress response, and how this change would affect both neural and behavioral outputs following exposure to an acute stress.

The present study used a non-invasive manipulation of the HPA that maintains near normal diurnal changes in plasma CORT, but interferes with the neuroendocrine stress response. This model is a variant of more classic approaches used in adrenalectomized rats (41, 42). In our model, mice treated with a low-dose (25 μg/ml) of CORT in the drinking water for 4 weeks show normal growth curves (23) and show a diurnal rhythm in plasma CORT that is somewhat higher, and peaks slightly later, than CORT in unmanipulated mice (**Figure 1**). In the present study, we further report that this model results in the shutdown of the normal endocrine response to forced-swim stress, with no increase in plasma CORT observed following the stress (**Figure 2**). This is likely due to both peripheral, as we have previously demonstrated that it also leads to atrophy of the adrenal gland (23, 25), as well as central effects, since we observed a decrease in PVN CRH mRNA (**Figure 3**). Given this altered plasma CORT response, the purpose of the second part of the study was to determine if disruption of the HPA response would result in differential neural and behavioral responses to acute stress.

Acute stress results in a sequence of neural and behavioral responses, and perturbations of these normal responses may indicate underlying faults in the neural and endocrine systems regulating stress reactivity. Following stress, brain *c-Fos* expression is increased in the PFC and HIPP, as well as in a number of other brain areas (43–45), suggesting increased neuronal activity. However, the consequences of altered HPA function for stress-induced *c-Fos* expression are unclear. Some reports indicate that ADX has no effect on acute stress-induced *c-Fos* mRNA (46), while others describe increases in stress-induced *c-Fos* expression following ADX (47, 48). Given these somewhat conflicting reports, we chose to explore both neural and behavioral responses in a model of HPA dysfunction. In our model, the normally observed increase in plasma CORT following stress is abolished (**Figure 2**), and levels of PVN CRH mRNA are reduced (**Figure 3**). This led us to posit that normal neural responses to stress would also be blunted, given that the primary neuroendocrine output of the stress response is greatly reduced. However, our hypothesis was proven incorrect, and instead we observed increased neural responses in the brain regions we explored (**Figure 4**).

Following stress, many brain regions in addition to the PFC and HIPP show increased activity (2, 3, 46, 49), forming an almost brain-wide network from the brain stem through to the forebrain. For instance, in response to a novel environmental stressor, noradrenergic neurons of the locus ceruleus (LC) rapidly increase firing rate. These cells are known to project to forebrain regions such as the PFC and HIPP where they play a largely excitatory role (50, 51). Many of these same areas are also intimately involved in the negative feedback aspect of the stress response, with activation of the GR serving to reduce their activity. For example, in ADX rats, a single bolus injection of CORT leads to a decrease in *c-Fos* expression in the dorsal HIPP within 45 min (52). In the present study, we determined that even though our model results in decreased plasma CORT in response to stress (**Figure 2**), neural activation (as measured by *c-Fos* mRNA) is significantly increased in the PFC, as well as the dorsal and ventral hippocampus (dHipp and vHipp; **Figure 4**). These novel and unexpected findings have led us to hypothesize that the lack of a normal endogenous CORT response in exogenous CORT-treated animals may deprive these circuits of a key negative feedback signal. Such attenuation of neural activity by glucocorticoids has been observed in LC noradrenergic neurons, where activation of the GR in LC neurons decreases tyrosine hydroxylase mRNA and presumably reduces neuronal activity (53). This leads to a possible mechanism that explains the observed increased *c-Fos* expression in the mPFC and hippocampus in CORT-treated animals: i.e., it is possible that without the inhibitory drive of CORT to the LC following a stressor, mPFC and hippocampal activity is increased above "normal" levels via overstimulation by noradrenergic LC afferents to

these regions. Such an outcome could be tested in future studies by specifically manipulating different components of this circuit, with or without chronic CORT treatment.

Given the obvious differences in both hormonal and neural responses to stress in CORT-treated mice, and to clarify the functional consequences of these altered responses, we next wanted to explore changes in behavioral outputs following stress. It is well documented that acute stress can lead to increased anxiety-like behaviors in rodents on a variety of measures, including the elevated plus maze, open field, and social avoidance (54–58). These alterations in behavior are mediated by both neural and hormonal changes during and following the stressor. To determine the behavioral consequences of a blunted hormonal stress response, but exaggerated neural stress response, we analyzed behavioral changes in the novel environment of an open field following a forced-swim stress (**Figure 5**). Rearing in a novel open field can be considered a form of exploratory behavior (59), associated with positive affective states and thus indicative of low anxiety (60). Our results indicate that in response to an acute stressor, animals chronically treated with CORT fail to display the normally observed decrease in rearing behavior. From this, we conclude that CORT treatment reduces anxiety responses normally elicited by a novel acute stress. Similarly, self-grooming behavior in rodents has been associated with high levels of anxiety (61, 62). In the present study, we observe lower duration of grooming in CORT-treated animals compared to those treated with vehicle in response to an acute stress. Chronic CORT-treated animals resemble non-stressed animals in that grooming duration does not significantly increase following stress. Thus, we conclude that the failure to increase grooming duration following acute stress in CORT-treated compared to vehicle-treated mice again suggests a lower anxiety state in CORT-treated mice. It is important to note that we did not find behavioral differences between the vehicle and CORT groups in the FST (**Figure 5A**), suggesting the neural and behavioral responses after FST are not simply due to differences in amount of exercise or stress coping strategies. Gourley et al. noted a slight increase in immobility in the tail suspension test using a similar model (63), but it is important to note that their results used CORT hemisuccinate (that may alter bioavailability), and a 3-day "washout" period, making direct comparison difficult as our mice were still being treated with CORT at time of testing. We conclude that disrupting the HPA axis using this model of chronic CORT in the drinking water blocks normal behavioral responses to acute stress.

The behavior of CORT-treated mice following acute stress is statistically indistinguishable from non-stressed mice with respect to grooming and rearing behaviors in the open field (**Figure 5**). On the other hand, activity in the PFC and HIPP clearly shows significant increase in *c-Fos* expression, to a level greater than both stressed vehicle mice as well as non-stressed vehicle or CORT mice (**Figure 4**). Thus, there appears to be a mismatch between the neural and behavioral responses to stress in CORT-treated mice that have a dysregulated HPA. It is intriguing to consider the ramifications of such "inappropriate" behavioral and neural responses following stress. One interpretation could be that the inability of CORT-treated mice to increase plasma CORT levels following a stressor results in a protective phenotype that does not show increases in anxiety-like behavior. However, such resilience should not necessarily be construed as a beneficial consequence to the organism, as blunted reactions to stress could be maladaptive. Acute environmental stressors produce necessary neurobehavioral responses that improve chances for survival in challenging environments. For instance, in many cases, memory and learning processes are increased by acute stress (64–66), with the presumed benefit of allowing for future avoidance of the stressor or context in which the stressor occurred (67). Restated, behavioral, and physiological phenotypes normally expressed following exposure to acute stress are thought to be adaptive. Thus, reduction of these behaviors by HPA dysregulation observed here could be described as maladaptive. This suggests it is equally plausible to consider the alternative interpretation: that the lack of a behavioral response following a stressor, in association with increased activity in the PFC and HIPP, may have negative consequences because the appropriate allostatic mediators are not being adequately mobilized, and/or their termination is not being efficiently regulated. Perhaps in the short term, such responses have little negative impact, but repeated stress exposure without the normal hormonal, neural, or behavioral responses may contribute to increased allostatic load and eventual long-term negative outcomes.

The results of our study suggest the low-dose CORT model (without ADX) may be useful in the investigation of disorders in which a dysregulation of the HPA axis, or other aspects of the stress response, are observed, such as PTSD, depression, and anxiety disorders. As we have shown that many gross markers of adrenal status recover after removal of the CORT treatment (25), the reversibility of this model could provide insight into long-term effects of short-term HPA dysregulation. By devising models that can be dissected both anatomically and temporally, we may gain increased understanding of the neural and behavioral underpinnings of complex neuropsychiatric disorders.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fpsyt.2015.00031/abstract

REFERENCES

1. Hill MN, Tasker JG. Endocannabinoid signaling, glucocorticoid-mediated negative feedback, and regulation of the hypothalamic-pituitary-adrenal axis. *Neuroscience* (2012) **204**:5–16. doi:10.1016/j.neuroscience.2011.12.030
2. Herman JP, Cullinan WE. Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. *Trends Neurosci* (1997) **20**:78–84. doi:10.1016/S0166-2236(96)10069-2
3. Herman JP, Figueiredo H, Mueller NK, Ulrich-Lai Y, Ostander MM, Choi DC, et al. Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamic-pituitary-adrenocortical responsiveness. *Front Neuroendocrinol* (2003) **24**:151–80. doi:10.1016/j.yfrne.2003.07.001
4. Ziegler DR, Herman JP. Neurocircuitry of stress integration: anatomical pathways regulating the hypothalamo-pituitary-adrenocortical axis of the rat. *Integr Comp Biol* (2002) **42**(3):541–51. doi:10.1093/icb/42.3.541
5. Chowdrey HS, Larsen PJ, Harbuz MS, Jessop DS, Aguilera G, Eckland DJ, et al. Evidence for arginine vasopressin as the primary activator of the HPA axis during adjuvant-induced arthritis. *Br J Pharmacol* (1995) **116**(5):2417–24. doi:10.1111/j.1476-5381.1995.tb15089.x
6. Stephens MA, Wand G. Stress and the HPA axis: role of glucocorticoids in alcohol dependence. *Alcohol Res* (2012) **34**(4):468–83.

7. Beuschlein F, Fassnacht M, Klink A, Allolio B, Reincke M. ACTH-receptor expression, regulation and role in adrenocortical tumor formation. *Eur J Endocrinol* (2001) **144**(3):199–206. doi:10.1530/eje.0.1440199
8. Macfarlane DP, Forbes S, Walker BR. Glucocorticoids and fatty acid metabolism in humans: fuelling fat redistribution in the metabolic syndrome. *J Endocrinol* (2008) **197**(2):189–204. doi:10.1677/JOE-08-0054
9. Hill MN, McLaughlin RJ, Bingham B, Shrestha L, Lee TT, Gray JM, et al. Endogenous cannabinoid signaling is essential for stress adaptation. *Proc Natl Acad Sci U S A* (2010) **107**(20):9406–11. doi:10.1073/pnas.0914661107
10. Hill MN, McLaughlin RJ, Pan B, Fitzgerald ML, Roberts CJ, Lee TT, et al. Recruitment of prefrontal cortical endocannabinoid signaling by glucocorticoids contributes to termination of the stress response. *J Neurosci* (2011) **31**(29):10506–15. doi:10.1523/JNEUROSCI.0496-11.2011
11. McEwen BS, Stellar E. Stress and the individual: mechanisms leading to disease. *Arch Intern Med* (1993) **153**:2093–101. doi:10.1001/archinte.153.18.2093
12. McEwen BS. Stress, adaptation, and disease. Allostasis and allostatic load. *Ann N Y Acad Sci* (1998) **840**:33–44. doi:10.1111/j.1749-6632.1998.tb09546.x
13. Cerqueira JJ, Pego JM, Taipa R, Bessa JM, Almeida OF, Sousa N. Morphological correlates of corticosteroid-induced changes in prefrontal cortex-dependent behaviors. *J Neurosci* (2005) **25**(34):7792–800. doi:10.1523/JNEUROSCI.1598-05.2005
14. McLaughlin KJ, Baran SE, Conrad CD. Chronic stress- and sex-specific neuromorphological and functional changes in limbic structures. *Mol Neurobiol* (2009) **40**(2):166–82. doi:10.1007/s12035-009-8079-7
15. Shansky RM, Hamo C, Hof PR, McEwen BS, Morrison JH. Stress-induced dendritic remodeling in the prefrontal cortex is circuit specific. *Cereb Cortex* (2009) **19**(10):2479–84. doi:10.1093/cercor/bhp003
16. Flandreau EI, Ressler KJ, Owens MJ, Nemeroff CB. Chronic overexpression of corticotropin-releasing factor from the central amygdala produces HPA axis hyperactivity and behavioral anxiety associated with gene-expression changes in the hippocampus and paraventricular nucleus of the hypothalamus. *Psychoneuroendocrinology* (2012) **37**(1):27–38. doi:10.1016/j.psyneuen.2011.04.014
17. Johnson SA, Fournier NM, Kalynchuk LE. Effect of different doses of corticosterone on depression-like behavior and HPA axis responses to a novel stressor. *Behav Brain Res* (2006) **168**(2):280–8. doi:10.1016/j.bbr.2005.11.019
18. Webster JI, Tonelli L, Sternberg EM. Neuroendocrine regulation of immunity. *Annu Rev Immunol* (2002) **20**:125–63. doi:10.1146/annurev.immunol.20.082401.104914
19. de Kloet CS, Vermetten E, Geuze E, Kavelaars A, Heijnen CJ, Westenberg HG. Assessment of HPA-axis function in posttraumatic stress disorder: pharmacological and non-pharmacological challenge tests, a review. *J Psychiatr Res* (2006) **40**(6):550–67. doi:10.1016/j.jpsychires.2005.08.002
20. Gotlib IH, Joormann J, Minor KL, Hallmayer J. HPA axis reactivity: a mechanism underlying the associations among 5-HTTLPR, stress, and depression. *Biol Psychiatry* (2008) **63**(9):847–51. doi:10.1016/j.biopsych.2007.10.008
21. Pariante CM, Lightman SL. The HPA axis in major depression: classical theories and new developments. *Trends Neurosci* (2008) **31**(9):464–8. doi:10.1016/j.tins.2008.06.006
22. Shea A, Walsh C, Macmillan H, Steiner M. Child maltreatment and HPA axis dysregulation: relationship to major depressive disorder and post traumatic stress disorder in females. *Psychoneuroendocrinology* (2005) **30**(2):162–78. doi:10.1016/j.psyneuen.2004.07.001
23. Karatsoreos IN, Bhagat SM, Bowles NP, Weil ZM, Pfaff DW, McEwen BS. Endocrine and physiological changes in response to chronic corticosterone: a potential model of the metabolic syndrome in mouse. *Endocrinology* (2010) **151**(5):2117–27. doi:10.1210/en.2009-1436
24. Bowles NP, Hill MN, Bhagat SM, Karatsoreos IN, Hillard CJ, McEwen BS. Chronic, noninvasive glucocorticoid administration suppresses limbic endocannabinoid signaling in mice. *Neuroscience* (2012) **204**:83–9. doi:10.1016/j.neuroscience.2011.08.048
25. Cassano AE, White JR, Penraat KA, Wilson CD, Rasmussen S, Karatsoreos IN. Anatomic, hematologic, and biochemical features of C57BL/6NCrl mice maintained on chronic oral corticosterone. *Comp Med* (2012) **62**(5):348–60.
26. Romeo RD, Karatsoreos IN, Jasnow AM, McEwen BS. Age- and stress-induced changes in corticotropin-releasing hormone mRNA expression in the paraventricular nucleus of the hypothalamus. *Neuroendocrinology* (2007) **85**(4):199–206. doi:10.1159/000102950
27. Jasnow AM, Schulkin J, Pfaff DW. Estrogen facilitates fear conditioning and increases corticotropin-releasing hormone mRNA expression in the central amygdala in female mice. *Horm Behav* (2006) **49**(2):197–205. doi:10.1016/j.yhbeh.2005.06.005
28. Karatsoreos IN, McEwen B. Stress and allostasis. In: Steptoe A, editor. *Handbook of Behavioral Medicine.* New York, NY: Springer (2010). p. 649–58.
29. McEwen BS. Protective and damaging effects of stress mediators. *N Engl J Med* (1998) **338**:171–9. doi:10.1056/NEJM199801153380307
30. McEwen BS. Physiology and neurobiology of stress and adaptation: central role of the brain. *Physiol Rev* (2007) **87**(3):873–904. doi:10.1152/physrev.00041.2006
31. Karatsoreos IN, McEwen BS. Psychobiological allostasis: resistance, resilience and vulnerability. *Trends Cogn Sci* (2011) **15**(12):576–84. doi:10.1016/j.tics.2011.10.005
32. Karatsoreos IN, McEwen BS. Resilience and vulnerability: a neurobiological perspective. *F1000Prime Rep* (2013) **5**:13. doi:10.12703/P5-13
33. McEwen BS, Wingfield JC. The concept of allostasis in biology and biomedicine. *Horm Behav* (2003) **43**(1):2–15. doi:10.1016/S0018-506X(02)00024-7
34. McEwen BS. Central effects of stress hormones in health and disease: understanding the protective and damaging effects of stress and stress mediators. *Eur J Pharmacol* (2008) **583**(2–3):174–85. doi:10.1016/j.ejphar.2007.11.071
35. Rosmond R. Stress induced disturbances of the HPA axis: a pathway to Type 2 diabetes? *Med Sci Monit* (2003) **9**(2):RA35–9.
36. Bremner JD, Licinio J, Darnell A, Krystal JH, Owens MJ, Southwick SM, et al. Elevated CSF corticotropin-releasing factor concentrations in posttraumatic stress disorder. *Am J Psychiatry* (1997) **154**(5):624–9. doi:10.1176/ajp.154.5.624
37. Baker DG, West SA, Nicholson WE, Ekhator NN, Kasckow JW, Hill KK, et al. Serial CSF corticotropin-releasing hormone levels and adrenocortical activity in combat veterans with posttraumatic stress disorder. *Am J Psychiatry* (1999) **156**(4):585–8.
38. Yehuda R, Bierer LM, Schmeidler J, Aferiat DH, Breslau I, Dolan S. Low cortisol and risk for PTSD in adult offspring of holocaust survivors. *Am J Psychiatry* (2000) **157**(8):1252–9. doi:10.1176/appi.ajp.157.8.1252
39. Cohen H, Kozlovsky N, Savion N, Matar MA, Loewenthal U, Loewenthal N, et al. An association between stress-induced disruption of the hypothalamic-pituitary-adrenal axis and disordered glucose metabolism in an animal model of post-traumatic stress disorder. *J Neuroendocrinol* (2009) **21**(11):898–909. doi:10.1111/j.1365-2826.2009.01913.x
40. Cohen H, Zohar J, Gidron Y, Matar MA, Belkind D, Loewenthal U, et al. Blunted HPA axis response to stress influences susceptibility to posttraumatic stress response in rats. *Biol Psychiatry* (2006) **59**(12):1208–18. doi:10.1016/j.biopsych.2005.12.003
41. Jacobson L, Akana SF, Cascio CS, Shinsako J, Dallman MF. Circadian variations in plasma corticosterone permit normal termination of adrenocorticotropin responses to stress. *Endocrinology* (1988) **122**(4):1343–8. doi:10.1210/endo-122-4-1343
42. Jacobson L, Akana SF, Cascio CS, Scribner K, Shinsako J, Dallman MF. The adrenocortical system responds slowly to removal of corticosterone in the absence of concurrent stress. *Endocrinology* (1989) **124**(5):2144–52. doi:10.1210/endo-124-5-2144
43. Nagahara AH, Handa RJ. Age-related changes in c-Fos mRNA induction after open-field exposure in the rat brain. *Neurobiol Aging* (1997) **18**(1):45–55. doi:10.1016/S0197-4580(96)00166-2
44. Mikkelsen JD, Larsen MH. Effects of stress and adrenalectomy on activity-regulated cytoskeleton protein (Arc) gene expression. *Neurosci Lett* (2006) **403**(3):239–43. doi:10.1016/j.neulet.2006.04.040
45. Hoffman AN, Anouti DP, Lacagnina MJ, Nikulina EM, Hammer RP Jr, Conrad CD. Experience-dependent effects of context and restraint stress on corticolimbic c-Fos expression. *Stress* (2013) **16**(5):587–91. doi:10.3109/10253890.2013.804505
46. Helmreich DL, Cullinan WE, Watson SJ. The effect of adrenalectomy on stress-induced c-Fos mRNA expression in the rat brain. *Brain Res* (1996) **706**(1):137–44. doi:10.1016/0006-8993(95)01215-X
47. Imaki T, Xiao-Quan W, Shibasaki T, Yamada K, Harada S, Chikada N, et al. Stress-induced activation of neuronal activity and corticotropin-releasing factor gene expression in the paraventricular nucleus is modulated by glucocorticoids in rats. *J Clin Invest* (1995) **96**(1):231–8. doi:10.1172/JCI118026
48. Fevurly RD, Spencer RL. Fos expression is selectively and differentially regulated by endogenous glucocorticoids in the paraventricular nucleus of the

hypothalamus and the dentate gyrus. *J Neuroendocrinol* (2004) **16**(12):970–9. doi:10.1111/j.1365-2826.2004.01257.x
49. Cullinan WE, Herman JP, Battaglia DF, Akil H, Watson SJ. Pattern and time course of immediate early gene expression in rat brain following acute stress. *Neuroscience* (1995) **64**(2):477–505. doi:10.1016/0306-4522(94)00355-9
50. Samuels ER, Szabadi E. Functional neuroanatomy of the noradrenergic locus coeruleus: its roles in the regulation of arousal and autonomic function part I: principles of functional organisation. *Curr Neuropharmacol* (2008) **6**(3):235–53. doi:10.2174/157015908785777229
51. Stanford SC. Central noradrenergic neurones and stress. *Pharmacol Ther* (1995) **68**(2):297–242. doi:10.1016/0163-7258(95)02010-1
52. Hansson AC, Fuxe K. Time-course of immediate early gene expression in hippocampal subregions of adrenalectomized rats after acute corticosterone challenge. *Brain Res* (2008) **1215**:1–10. doi:10.1016/j.brainres.2008.03.080
53. Makino S, Smith MA, Gold PW. Regulatory role of glucocorticoids and glucocorticoid receptor mRNA levels on tyrosine hydroxylase gene expression in the locus coeruleus during repeated immobilization stress. *Brain Res* (2002) **943**(2):216–23. doi:10.1016/S0006-8993(02)02647-1
54. Anthony TE, Dee N, Bernard A, Lerchner W, Heintz N, Anderson DJ. Control of stress-induced persistent anxiety by an extra-amygdala septohypothalamic circuit. *Cell* (2014) **156**(3):522–36. doi:10.1016/j.cell.2013.12.040
55. Ciccocioppo R, de Guglielmo G, Hansson AC, Ubaldi M, Kallupi M, Cruz MT, et al. Restraint stress alters nociceptin/orphanin FQ and CRF systems in the rat central amygdala: significance for anxiety-like behaviors. *J Neurosci* (2014) **34**(2):363–72. doi:10.1523/JNEUROSCI.2400-13.2014
56. Haller J, Bakos N. Stress-induced social avoidance: a new model of stress-induced anxiety? *Physiol Behav* (2002) **77**(2–3):327–32. doi:10.1016/S0031-9384(02)00860-0
57. Morilak DA, Barrera G, Echevarria DJ, Garcia AS, Hernandez A, Ma S, et al. Role of brain norepinephrine in the behavioral response to stress. *Prog Neuropsychopharmacol Biol Psychiatry* (2005) **29**(8):1214–24. doi:10.1016/j.pnpbp.2005.08.007
58. Varlinskaya EI, Spear LP. Increases in anxiety-like behavior induced by acute stress are reversed by ethanol in adolescent but not adult rats. *Pharmacol Biochem Behav* (2012) **100**(3):440–50. doi:10.1016/j.pbb.2011.10.010
59. Crusio WE. Genetic dissection of mouse exploratory behaviour. *Behav Brain Res* (2001) **125**(1–2):127–32. doi:10.1016/S0166-4328(01)00280-7
60. Alcaro A, Panksepp J. The SEEKING mind: primal neuro-affective substrates for appetitive incentive states and their pathological dynamics in addictions and depression. *Neurosci Biobehav Rev* (2011) **35**(9):1805–20. doi:10.1016/j.neubiorev.2011.03.002
61. Spruijt BM, van Hooff JA, Gispen WH. Ethology and neurobiology of grooming behavior. *Physiol Rev* (1992) **72**(3):825–52.
62. Fentress JC. Expressive contexts, fine structure, and central mediation of rodent grooming. *Ann N Y Acad Sci* (1988) **525**:18–26. doi:10.1111/j.1749-6632.1988.tb38592.x
63. Gourley SL, Wu FJ, Kiraly DD, Ploski JE, Kedves AT, Duman RS, et al. Regionally specific regulation of ERK MAP kinase in a model of antidepressant-sensitive chronic depression. *Biol Psychiatry* (2008) **63**(4):353–9. doi:10.1016/j.biopsych.2007.07.016
64. Popoli M, Yan Z, McEwen BS, Sanacora G. The stressed synapse: the impact of stress and glucocorticoids on glutamate transmission. *Nat Rev Neurosci* (2012) **13**(1):22–37. doi:10.1038/nrn3138
65. Yuen EY, Liu W, Karatsoreos IN, Feng J, McEwen BS, Yan Z. Acute stress enhances glutamatergic transmission in prefrontal cortex and facilitates working memory. *Proc Natl Acad Sci U S A* (2009) **106**(33):14075–9. doi:10.1073/pnas.0906791106
66. Yuen EY, Liu W, Karatsoreos IN, Ren Y, Feng J, McEwen BS, et al. Mechanisms for acute stress-induced enhancement of glutamatergic transmission and working memory. *Mol Psychiatry* (2011) **16**(2):156–70. doi:10.1038/mp.2010.50
67. Cahill L, McGaugh JL. Mechanisms of emotional arousal and lasting declarative memory. *Trends Neurosci* (1998) **21**(7):294–9. doi:10.1016/S0166-2236(97)01214-9

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Repeated exposure to conditioned fear stress increases anxiety and delays sleep recovery following exposure to an acute traumatic stressor

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Repeated stressor exposure can sensitize physiological responses to novel stressors and facilitate the development of stress-related psychiatric disorders including anxiety. Disruptions in diurnal rhythms of sleep–wake behavior accompany stress-related psychiatric disorders and could contribute to their development. Complex stressors that include fear-eliciting stimuli can be a component of repeated stress experienced by human beings, but whether exposure to repeated fear can prime the development of anxiety and sleep disturbances is unknown. In the current study, adult male F344 rats were exposed to either control conditions or repeated contextual fear conditioning for 22 days followed by exposure to no, mild (10), or severe (100) acute uncontrollable tail shock stress. Exposure to acute stress produced anxiety-like behavior as measured by a reduction in juvenile social exploration and exaggerated shock-elicited freezing in a novel context. Prior exposure to repeated fear enhanced anxiety-like behavior as measured by shock-elicited freezing, but did not alter social exploratory behavior. The potentiation of anxiety produced by prior repeated fear was temporary; exaggerated fear was present 1 day but not 4 days following acute stress. Interestingly, exposure to acute stress reduced rapid eye movement (REM) and non-REM (NREM) sleep during the hours immediately following acute stress. This initial reduction in sleep was followed by robust REM rebound and diurnal rhythm flattening of sleep/wake behavior. Prior repeated fear extended the acute stress-induced REM and NREM sleep loss, impaired REM rebound, and prolonged the flattening of the diurnal rhythm of NREM sleep following acute stressor exposure. These data suggest that impaired recovery of sleep/wake behavior following acute stress could contribute to the mechanisms by which a history of prior repeated stress increases vulnerability to subsequent novel stressors and stress-related disorders.

Keywords: REM, NREM, anxiety, diurnal rhythm, conditioning, classical, sleep, chronic stress

INTRODUCTION

Although acute activation of the stress response evolved to enhance chances of survival, excessive, chronic, or repeated activation of the stress response can negatively impact central and peripheral physiological systems (1–3) and is a significant risk factor for the development of stress-related mental illness including depression, anxiety, and post-traumatic stress disorder [PTSD; (4–6)]. One way that repeated stressor exposure could facilitate psychiatric disorders is by sensitizing responses to novel stressors. Indeed, individuals with PTSD display exaggerated startle (7–9), autonomic (10, 11), and in some cases hypothalamic–pituitary–adrenal axis (12, 13) responses to aversive stimuli. Similarly, rodents exposed to repeated stressors can display exaggerated hormonal (14–16), autonomic (17, 18) and neuronal (19, 20), responses to acute, novel stressors. These exaggerated responses to novel stressors in rodents can occur concordantly with the development of anxiety- and depression-like behaviors (21, 22).

Disruptions in diurnal rhythms of sleep–wake behavior and specific alterations in sleep architecture accompany stress-related psychiatric disorders and could contribute to their development (23–27). In fact, the Diagnostic and Statistical Manual for Mental Disorders lists sleep disturbance as diagnostic criteria for several types of anxiety disorders, and epidemiological studies reveal that insomnia is a risk factor for depression (28). Sleep architecture can be characterized by measuring stages of sleep using electroencephalogram (EEG). A typical night of sleep consists of episodes of non-rapid eye movement (NREM) sleep, which includes slow-wave sleep and rapid eye movement (REM) sleep. Individuals suffering from panic disorder have disruptions in sleep that include reduced percent (%) time spent in NREM sleep (29, 30). Depressed patients generally display increased % REM, sleep fragmentation, and reduced % NREM (31, 32). Disturbed slow wave (33) and REM (34–37) sleep is also considered a hallmark symptom (38) and post-trauma predictor (39) of PTSD.

In rodents, stressor exposure can disrupt REM and NREM sleep and flatten diurnal rhythms of sleep/wake behavior [see Ref. (40, 41) for reviews]. Finally, manipulations of the sleep/wake cycle can alleviate depression symptoms (42, 43). Together, these data suggest that factors that increase vulnerability to sleep disruption could contribute to the development of stress-related psychiatric disorders.

Stressors that are unpredictable and uncontrollable are the most potent in terms of their deleterious consequences on emotion (44) and sleep (41). Rats exposed to a series of uncontrollable tail shocks, but not an equal number and intensity of controllable tail shocks, for example, display behaviors resembling anxiety and depression including a reduction in social exploratory behavior (45), an increase in fear conditioning (46), and a deficit in goal-directed learning in a shuttle-box escape task (44). Exposure to this same uncontrollable stressor can also flatten diurnal rhythms of activity and physiology (47), and increase sleep fragmentation and suppress REM (Thompson et al., unpublished). Similarly, repeated uncontrollable foot shock stress reduces overall time spent in REM in mice during the 20 h period following each uncontrollable shock session or re-exposure to the shock context (48). Interestingly, stress-buffering manipulations such as voluntary exercise prevent both the anxiety- and depression-like behavioral (49–51) and sleep-disrupting consequences of uncontrollable stress in rats. Although it is clear that stress can impact sleep depending on the nature of the stressor (41), whether a history of repeated stressor exposure can sensitize sleep disruption in response to a novel, acute uncontrollable stressor remains unknown.

In the current study, adult male F344 rats were exposed to control conditions or repeated contextual fear conditioning for 22 days followed by exposure to no, mild (10), or severe (100) acute uncontrollable tail shock stress. Complex stressors that include exposure to fear-eliciting stimuli can be a component of repeated stress experienced by human beings. We have previously reported that repeated exposure to fear conditioning can sensitize physiological responses to acute uncontrollable stress and exacerbate uncontrollable stress-induced disruptions in diurnal rhythms of heart rate (HR) and core body temperature (CBT) (18). The goal of the current study was to determine whether repeated exposure to conditioned fear can also prime the development of anxiety and sleep disturbances following exposure to an acute uncontrollable stressor.

MATERIALS AND METHODS

ANIMALS

A total of 141 adult, male F344 rats (Harlan Laboratories) weighing 200–230 g upon arrival were housed under controlled temperature (22°C) and humidity. The animals were maintained on a 12:12 h light/dark cycle (lights on 7:00 a.m. to 7:00 p.m). All rats were single housed in Nalgene Plexiglas cages (45 cm × 25.2 cm × 14.7 cm) and were allowed to acclimate to the housing conditions for 1 week before start of experimental procedures. Rats had *ad libitum* access to food and water and were weighed three times per week. All experimental procedures were performed during the inactive (light) phase of the light:dark cycle and animals were handled during the 1 week acclimation period. Animal discomfort was minimized during all procedures. Experimental protocols for these studies were approved by the University of Colorado Animal Care and Use Committee.

REPEATED FEAR

Exposure to repeated contextual fear was performed as previously described in detail (18, 52) Briefly, rats were placed into a conditioning chamber (46 cm × 20.7 cm × 20 cm) on day 0 for 5 min in order to acquire a memory of the context, after which rats received three, 2 s, 1.5 mA foot shocks (1 min ITI). This initial contextual fear conditioning occurred at 1000 h. Rats were returned to their home cages immediately following initial conditioning and after every subsequent re-exposure to the conditioned context. Starting 24 h following initial conditioning (day 1), rats were repeatedly exposed to the conditioned context twice a day for 22 days: once in the a.m. and once in the p.m. Each exposure was 20 min in duration and occurred between 8:00 and 12:00 h (a.m. session) and 12:00 and 5:00 h (p.m. session). The time of each a.m. and p.m. exposure was chosen randomly in order to reduce predictability. Freezing, defined by the absence of movement except that required for respiration, was scored during each re-exposure session using a random sampling procedure, whereby rats were either scored as freezing or not freezing every 10 s. To prevent extinction of fear, minimal numbers of re-instatement foot shocks were used to re-instate contextual conditioned fear. When average freezing during an a.m. session fell below 50%, all rats were administered a single re-instatement foot shock (2 s, 1.5 mA) at the end of the p.m. re-exposure session.

INESCAPABLE TAIL SHOCK STRESS

After 22 days of no repeated fear or repeated fear exposure, rats either remained in their home cages and were not exposed to acute stress (0 tail shocks), or were exposed to 10 or 100 inescapable tail shocks as previously described (18). On the day of exposure to tail shock, rats assigned to the tail shock groups were transported to a separate room, placed in Broome-style Plexiglas restraining tubes (23.4 cm long and 7.0 cm in diameter), and exposed to 10 or 100, 5 s, 1.5 mA inescapable tail shocks. Shocks were delivered at a variable-60 s ITI between 8:00 a.m. and 11:00 a.m. Rats were immediately returned to their home cages following termination of the appropriate number of shocks (10 or 100). Inescapable tail shock was used as the acute novel stressor in these experiments because it produces reliable behaviors in rodents resembling human symptoms of stress-related psychiatric disorders (44, 45), and we have previously reported that prior repeated fear stress sensitizes physiological responses to tail shock stress (18).

BEHAVIORAL TESTING

Measures of juvenile social exploration and shock-elicited freezing were obtained sequentially as previously described (53). Testing for baseline juvenile social exploration occurred 1 week prior to uncontrollable stress. During social exploration testing, each adult experimental subject was placed into separate plastic cages identical to their home cages with bedding and a plastic, filter-top lid between 7:00 a.m. and 8:00 a.m. After 1 h, a 28–32-day-old male juvenile was introduced to the cage for 3 min and exploratory behaviors (sniffing, pinning, and allogrooming) were timed by an observer blind to treatment. After the test, the juvenile was

removed and the experimental rat was returned to the home cage. Baseline testing was used to reduce neophobia to the social exploration procedure.

One week after baseline testing, and either 1 or 4 days following 0, 10, or 100 tail shocks, rats were again tested for social exploratory behaviors as described for the baseline test. Different juvenile rats were used for the two social exploration tests, so that experimental rats were not exposed repeatedly to the same juvenile. Social exploration occurred prior to shock-elicited freezing so that shock administered during the fear test would not interfere with social exploration behavior.

Following the completion of social exploration testing, rats were transferred to novel, brightly lit chambers that differed in size, lighting, odor, and background noise from the conditioning chambers used for repeated fear stress. Freezing behavior was observed for 10 min immediately after placement of the rats into the chambers. Rats then received two, 1 s, foot shocks (0.7 mA, 1 min ITI) followed by a 20 min, post-shock freezing observation period. Freezing immediately following shock presentation is a measure of fear conditioned to cues present in the shuttle box (54). Reduction in juvenile social exploration (45, 55) and enhanced shock-elicited freezing (56, 57) represent rodent analogs of social- and fear-related anxiety behaviors, respectively.

BIOTELEMETRY SURGERIES

F40-EET biotelemetry transmitters (Data Sciences International, St. Paul, MN, USA) were implanted into animals used in Experiment 3 as previously described (18, 47, 52). Following ketamine (i.p. 75.0 mg/kg), and medetomidine (i.p. 0.5 mg/kg) anesthesia, a midline incision was made approximately 5.0 cm in length on the ventral abdominal wall. Biopotential leads were passed through the ventral abdominal wall and then the transmitter was sutured to the ventral abdominal wall. The EEG leads were placed as previously described in telemetry studies of mice (58, 59). Briefly, insulated leads were passed subcutaneously to the base of the skull, where they were attached to pan head stainless steel screws (Plastics One Inc.), which served as EEG recording electrodes. Screws were placed according to the Rat Brain Atlas in Stereotaxic Coordinates by Paxinos and Watson (60) at anterior 2.0 mm; lateral 2.5 mm and posterior 5.5 mm; lateral 3.0 mm from Bregma using standard stereotaxic methods (61). Screws and leads were embedded in dental acrylic to ensure the integrity of the recording signal. Immediately following surgery, rats were given meloxicam (1.0 mg/kg s.c.) for analgesia after which they recovered on a heating pad at 37°C until ambulatory. Once ambulatory, rats were returned to their home cages and given one 2.0 mg rimadyl tablet (Bio-Serv) and several fruity bites (Bio-Serv). Animals were allowed to recover for 1 week before the start of repeated exposure to conditioned fear.

BIOTELEMETRY DATA ACQUISITION AND ANALYSIS

The F40-EET transmitter (DSI) allows *in vivo* real-time measurement of locomotor activity (LA), HR, CBT, and EEG in freely moving animals. Biotelemetry recordings were acquired/analyzed using Dataquest ART 4.3 Gold Acquisition/Analysis Software (Data Sciences International, St. Paul, MN, USA), as previously described (18, 52). Analyses of the sleep/wake cycles were performed using the automated Neuroscore 2.1.0 software (Data Sciences International, St. Paul, MN, USA). The trace EEG signal was subjected to fast Fourier Transformation (FFT), yielding spectra between 0.5 and 30 Hz in 0.5-Hz frequency bins. The delta frequency band was defined at 0.5–4.5 Hz and the theta frequency band was defined as 6.0–9.0 Hz, as previously described (59). Arousal state was scored in 10-s epochs and classified as NREM, REM, or wake on the basis of state-dependent changes in multiple parameters, including the EEG, LA, HR, and body temperature, as previously described (59, 62). Wakefulness was defined on the basis of a low amplitude, mixed frequency EEG (delta≈theta) accompanied by body movements (i.e., activity), and increases in body temperature. NREM sleep was identified by increased absolute EEG amplitude with integrated values for the delta frequency band greater than those for the theta frequency and lack of body movements. Body temperature declines upon entry into NREM sleep until it reaches a regulated asymptote. REM was characterized by a low amplitude EEG with integrated values for the delta frequency band less than those for the theta frequency band. Any epochs containing artifact or electrical noise were tagged and excluded from subsequent spectral analysis. All sleep scoring was performed by an individual blind to treatment condition of the animal.

EXPERIMENTAL DESIGN

Experiment 1

Experiment 1 was designed to test the hypothesis that prior repeated fear stress sensitizes anxiety responses to novel acute stressor exposure. Rats were randomly assigned to the following groups: no repeated fear (home cage)/0 tail shocks ($n = 7$); home cage/10 tail shocks ($n = 8$); home cage/100 tail shocks ($n = 8$); repeated fear/0 tail shocks ($n = 8$); repeated fear/10 tail shocks ($n = 8$); repeated fear/100 tail shocks ($n = 7$). All rats were tested for anxiety-like behavior using social exploration and shock-elicited freezing 24 h following tail shock exposure. **Figure 1A** shows the sequence of events followed during Experiment 1.

Experiment 2

Experiment 2 explored whether acute stress-induced anxiety-like behavior persisted longer following acute stress in rats previously exposed to repeated fear compared to home cage rats. Rats previously exposed to home cage or repeated fear conditions were exposed to 0 or 100 tail shocks and were then tested for anxiety-like behaviors either 1 or 4 days later. Groups containing home cage and repeated fear rats not exposed to tail shock (the 0 tail shock groups) were split so that half of the rats were tested with the 1-day group and the other half were tested with the 4-day group. No time-dependent effects were noted between the 0 tail shock rats tested at the 1 and 4-day time points, so the time points were combined. Therefore, the home cage/0 tail shock and repeated fear/0 tail shock groups include rats tested at both the day 1 and day 4 time points. The following groups were tested: Home cage/0 ($n = 8$); home cage/1 day after 100 tail shocks ($n = 8$); home cage/4 days after 100 tail shocks ($n = 8$); repeated fear/0 ($n = 8$); repeated fear/1 day after 100 tail shocks ($n = 7$); repeated fear/4 days after 100 tail shocks ($n = 8$). A time line for the procedures used in Experiment 2 can be found in **Figure 1B**.

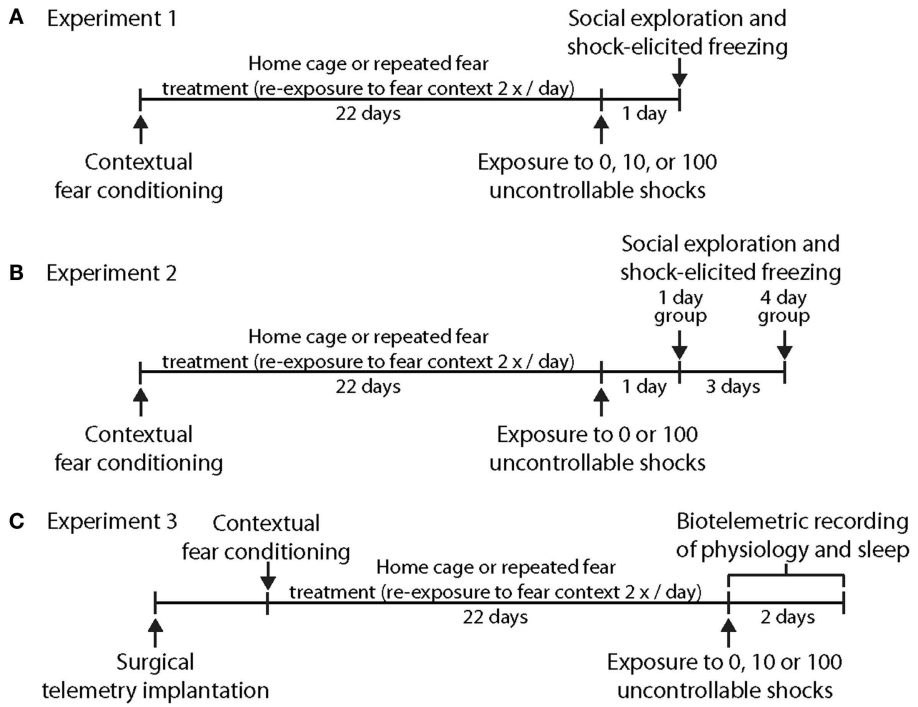

FIGURE 1 | Time lines depicting the series of events used in Experiment 1 (A), Experiment 2 (B), and Experiment 3 (C).

Experiment 3

Rats implanted with F40-EET transmitters were exposed to home cage or repeated fear conditions for 22 days, followed by 0, 10, or 100 tails shocks. Data were recorded starting 1 week after telemetry implantation and continued throughout the remainder of the experiment. Body weight, freezing behavior during repeated fear stress, HR, body temperature, and activity obtained from the rats used in this experiment have been published previously (18). Here, EEG and physiological data were analyzed starting at clock time 1:00 p.m. (~2 h following the termination of tail shocks) and continued for 3 days thereafter. We waited approximately 2 h following the termination of tail shock stress in order to avoid the disruption in the telemetry signal produced by moving the rats from the stress induction room back to their home cages. Moreover, starting the analyses at the same clock time for all animals eased analyses of the data. Twelve of the initial 48 rats used in the study were dropped from analyses due to loss or interference with the EEG signal, yielding the following groups: home cage/0 tail shocks ($n = 5$); home cage/10 tail shocks ($n = 7$); home cage/100 tail shocks ($n = 6$); repeated fear/0 tail shocks ($n = 5$); repeated fear/10 tail shocks ($n = 7$); repeated fear/100 tail shocks ($n = 6$). A time line for the procedures used in Experiment 3 can be found in **Figure 1C**.

STATISTICAL ANALYSIS

Body weight data were analyzed using repeated measures ANOVA. Average time spent exploring during the 3 min social exploration test and average % freezing during the 20 min post-shock freezing period in Experiment 1 were analyzed using 2 (home cage, repeated fear) × 3 (0, 10, 100 tail shocks) ANOVAs or, for Experiment 2, 2 (home cage, repeated fear) × 3 (no acute stress, 1 day after 100 tail shocks, 4 days after 100 tail shocks) ANOVAs. Percent time spent in REM, NREM, and wake during the remaining 6 h of the light cycle starting approximately 2 h following termination of acute stress were collapsed into 1 h blocks and analyzed with 2 (home cage, repeated fear) × 3 (0, 10, 100 tail shocks) repeated measures ANOVA. Subsequent percent time spent in REM, NREM, and wake were collapsed into 12 h blocks and compared with 2 × 3 ANOVAs. Light and dark cycles were analyzed independently. Diurnal differences of average % REM, % NREM, and % wake, calculated by subtracting the average dark cycle value from the average light cycle value, were compared using 2 × 3 ANOVAs. Fisher's PLSD *post hoc* analyses were used as appropriate. Group means were considered different when $p < 0.05$.

RESULTS

FREEZING DATA AND BODY WEIGHT

Rats exposed to repeated conditioned fear stress for 22 days displayed freezing behavior upon each re-exposure to the conditioned context (**Figure 2A**). Rats in both Experiments 1 and 2 required 6 foot shocks to maintain levels of freezing above 50%. Body weights of rats used in Experiments 1 and 2 are shown in **Figures 2B,C**, respectively. Both groups gained weight over time [Experiment 1, $F(8, 352) = 467.1$; $p < 0.0001$; Experiment 2, $F(8, 360) = 704.5$; $p < 0.0001$], but rats exposed to repeated fear stress gained less weight over time compared to rats exposed to home cage treatment [Experiment 1, $F(8, 352) = 57.56$; $p < 0.0001$; Experiment 2, $F(8, 360) = 37.97$; $p < 0.0001$]. Freezing and body weight data from rats used in Experiment 3 have been published previously (18) and thus are not shown.

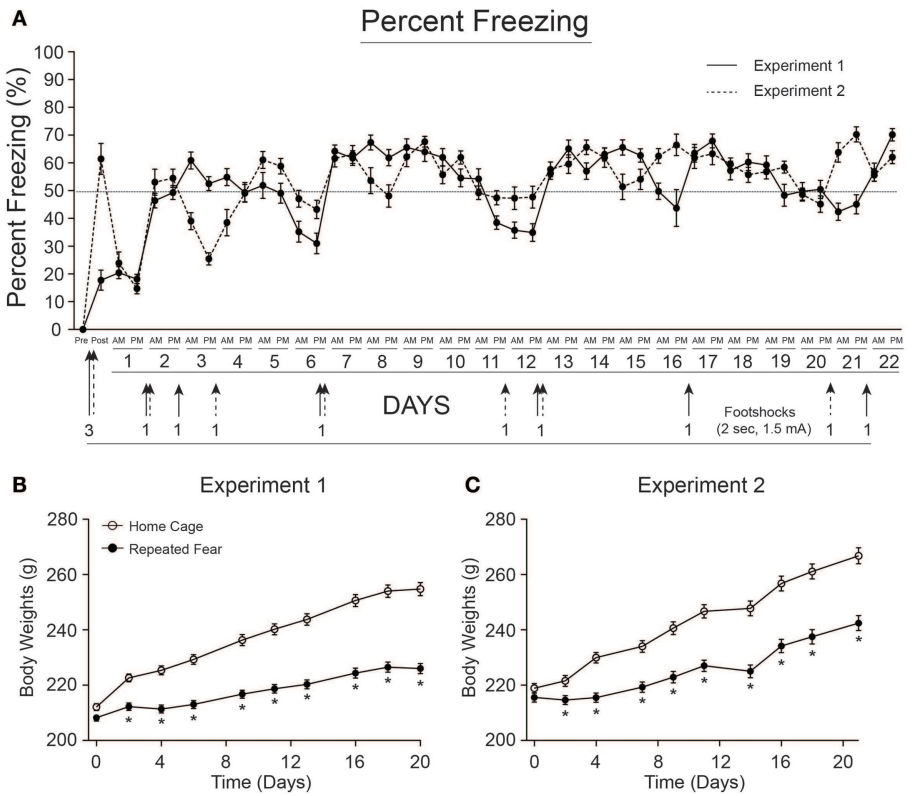

FIGURE 2 | Freezing behavior and body weights for rats used in Experiments 1 and 2. **(A)** Freezing was scored before (pre) and after (post) administration of 3 foot shocks during contextual fear conditioning on day 0. Rats were re-exposed to the conditioned context twice a day, early (a.m.), or late (p.m.) during the light cycle, for 22 days. Re-instatement shocks (denoted by arrows) were administered at the end of the p.m. session when average freezing fell below 50%. The number of shocks administered is noted next to each arrow. Rats in both Experiment 1 **(B)** and Experiment 2 **(C)** exposed to repeated fear stress gained less weight over time relative to home cage control rats. $*p < 0.05$ relative to home cage control.

PRIOR EXPOSURE TO REPEATED FEAR STRESS INCREASES ANXIETY AS MEASURED BY SHOCK-ELICITED FREEZING

Twenty four hours following exposure to 0, 10, or 100 tail shocks, rats used in Experiment 1 were tested for social exploratory behavior and shock-elicited fear. Consistent with prior reports (50, 53, 55), exposure to acute stress reduced social exploration [$F(2, 40) = 8.48$; $p = 0.0008$; **Figure 3A**] and increased shock-elicited freezing [$F(2, 40) = 3.54$; $p = 0.03$; **Figure 3B**]. Only 100 tail shocks reduced social exploration ($p = 0.02$) and increased fear ($p = 0.01$). The reduction in social exploration ($p = 0.07$) and the increase in fear ($p = 0.07$) following 10 shocks failed to reach significance. A history of repeated fear had no impact on social exploratory behavior [$F(1, 40) = 1.13$; $p > 0.05$], thus acute stress reduced social exploratory behavior equally regardless of history of repeated fear. In contrast, rats exposed to repeated fear displayed more fear than rats exposed to home cage treatment [$F(1, 40) = 3.82$; $p < 0.05$]. These effects occurred in the absence of gross changes in LA. Neither acute tail shock stress [$F(2, 40) = 1.97$; $p > 0.05$] nor repeated fear [$F(1, 40) = 1.62$; $p > 0.05$] altered the number of spontaneous cage crosses during social exploration testing (data not shown).

In Experiment 2, rats exposed to repeated fear or home cage treatments were tested for anxiety-like behavior either 1 or 4 days following 0 or 100 tail shocks. Results similar to those observed in Experiment 1 were seen here. Exposure to acute stress reduced social exploration [$F(2, 41) = 9.38$; $p = 0.0004$; **Figure 4A**] and increased shock-elicited freezing [$F(2, 41) = 4.75$; $p = 0.01$; **Figure 4B**]. Regardless of history of prior repeated fear exposure, a reduction in social exploratory behavior was observed both 1 ($p = 0.003$) and 4 ($p = 0.0002$) days following tail shock stress, whereas the increase in shock-elicited freezing was only present when rats were tested 1 day following tail shock ($p = 0.01$). Although a history of repeated fear had no impact on social exploration [$F(1, 41) = 0.007$; $p > 0.05$], exaggerated shock-elicited freezing was again observed in rats exposed to repeated fear stress [$F(1, 41) = 9.52$; $p = 0.004$]. This exaggerated fear produced by repeated fear stress relative to home cage treatment was temporary. Exaggerated fear produced by repeated fear stress was present in rats not exposed to acute tail shock stress ($p = 0.01$) and 1 day ($p = 0.02$), but not 4 days ($p > 0.05$), following acute tail shock stress. Again, neither acute tail shock stress [$F(2, 41) = 3.07$; $p > 0.05$] nor repeated fear [$F(1, 41) = 0.16$; $p > 0.05$] altered the

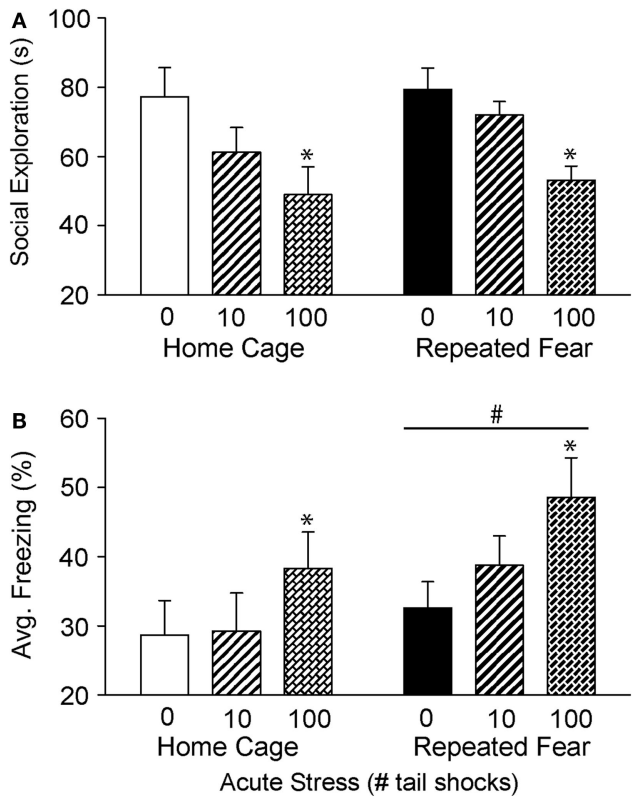

FIGURE 3 | Effects of prior repeated fear stress on anxiety behavior following mild or severe acute stress. Following 22 days of no repeated fear (home cage) or repeated fear stress (repeated fear), rats were exposed to no acute stress (0), mild (10 uncontrollable tail shocks; 10), or severe (100 uncontrollable tail shocks; 100) acute stress. Juvenile social exploration **(A)** and shock-elicited freezing **(B)** were measured 24 h later in a novel environment. *$p < 0.05$ relative to 0 groups; #main effect of repeated fear stress ($p < 0.05$).

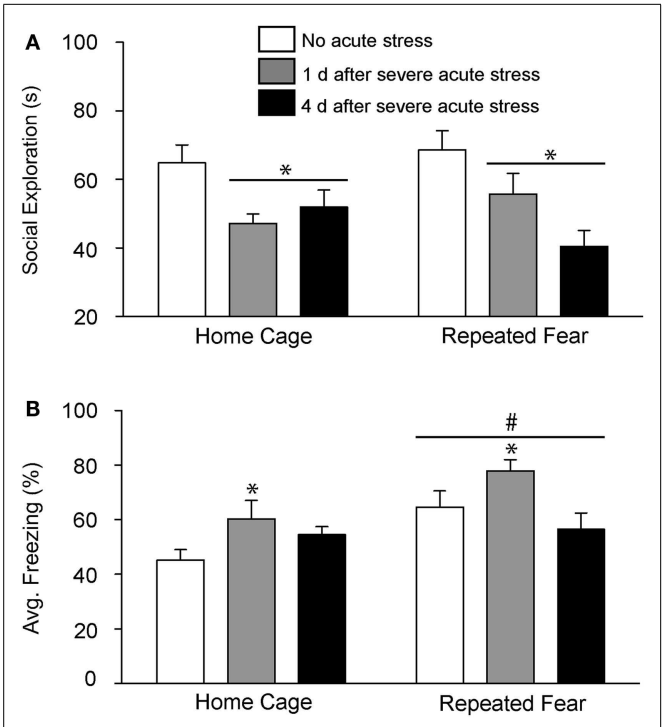

FIGURE 4 | Effects of prior repeated fear stress on persistence of anxiety behavior following severe acute stress. Following 22 days of no repeated fear (home cage) or repeated fear stress (repeated fear), rats were either exposed to no acute stress or to 100 uncontrollable tail shocks (acute severe stress). Juvenile social exploration **(A)** and shock-elicited freezing **(B)** were measured either 1 or 4 days later in a novel environment. *$p < 0.05$ relative to 0 groups; #main effect of repeated fear stress ($p < 0.05$).

number of cage crosses during social exploration testing (data not shown).

REPEATED FEAR PROLONGS REM AND NREM SLEEP LOSS IMMEDIATELY FOLLOWING ACUTE STRESS

The % time spent in REM, NREM, and wake during the remaining 6 h of the light cycle starting approximately 2 h following the termination of acute tail shock stress are shown in **Figure 5**. Acute stress reduced % REM (**Figures 5A,B**) and % NREM (**Figures 5C,D**) in both home cage and repeated fear groups during the first few hours following acute stress. The reduction in % REM and % NREM following acute stress persisted longer in rats that had been previously exposed to repeated fear stress compared to home cage treatment. This was especially true for % REM. In contrast to the effect of acute stress in home cage rats, which only persisted for 2 h, both 10 and 100 tail shocks suppressed % REM in repeatedly stressed rats for 5 h. These results were confirmed with repeated measures ANOVA, which revealed a significant main effect of acute stress [$F(2, 30) = 4.22$; $p = 0.02$] and significant interactions between acute stress and time ($10, 150) = 2.5$; $p = 0.008$) and acute stress, time, and repeated fear [$F(10, 150) = 2.41$; $p = 0.01$] on % REM; and significant main effects of repeated fear [$F(1, 30) = 5.45$; $p = 0.02$], acute stress [$F(2, 30) = 5.6$; $p = 0.02$], and significant interactions between repeated fear and time [$F(5, 150) = 2.68$; $p = 0.02$] and acute stress and time [$F(10, 150) = 4.29$; $p < 0.0001$] on % NREM. The acute stress-induced reduction in REM and NREM sleep was accompanied by a detectible increase in wakefulness (**Figures 5E,F**). ANOVA revealed significant main effects of repeated fear stress [$F(1, 30) = 7.32$; $p = 0.01$] and acute stress [$F(2, 30) = 6.9$; $p = 0.005$]; and a significant interaction between acute stress and time [$F(10, 150) = 3.08$; $p < 0.0001$]. The home cage and repeated fear groups not exposed to acute stress (the 0 groups) did not differ in any parameter measured. See graphs for results of *post hoc* comparisons.

PRIOR EXPOSURE TO REPEATED FEAR IMPAIRS REM REBOUND FOLLOWING ACUTE STRESS

The % REM, % NREM, and % wake during the 48 h following the beginning of the first night cycle after acute stress are shown in 12 h blocks in **Figure 6**. Consistent with REM rebound following periods of REM sleep loss (63) and stress (64, 65), acute stress increased % REM during the first [$F(2, 30) = 14.8$; $p < 0.0001$; **Figure 6A**] and second [$F(2, 30) = 3.97$; $p = 0.03$;

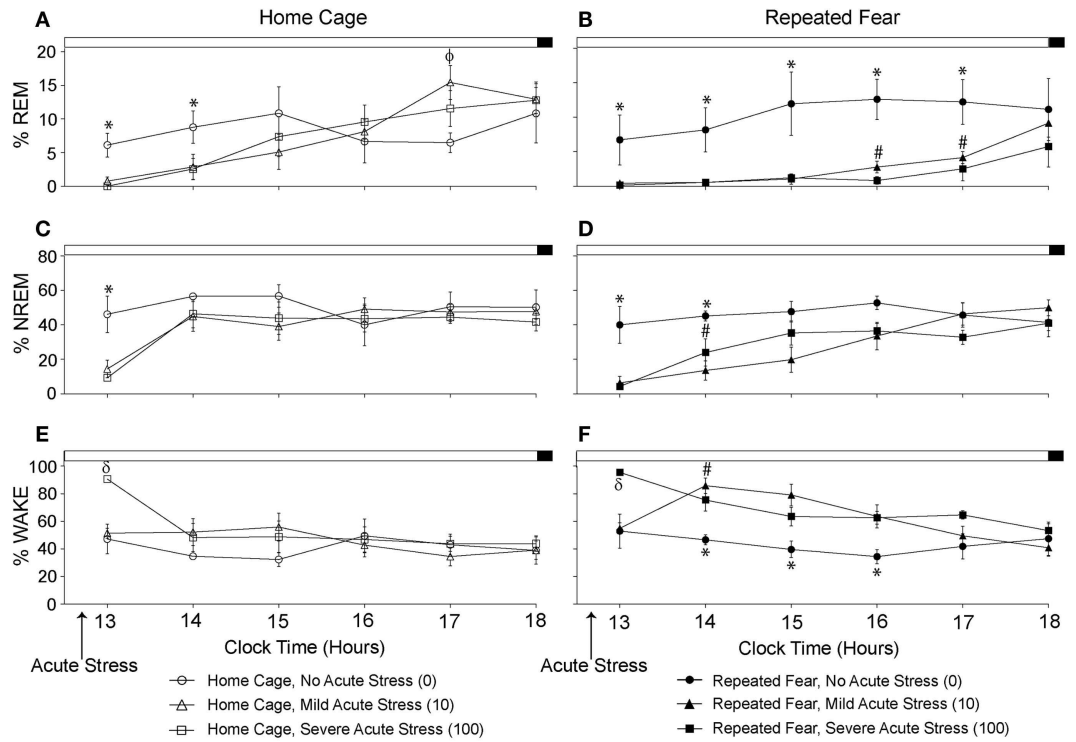

FIGURE 5 | Effects of repeated fear stress on sleep/wake behavior during the remainder of the light cycle following acute stressor exposure. Following 22 days of no repeated fear (home cage) or repeated fear stress (repeated fear), rats were exposed to no acute stress (0), mild (10 uncontrollable tail shocks; 10), or severe (100 uncontrollable tails shocks; 100) acute stress. Percent time spent in rapid eye movement [REM; **(A,B)**] sleep, non-rapid eye movement [NREM; **(C,D)**] sleep, and wake **(E,F)** were determined in the home cage during the remainder of the light cycle starting approximately 2 h following the termination of acute tail shock stress. $*p < 0.05$ relative to respective 10 and 100 groups; $\#p < 0.05$ relative to home cage/10 and home cage/100 groups; $\phi p < 0.05$ relative to home cage/0 group; $\delta p < 0.05$ relative to respective 0 and 10 groups.

Figure 6C] 12 h dark cycles following acute stress. The % REM in the light cycle was not impacted by acute or repeated fear stress (**Figures 6B,D**); however, the effect of acute stress on % REM in the second light cycle following acute stress just missed significance ($p = 0.06$). Repeated fear impaired the REM rebound that occurred in acute stress groups during the first dark cycle that followed acute stress (**Figure 6A**). This was confirmed with ANOVA, which revealed a significant main effect of repeated fear [$F(1, 30) = 8.18$; $p = 0.007$] and a significant interaction between acute stress and repeated fear [$F(2, 30) = 4.75$; $p = 0.01$; see **Figure 6A** for results of *post hoc* tests]. At no time did the home cage and repeated fear groups not exposed to acute stress differ in their % REM. Neither repeated fear nor acute stress altered % NREM (**Figures 6E–H**).

The increase in % REM following acute stress was paralleled by a reduction in wakefulness (**Figures 6I–L**). Both acute stress [$F(2, 30) = 5.59$; $p = 0.008$] and repeated fear [$F(1, 31) = 5.6$; $p = 0.02$] increased % wake during the first dark cycle following acute stressor exposure (**Figure 6I**). At no other time following acute stress was % wake altered by acute or repeated fear stress. The % REM, % NREM, and % wake in all groups resembled control values after the second light cycle that followed acute stress, thus these data are not shown.

PRIOR EXPOSURE TO REPEATED FEAR PROLONGS THE FLATTENING OF THE DIURNAL RHYTHM OF NREM SLEEP FOLLOWING ACUTE STRESS

To determine the impact of repeated fear and acute uncontrollable stress on the diurnal rhythms of sleep/wake behavior, the diurnal difference (dark–light) of % REM, % NREM, and % wake were compared between groups following acute stressor exposure. Consistent with greater REM rebound in the light cycle observed in the home cage rats following acute stress (**Figure 6A**), both repeated fear [$F(1, 30) = 5.67$; $p = 0.02$] and acute stress [$F(2, 30) = 10.37$; $p = 0.0004$] reduced the diurnal difference of % REM (**Figure 7A**). The flattening of the diurnal rhythm of % REM sleep was present during the first, but was gone by the second (**Figure 7B**), 24 h period following acute stress. Acute stress reduced the diurnal difference of % NREM sleep [$F(2, 30) = 14.3$; $p < 0.0001$; **Figure 7C**] and % wake [$F(2, 30) = 18.77$; $p < 0.0001$; **Figure 7E**] during the first 24 h period following acute stress in both home cage and repeated fear-exposed rats. During the second 24 h period following acute stress, significant interactions between repeated fear and acute stress revealed that the flattening of the diurnal rhythm of both % NREM sleep [$F(2, 30) = 4.88$; $p = 0.01$; **Figure 7D**] and % wake [$F(2,30) = 5.44$; $p = 0.009$; **Figure 7F**] persisted longer following acute stress in rats previously exposed to repeated fear. See **Figure 7** for results of *post hoc* tests.

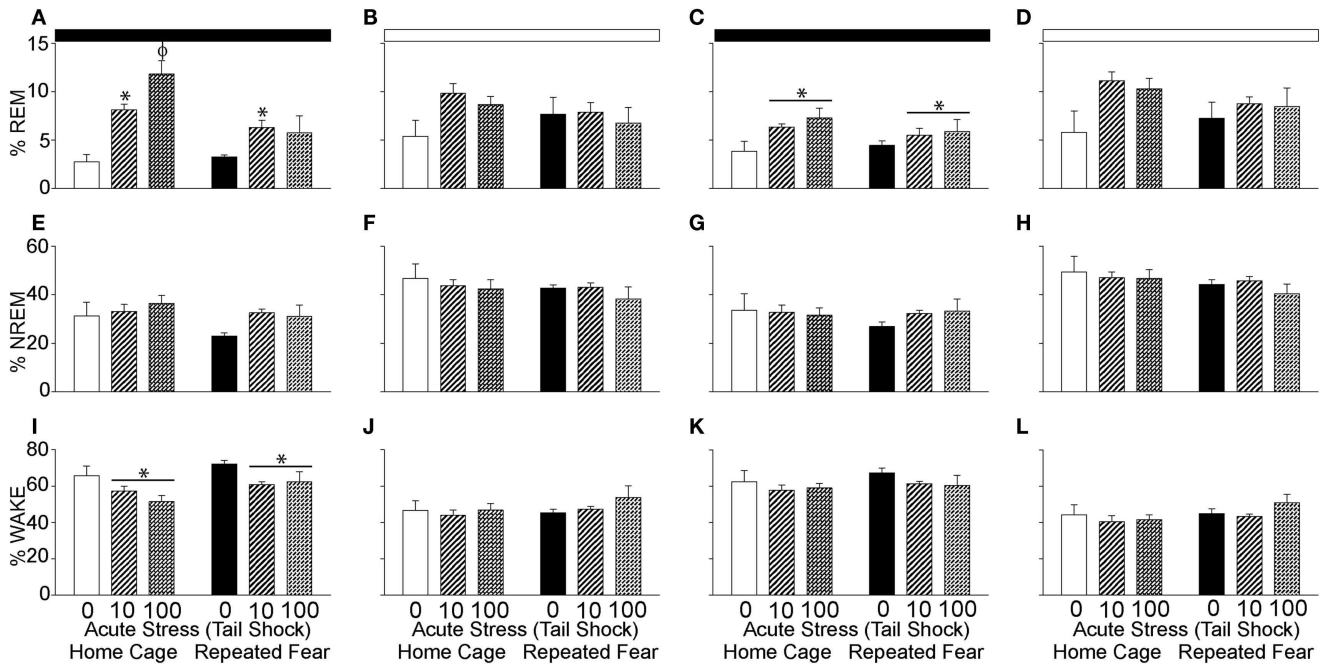

FIGURE 6 | Effects of repeated fear and acute stress on diurnal pattern of undisturbed home cage sleep/wake behavior. Following 22 days of no repeated fear (home cage) or repeated fear stress (repeated fear), rats were exposed to no acute stress (0), mild (10 uncontrollable tail shocks; 10), or severe (100 uncontrollable tails shocks; 100) acute stress. Percent time spent in rapid eye movement [REM; **(A–D)**] sleep, non-rapid eye movement [NREM; **(E–H)**] sleep, and wake **(I–L)** were determined in the home cage for 48 consecutive hours starting the dark cycle immediately following the day of acute stressor exposure. Averaged dark and light cycle data are reported in 12 h blocks. $*p < 0.05$ relative to 0 groups (lines represent main effects of acute stress); $\phi p < 0.05$ relative to all other groups. Dark bar represents 12 h dark cycle; white bar represents 12 h light cycle.

DISCUSSION

Here, we report that prior exposure to a repeated emotional stressor can increase anxiety-like behavior and induce prolonged sleep disruption following exposure to a subsequent acute, novel stressor. These observations add to our prior observations that repeated exposure to conditioned fear stress can sensitize HR and CBT responses to acute severe stress (18). Taken together, these data indicate that repeated exposure to conditioned fear stress can produce behaviors in rodents that resemble characteristics of stress-related psychiatric disorders, including sensitized autonomic responses (18), enhanced fear learning (**Figures 3B** and **4B**), and prolonged stress-induced flattening of biological rhythms including NREM sleep (**Figure 7D**) following exposure to a novel, acute uncontrollable stressor.

Exposure to severe (100 tail shocks), but not mild (10 tail shocks), acute uncontrollable stress reduced social exploratory behavior and increased freezing immediately following administration of foot shocks during fear conditioning in a novel environment (**Figures 3** and **4**). The observation that mild acute stress was insufficient to elicit anxiety-like behavior is consistent with prior work showing that greater than 50 uncontrollable tail shocks are required to activate serotonin (5-HT) neurons in the dorsal raphe nucleus (66); the putative mechanism by which acute severe stress transiently enhances fear conditioning (67, 68) and reduces social exploration (45). Repeated fear stress neither sensitized (**Figure 3A**) nor prolonged (**Figure 4A**) the effect of acute uncontrollable stress on social exploration. In contrast, a history of repeated fear stress enhanced shock-elicited freezing, regardless of acute stressor exposure (**Figures 3B** and **4B**). These data indicate that like a history of acute stress (50, 69, 70), prior exposure to repeated fear stress can enhance the acquisition of a new fear memory. Similarly, clinical data indicate that patients with PTSD respond in an exaggerated manner to novel fear-eliciting stimuli and remember these aversive stimuli better than trauma-exposed controls without PTSD (10).

The fact that repeated fear stress increased anxiety-like behavior as measured by shock-elicited freezing but not social avoidance suggests that repeated fear stress impacts various anxiety-related behaviors differently. It is unlikely that a history of foot shock during repeated fear sensitized the anxiety response to subsequent foot shock during anxiety testing, because repeated exposure to homotypic stimuli typically lead to habituation, not sensitization, of the stress response to that stimuli (20, 71). Although the exaggerated fear and social avoidance produced by acute uncontrollable stress are thought to have similar mechanisms involving 5-HT and the amygdala (55, 72), repeated fear stress might impact a neural substrate capable of modulating fear- and not social-related anxiety behaviors. Although the hippocampus is an attractive candidate because of its involvement in contextual fear conditioning (73) and sensitivity to repeated stress (74), enhanced fear learning produced

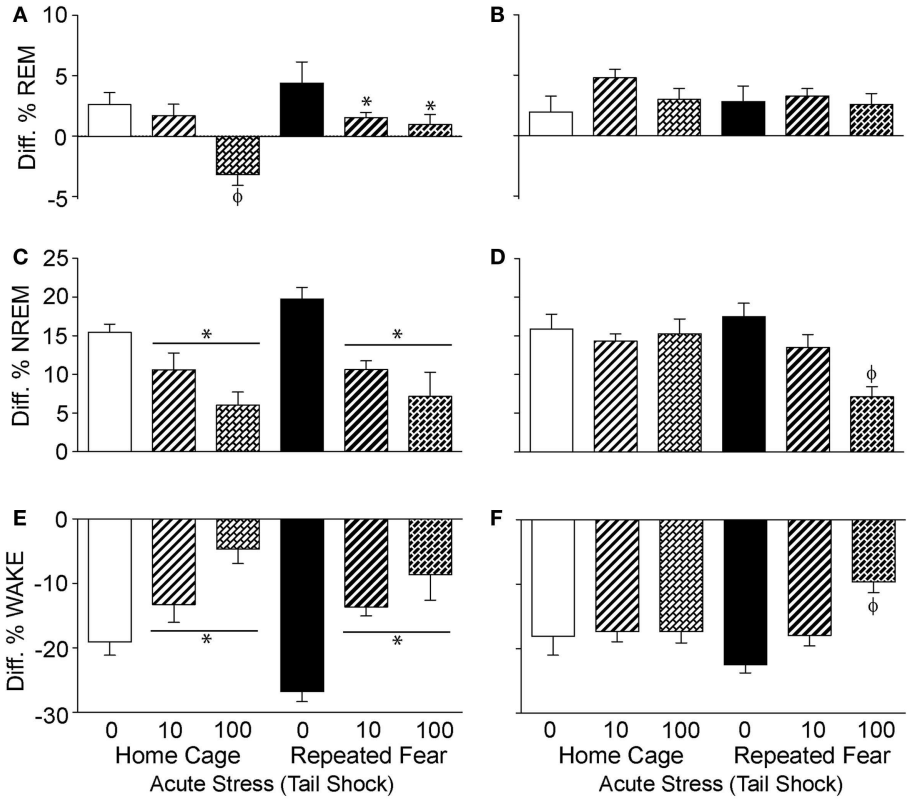

FIGURE 7 | Effects of repeated fear and acute stress on diurnal difference of sleep/wake behavior. Following 22 days of no repeated fear (home cage) or repeated fear stress (repeated fear), rats were exposed to no acute stress (0), mild (10 uncontrollable tail shocks; 10), or severe (100 uncontrollable tails shocks; 100) acute stress. Diurnal difference of % rapid eye movement [REM; **(A,B)**] sleep, non-rapid eye movement [NREM; **(C,D)**] sleep, and wake **(E,F)** were calculated by subtracting light cycle values from dark cycle values of sleep parameters measured in the home cage for 48 h starting the dark (active) cycle immediately following acute stressor exposure. The diurnal differences calculated from the first 24 h period starting the first active (dark) cycle immediately following acute stress are shown in the left panel **(A,C,E)**. The diurnal differences calculated from the second 24 h period following acute stress are shown in the right panel **(B,D,F)**. $*p < 0.05$ relative to respective 0 groups (lines represent main effects of acute stress); $\phi p < 0.05$ relative to all other groups.

by repeated fear stress was observed immediately following shock administration during conditioning in a novel context, a time during which freezing is independent of the hippocampus (75). The prefrontal cortex is an important emotional control region that is known to undergo structural remodeling following repeated stress (76, 77) and can modulate fear expression (78). Thus, the prefrontal cortex could be a structure through which repeated fear acts to enhance fear-related anxiety-like behavior. Consistent with this possibility are the observations of functional and structural deficits in the PFC of patients suffering from PTSD and depression (79–82).

Rats exposed to repeated conditioned fear had similar sleep patterns to rats exposed to home cage treatment in the absence of tail shock (the acute stress 0 groups), suggesting that repeated exposure to conditioned fear may not by itself impact sleep. This is surprising considering that both re-exposure to a cue (83) or a context (84) previously associated with a foot shock has been reported to reduce % REM sleep during the inactive cycle. One reason for this discrepancy could be that the impact of fear conditioning on sleep habituates with time. Indeed, Kant et al. (85) reported that although total sleep time is initially reduced during repeated daily exposure to foot shock stress in the home cage, total sleep time returns to baseline levels by the 7th day of stress. This explanation seems unlikely, however, considering that (1) the return to baseline sleep time following repeated foot shock reported in Ref. (85) was accompanied by a disruption of the normal diurnal rhythm of sleep and no such diurnal disruption following repeated fear stress was observed in the current study and (2) physiological and fear responses to the conditioned context were maintained throughout the duration of the study (18). Moreover, although extinction of fear reduces the impact of re-exposure to a contextual conditioned stimulus on REM sleep (84), fear extinction was prevented in the current study by administration of foot shocks when average freezing levels dropped below 50%. An alternative explanation could be the strain of rat used in the study. Tang et al. (64) reported that relative to Lewis and Wistar rats, which exhibit a reduction in % REM following re-exposure to a conditioned context, F344 rats display no such reduction. In fact, F344 rats displayed the

greatest fear response and an increase in % REM during the dark cycle following both acquisition and expression of contextual fear conditioning (86). The possibility remains, however, that repeated fear did disrupt sleep, but that disrupted sleep had resolved by the time analyses of sleep patterns began 24 h following the last exposure to the contextual CS. Indeed, Moreau et al. (87) report that changes in % REM sleep produced by several weeks of mild stress disappeared progressively following termination of stress (87).

In contrast to the lack of observed effect of repeated fear stress on sleep, exposure to acute tail shock stress clearly impacted sleep. Similar to prior reports (88), acute stress reduced both % REM and % NREM sleep and increased % wake during the hours immediately following acute stressor exposure. These data are consistent with our prior work reporting that this same stressor increases activity, HR, and CBT for several hours following stress (18, 47). Compared to home cage rats exposed to acute stress, rats previously exposed to repeated fear displayed a prolonged reduction in % REM and % NREM sleep for hours following stress. In fact, rats not exposed to repeated fear began to show signs of REM recovery by 5 h post-acute stress (**Figure 5**), a time point during which % REM of rats exposed to repeated fear stress was still suppressed. Similar to the effect of acute stress observed in the current study, victims of acute traumatic injury display increased wake time following trauma (39). Interestingly, those trauma victims who also have disrupted REM sleep (increased frequency but very short duration REM sleep bouts) were also more likely to develop PTSD (39). Consistent with these clinical data, acute stress impacted REM sleep of rats previously exposed to repeated fear to a greater extent than home cage control rats, and this suppressed REM sleep was associated with the most robust shock-elicited freezing.

Rapid eye movement rebound [increased REM sleep after periods of REM suppression; (63)] has been reported to follow stressor exposure (64) and has been argued to represent an adaptive response to stress [for a review, see Ref. (65)]. Significant REM rebound was observed in home cage rats during several dark periods following acute stressor exposure (**Figures 5A** and **6C**). In contrast, and despite the prolonged loss of REM sleep produced by acute stress in the rats exposed to repeated fear, rats exposed to repeated fear had impaired REM rebound (**Figure 6A**). Suppressed REM rebound following acute stress in rats exposed to repeated fear could thus represent a maladaptive response to acute novel stress that could contribute to vulnerability to stress-related disorders.

The mechanisms underlying REM rebound following stress have been reviewed (65) and could involve serotonin (88), corticosterone (89), prolactin (65, 90), or the central nucleus of the amygdala (91). Further work will be required to elucidate the mechanisms by which repeated fear exposure impairs REM rebound. It should be mentioned, however, that repeated fear stress does not result in exaggerated corticosterone responses to acute tail shock stress (18). Thus, differences in circulating corticosterone are unlikely to be involved in the observed suppression of the REM rebound.

In addition to prolonging sleep disruption immediately following acute stress and suppressing REM rebound, repeated fear stress produced a protracted disruption in the diurnal rhythm of NREM sleep elicited by acute stress. We have previously reported that prior repeated fear stress prolongs the acute stress-induced disruption of the diurnal rhythms of HR and CBT (18). Here, we extend those observations to include disruptions in diurnal rhythms of NREM. Although no differences in total % NREM were observed following exposure to repeated or acute stress (**Figure 6**), prolonged damping of diurnal rhythms could itself be a precursor to mood disorders (92, 93). Indeed, disruption of sleep/wake cycles, such as occurs with seasonal affective disorders (94) and shift work (95, 96), can trigger mood-related problems in vulnerable individuals.

In conclusion, we present evidence that exposure to repeated fear stress increases selective measures of anxiety-like behavior, prolongs sleep disruption including REM and NREM suppression and NREM diurnal disruption, and impairs REM rebound following exposure to an acute, novel stressor. Sensitization of sleep disruption following acute stressors could contribute to the mechanisms by which a history of repeated stress leads to vulnerability to stress-related psychiatric disorders including anxiety.

AUTHOR CONTRIBUTIONS

Benjamin N. Greenwood and Robert S. Thompson conducted experiments, collected data, and co-authored the manuscript. Benjamin N. Greenwood, Robert S. Thompson, Mark R. Opp, and Monika Fleshner designed the experiments and analyzed data. Mark R. Opp and Monika Fleshner edited the final manuscript.

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REFERENCES

1. Sapolsky RM. Why stress is bad for your brain. *Science* (1996) **273**:749–50. doi:10.1126/science.273.5276.749
2. Vanitallie TB. Stress: a risk factor for serious illness. *Metabolism* (2002) **51**:40–5. doi:10.1053/meta.2002.33191
3. Swaab DF, Bao AM, Lucassen PJ. The stress system in the human brain in depression and neurodegeneration. *Ageing Res Rev* (2005) **4**:141–94. doi:10.1016/j.arr.2005.03.003
4. Sapolsky RM. Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. *Arch Gen Psychiatry* (2000) **57**:925–35. doi:10.1001/archpsyc.57.10.925
5. Yehuda R. Biology of posttraumatic stress disorder. *J Clin Psychiatry* (2001) **62**(Suppl 17):41–6.
6. van Praag HM. Can stress cause depression? *World J Biol Psychiatry* (2005) **6**(Suppl 2):5–22. doi:10.1080/15622970510030018
7. Orr SP, Lasko NB, Shalev AY, Pitman RK. Physiologic responses to loud tones in Vietnam veterans with posttraumatic stress disorder. *J Abnorm Psychol* (1995) **104**:75–82. doi:10.1037/0021-843X.104.1.75
8. Morgan CA III, Doran A, Steffian G, Hazlett G, Southwick SM. Stress-induced deficits in working memory and visuo-constructive abilities in special operations soldiers. *Biol Psychiatry* (2006) **60**:722–9. doi:10.1016/j.biopsych.2006.04.021
9. Stam R. PTSD and stress sensitisation: a tale of brain and body Part 1: human studies. *Neurosci Biobehav Rev* (2007) **31**:530–57. doi:10.1016/j.neubiorev.2006.11.010
10. Orr SP, Metzger LJ, Lasko NB, Macklin ML, Peri T, Pitman RK. De novo conditioning in trauma-exposed individuals with and without posttraumatic stress disorder. *J Abnorm Psychol* (2000) **109**:290–8. doi:10.1037/0021-843X.109.2.290
11. Orr SP, Metzger LJ, Lasko NB, Macklin ML, Hu FB, Shalev AY, et al. Physiologic responses to sudden, loud tones in monozygotic twins discordant for combat

exposure: association with posttraumatic stress disorder. *Arch Gen Psychiatry* (2003) **60**:283–8. doi:10.1001/archpsyc.60.3.283
12. Heim C, Newport DJ, Heit S, Graham YP, Wilcox M, Bonsall R, et al. Pituitary-adrenal and autonomic responses to stress in women after sexual and physical abuse in childhood. *JAMA* (2000) **284**:592–7. doi:10.1001/jama.284.5.592
13. Morris MC, Compas BE, Garber J. Relations among posttraumatic stress disorder, comorbid major depression, and HPA function: a systematic review and meta-analysis. *Clin Psychol Rev* (2012) **32**:301–15. doi:10.1016/j.cpr.2012.02.002
14. Bhatnagar S, Dallman M. Neuroanatomical basis for facilitation of hypothalamic-pituitary-adrenal responses to a novel stressor after chronic stress. *Neuroscience* (1998) **84**:1025–39. doi:10.1016/S0306-4522(97)00577-0
15. Fernandes GA, Perks P, Cox NK, Lightman SL, Ingram CD, Shanks N. Habituation and cross-sensitization of stress-induced hypothalamic-pituitary-adrenal activity: effect of lesions in the paraventricular nucleus of the thalamus or bed nuclei of the stria terminalis. *J Neuroendocrinol* (2002) **14**:593–602. doi:10.1046/j.1365-2826.2002.00819.x
16. Bhatnagar S, Vining C. Facilitation of hypothalamic-pituitary-adrenal responses to novel stress following repeated social stress using the resident/intruder paradigm. *Horm Behav* (2003) **43**:158–65. doi:10.1016/S0018-506X(02)00011-9
17. Bhatnagar S, Mitchell JB, Betito K, Boksa P, Meaney MJ. Effects of chronic intermittent cold stress on pituitary adrenocortical and sympathetic adrenomedullary functioning. *Physiol Behav* (1995) **57**:633–9. doi:10.1016/0031-9384(94)00161-8
18. Thompson RS, Strong PV, Clark PJ, Maslanik TM, Wright KP Jr, Greenwood BN, et al. Repeated fear-induced diurnal rhythm disruptions predict PTSD-like sensitized physiological acute stress responses in F344 rats. *Acta Physiol* (2014) **211**(2):447–65. doi:10.1111/apha.12239
19. Cuadra G, Zurita A, Lacerra C, Molina V. Chronic stress sensitizes frontal cortex dopamine release in response to a subsequent novel stressor: reversal by naloxone. *Brain Res Bull* (1999) **48**:303–8. doi:10.1016/S0361-9230(98)00179-8
20. Weinberg MS, Bhatt AP, Girotti M, Masini CV, Day HE, Campeau S, et al. Repeated ferret odor exposure induces different temporal patterns of same-stressor habituation and novel-stressor sensitization in both hypothalamic-pituitary-adrenal axis activity and forebrain c-fos expression in the rat. *Endocrinology* (2009) **150**:749–61. doi:10.1210/en.2008-0958
21. Murua VS, Gomez RA, Andrea ME, Molina VA. Shuttle-box deficits induced by chronic variable stress: reversal by imipramine administration. *Pharmacol Biochem Behav* (1991) **38**:125–30. doi:10.1016/0091-3057(91)90599-W
22. Muscat R, Papp M, Willner P. Reversal of stress-induced anhedonia by the atypical antidepressants, fluoxetine and maprotiline. *Psychopharmacology* (1992) **109**:433–8. doi:10.1007/BF02247719
23. Papadimitriou GN, Linkowski P. Sleep disturbance in anxiety disorders. *Int Rev Psychiatry* (2005) **17**:229–36. doi:10.1080/09540260500104524
24. Taylor DJ, Lichstein KL, Durrence HH, Reidel BW, Bush AJ. Epidemiology of insomnia, depression, and anxiety. *Sleep* (2005) **28**:1457–64.
25. Armitage R. Sleep and circadian rhythms in mood disorders. *Acta Psychiatr Scand Suppl* (2007) **433**:104–15. doi:10.1111/j.1600-0447.2007.00968.x
26. Novati A, Roman V, Cetin T, Hagewoud R, Den Boer JA, Luiten PG, et al. Chronically restricted sleep leads to depression-like changes in neurotransmitter receptor sensitivity and neuroendocrine stress reactivity in rats. *Sleep* (2008) **31**:1579–85.
27. Baron KG, Reid KJ. Circadian misalignment and health. *Int Rev Psychiatry* (2014) **26**:139–54. doi:10.3109/09540261.2014.911149
28. Riemann D, Berger M, Voderholzer U. Sleep and depression – results from psychobiological studies: an overview. *Biol Psychol* (2001) **57**:67–103. doi:10.1016/S0301-0511(01)00090-4
29. Stein MB, Enns MW, Kryger MH. Sleep in nondepressed patients with panic disorder: II. Polysomnographic assessment of sleep architecture and sleep continuity. *J Affect Disord* (1993) **28**:1–6. doi:10.1016/0165-0327(93)90071-Q
30. Arriaga F, Paiva T, Matos-Pires A, Cavaglia F, Lara E, Bastos L. The sleep of non-depressed patients with panic disorder: a comparison with normal controls. *Acta Psychiatr Scand* (1996) **93**:191–4. doi:10.1111/j.1600-0447.1996.tb10630.x
31. Benca RM, Obermeyer WH, Thisted RA, Gillin JC. Sleep and psychiatric disorders. A meta-analysis. *Arch Gen Psychiatry* (1992) **49**:651–68. doi:10.1001/archpsyc.1992.01820080059010
32. Seifritz E. Contribution of sleep physiology to depressive pathophysiology. *Neuropsychopharmacology* (2001) **25**:S85–8. doi:10.1016/S0893-133X(01)00319-0
33. Neylan TC, Otte C, Yehuda R, Marmar CR. Neuroendocrine regulation of sleep disturbances in PTSD. *Ann N Y Acad Sci* (2006) **1071**:203–15. doi:10.1196/annals.1364.015
34. Ross RJ, Ball WA, Dinges DF, Kribbs NB, Morrison AR, Silver SM, et al. Rapid eye movement sleep disturbance in posttraumatic stress disorder. *Biol Psychiatry* (1994) **35**:195–202. doi:10.1016/0006-3223(94)91152-5
35. Mellman TA, Kumar A, Kulick-Bell R, Kumar M, Nolan B. Nocturnal/daytime urine noradrenergic measures and sleep in combat-related PTSD. *Biol Psychiatry* (1995) **38**:174–9. doi:10.1016/0006-3223(94)00238-X
36. Mellman TA. Psychobiology of sleep disturbances in posttraumatic stress disorder. *Ann N Y Acad Sci* (1997) **821**:142–9. doi:10.1111/j.1749-6632.1997.tb48275.x
37. Harvey AG, Jones C, Schmidt DA. Sleep and posttraumatic stress disorder: a review. *Clin Psychol Rev* (2003) **23**:377–407. doi:10.1016/S0272-7358(03)00032-1
38. Ross RJ, Ball WA, Sullivan KA, Caroff SN. Sleep disturbance as the hallmark of posttraumatic stress disorder. *Am J Psychiatry* (1989) **146**:697–707.
39. Mellman TA, Bustamante V, Fins AI, Pigeon WR, Nolan B. REM sleep and the early development of posttraumatic stress disorder. *Am J Psychiatry* (2002) **159**:1696–701. doi:10.1176/appi.ajp.159.10.1696
40. Pawlyk AC, Morrison AR, Ross RJ, Brennan FX. Stress-induced changes in sleep in rodents: models and mechanisms. *Neurosci Biobehav Rev* (2008) **32**:99–117. doi:10.1016/j.neubiorev.2007.06.001
41. Sanford LD, Suchecki D, Meerlo P. Stress, arousal, and sleep. *Curr Top Behav Neurosci* (2014). doi:10.1007/7854_2014_314
42. Dallaspezia S, Benedetti F. Chronobiological therapy for mood disorders. *Expert Rev Neurother* (2011) **11**:961–70. doi:10.1586/ern.11.61
43. Maturana MJ, Pudell C, Targa AD, Rodrigues LS, Noseda AC, Fortes MH, et al. REM sleep deprivation reverses neurochemical and other depressive-like alterations induced by olfactory bulbectomy. *Mol Neurobiol* (2014). doi:10.1007/s12035-014-8721-x
44. Maier SF, Watkins LR. Stressor controllability and learned helplessness: the roles of the dorsal raphe nucleus, serotonin, and corticotropin-releasing factor. *Neurosci Biobehav Rev* (2005) **29**:829–41. doi:10.1016/j.neubiorev.2005.03.021
45. Christianson JP, Paul ED, Irani M, Thompson BM, Kubala KH, Yirmiya R, et al. The role of prior stressor controllability and the dorsal raphe nucleus in sucrose preference and social exploration. *Behav Brain Res* (2008) **193**:87–93. doi:10.1016/j.bbr.2008.04.024
46. Maier SF. Role of fear in mediating shuttle escape learning deficit produced by inescapable shock. *J Exp Psychol Anim Behav Process* (1990) **16**:137–49. doi:10.1037/0097-7403.16.2.137
47. Thompson RS, Christianson JP, Maslanik TM, Maier SF, Greenwood BN, Fleshner M. Effects of stressor controllability on diurnal physiological rhythms. *Physiol Behav* (2013) **15**(112–113):32–9. doi:10.1016/j.physbeh.2013.02.009
48. Sanford LD, Yang L, Wellman LL, Liu X, Tang X. Differential effects of controllable and uncontrollable footshock stress on sleep in mice. *Sleep* (2010) **33**:621–30.
49. Moraska A, Fleshner M. Voluntary physical activity prevents stress-induced behavioral depression and anti-KLH antibody suppression. *Am J Physiol Regul Integr Comp Physiol* (2001) **281**:R484–9.
50. Greenwood BN, Foley TE, Day HE, Campisi J, Hammack SH, Campeau S, et al. Freewheel running prevents learned helplessness/behavioral depression: role of dorsal raphe serotonergic neurons. *J Neurosci* (2003) **23**:2889–98.
51. Greenwood BN, Spence KG, Crevling DM, Clark PJ, Craig WC, Fleshner M. Exercise-induced stress resistance is independent of exercise controllability and the medial prefrontal cortex. *Eur J Neurosci* (2013) **37**:469–78. doi:10.1111/ejn.12044
52. Thompson RS, Strong PS, Fleshner M. Physiological consequences of repeated exposures to conditioned fear. *Behav Sci* (2012) **2**:57–78. doi:10.3390/bs2020057
53. Greenwood BN, Loughridge AB, Sadaoui N, Christianson JP, Fleshner M. The protective effects of voluntary exercise against the behavioral consequences of uncontrollable stress persist despite an increase in anxiety following forced cessation of exercise. *Behav Brain Res* (2012) **233**:314–21. doi:10.1016/j.bbr.2012.05.017

54. Fanselow M, Lester L. A functional behavioristic approach to aversively motivated behavior: predatory imminence as a determinant of the topography of defensive behavior. In: Hillsdale NJ, Beecher RC, Bolles, MD, editors. *Evolution and Learning*. Hillsdale, NJ: Erlbaum (1988). p. 185–212.
55. Christianson JP, Ragole T, Amat J, Greenwood BN, Strong PV, Paul ED, et al. 5-hydroxytryptamine 2C receptors in the basolateral amygdala are involved in the expression of anxiety after uncontrollable traumatic stress. *Biol Psychiatry* (2010) **67**:339–45. doi:10.1016/j.biopsych.2009.09.011
56. Maier SF, Watkins LR. Stressor controllability, anxiety, and serotonin. *Cognit Ther Res* (1998) **22**:595–613. doi:10.1023/A:1018794104325
57. Greenwood BN, Strong PV, Brooks L, Fleshner M. Anxiety-like behaviors produced by acute fluoxetine administration in male Fischer 344 rats are prevented by prior exercise. *Psychopharmacology (Berl)* (2008) **199**:209–22. doi:10.1007/s00213-008-1167-y
58. Baracchi F, Opp MR. Sleep-wake behavior and responses to sleep deprivation of mice lacking both interleukin-1 beta receptor 1 and tumor necrosis factor-alpha receptor 1. *Brain Behav Immun* (2008) **22**:982–93. doi:10.1016/j.bbi.2008.02.001
59. Olivadoti MD, Opp MR. Effects of i.c.v. administration of interleukin-1 on sleep and body temperature of interleukin-6-deficient mice. *Neuroscience* (2008) **153**:338–48. doi:10.1016/j.neuroscience.2008.02.008
60. Paxinos G, Watson C. *The Rat Brain in Stereotaxic Coordinates*. Elsevier (2006).
61. Greenwood BN, Strong PV, Loughridge AB, Day HE, Clark PJ, Mika A, et al. 5-HT(2C) receptors in the basolateral amygdala and dorsal striatum are a novel target for the anxiolytic and antidepressant effects of exercise. *PLoS One* (2012) **7**:e46118. doi:10.1371/journal.pone.0046118
62. Morrow JD, Opp MR. Diurnal variation of lipopolysaccharide-induced alterations in sleep and body temperature of interleukin-6-deficient mice. *Brain Behav Immun* (2005) **19**:40–51. doi:10.1016/j.bbi.2004.04.001
63. Dement W. The effect of dream deprivation. *Science* (1960) **131**:1705–7. doi:10.1126/science.131.3415.1705
64. Tang X, Liu X, Yang L, Sanford LD. Rat strain differences in sleep after acute mild stressors and short-term sleep loss. *Behav Brain Res* (2005) **160**:60–71. doi:10.1016/j.bbr.2004.11.015
65. Suchecki D, Tiba PA, Machado RB. REM sleep rebound as an adaptive response to stressful situations. *Front Neurol* (2012) **3**:41. doi:10.3389/fneur.2012.00041
66. Takase LF, Nogueira MI, Bland ST, Baratta M, Watkins LR, Maier SF, et al. Effect of number of tailshocks on learned helplessness and activation of serotonergic and noradrenergic neurons in the rat. *Behav Brain Res* (2005) **162**(2):299–306. doi:10.1016/j.bbr.2005.04.008
67. Maier SF, Grahn RE, Kalman BA, Sutton LC, Wiertelak EP, Watkins LR. The role of the amygdala and dorsal raphe nucleus in mediating the behavioral consequences of inescapable shock. *Behav Neurosci* (1993) **107**:377–88. doi:10.1037/0735-7044.107.2.377
68. Maier SF, Grahn RE, Watkins LR. 8-OH-DPAT microinjected in the region of the dorsal raphe nucleus blocks and reverses the enhancement of fear conditioning and interference with escape produced by exposure to inescapable shock. *Behav Neurosci* (1995) **109**:404–12. doi:10.1037/0735-7044.109.3.404
69. Rau V, Decola JP, Fanselow MS. Stress-induced enhancement of fear learning: an animal model of posttraumatic stress disorder. *Neurosci Biobehav Rev* (2005) **29**:1207–23. doi:10.1016/j.neubiorev.2005.04.010
70. Baratta MV, Christianson JP, Gomez DM, Zarza CM, Amat J, Masini CV, et al. Controllable versus uncontrollable stressors bi-directionally modulate conditioned but not innate fear. *Neuroscience* (2007) **146**:1495–503. doi:10.1016/j.neuroscience.2007.03.042
71. Grissom N, Bhatnagar S. Habituation to repeated stress: get used to it. *Neurobiol Learn Mem* (2009) **92**:215–24. doi:10.1016/j.nlm.2008.07.001
72. Christianson JP, Greenwood BN. Stress-protective neural circuits: not all roads lead through the prefrontal cortex. *Stress* (2014) **17**:1–12. doi:10.3109/10253890.2013.794450
73. Rudy JW, Barrientos RM, O'Reilly RC. Hippocampal formation supports conditioning to memory of a context. *Behav Neurosci* (2002) **116**:530–8. doi:10.1037/0735-7044.116.4.530
74. McEwen BS. Plasticity of the hippocampus: adaptation to chronic stress and allostatic load. *Ann N Y Acad Sci* (2001) **933**:265–77. doi:10.1111/j.1749-6632.2001.tb05830.x
75. Kim JJ, Rison RA, Fanselow MS. Effects of amygdala, hippocampus, and periaqueductal gray lesions on short- and long-term contextual fear. *Behav Neurosci* (1993) **107**:1093–8. doi:10.1037/0735-7044.107.6.1093
76. Cook SC, Wellman CL. Chronic stress alters dendritic morphology in rat medial prefrontal cortex. *J Neurobiol* (2004) **60**:236–48. doi:10.1002/neu.20025
77. Goldwater DS, Pavlides C, Hunter RG, Bloss EB, Hof PR, Mcewen BS, et al. Structural and functional alterations to rat medial prefrontal cortex following chronic restraint stress and recovery. *Neuroscience* (2009) **164**:798–808. doi:10.1016/j.neuroscience.2009.08.053
78. Sierra-Mercado D, Padilla-Coreano N, Quirk GJ. Dissociable roles of prelimbic and infralimbic cortices, ventral hippocampus, and basolateral amygdala in the expression and extinction of conditioned fear. *Neuropsychopharmacology* (2011) **36**:529–38. doi:10.1038/npp.2010.184
79. Drevets WC, Price JL, Furey ML. Brain structural and functional abnormalities in mood disorders: implications for neurocircuitry models of depression. *Brain Struct Funct* (2008) **213**:93–118. doi:10.1007/s00429-008-0189-x
80. Koenigs M, Grafman J. Posttraumatic stress disorder: the role of medial prefrontal cortex and amygdala. *Neuroscientist* (2009) **15**:540–8. doi:10.1177/1073858409333072
81. Li L, Wu M, Liao Y, Ouyang L, Du M, Lei D, et al. Grey matter reduction associated with posttraumatic stress disorder and traumatic stress. *Neurosci Biobehav Rev* (2014) **43C**:163–72. doi:10.1016/j.neubiorev.2014.04.003
82. Meng Y, Qiu C, Zhu H, Lama S, Lui S, Gong Q, et al. Anatomical deficits in adult posttraumatic stress disorder: a meta-analysis of voxel-based morphometry studies. *Behav Brain Res* (2014) **270C**:307–15. doi:10.1016/j.bbr.2014.05.021
83. Jha SK, Brennan FX, Pawlyk AC, Ross RJ, Morrison AR. REM sleep: a sensitive index of fear conditioning in rats. *Eur J Neurosci* (2005) **21**:1077–80. doi:10.1111/j.1460-9568.2005.03920.x
84. Wellman LL, Yang L, Tang X, Sanford LD. Contextual fear extinction ameliorates sleep disturbances found following fear conditioning in rats. *Sleep* (2008) **31**:1035–42.
85. Kant GJ, Pastel RH, Bauman RA, Meininger GR, Maughan KR, Robinson TN III, et al. Effects of chronic stress on sleep in rats. *Physiol Behav* (1995) **57**:359–65. doi:10.1016/0031-9384(94)00241-V
86. Tang X, Yang L, Sanford LD. Rat strain differences in freezing and sleep alterations associated with contextual fear. *Sleep* (2005) **28**:1235–44.
87. Moreau JL, Scherschlicht R, Jenck F, Martin JR. Chronic mild stress-induced anhedonia model of depression; sleep abnormalities and curative effects of electroshock treatment. *Behav Pharmacol* (1995) **6**:682–7. doi:10.1097/00008877-199511000-00003
88. Rachalski A, Alexandre C, Bernard JF, Saurini F, Lesch KP, Hamon M, et al. Altered sleep homeostasis after restraint stress in 5-HTT knock-out male mice: a role for hypocretins. *J Neurosci* (2009) **29**:15575–85. doi:10.1523/JNEUROSCI.3138-09.2009
89. Bonnet C, Marinesco S, Debilly G, Kovalzon V, Cespuglio R. Influence of a 1-h immobilization stress on sleep and CLIP (ACTH(18-39)) brain contents in adrenalectomized rats. *Brain Res* (2000) **853**:323–9. doi:10.1016/S0006-8993(99)02313-6
90. Machado RB, Tufik S, Suchecki D. Chronic stress during paradoxical sleep deprivation increases paradoxical sleep rebound: association with prolactin plasma levels and brain serotonin content. *Psychoneuroendocrinology* (2008) **33**:1211–24. doi:10.1016/j.psyneuen.2008.06.007
91. Sanford LD, Parris B, Tang X. GABAergic regulation of the central nucleus of the amygdala: implications for sleep control. *Brain Res* (2002) **956**:276–84. doi:10.1016/S0006-8993(02)03552-7
92. Emens J, Lewy A, Kinzie JM, Arntz D, Rough J. Circadian misalignment in major depressive disorder. *Psychiatry Res* (2009) **168**:259–61. doi:10.1016/j.psychres.2009.04.009
93. Hasler BP, Buysse DJ, Kupfer DJ, Germain A. Phase relationships between core body temperature, melatonin, and sleep are associated with depression severity: further evidence for circadian misalignment in non-seasonal depression. *Psychiatry Res* (2010) **178**:205–7. doi:10.1016/j.psychres.2010.04.027
94. Howland RH. An overview of seasonal affective disorder and its treatment options. *Phys Sportsmed* (2009) **37**:104–15. doi:10.3810/psm.2009.12.1748

95. Cole RJ, Loving RT, Kripke DF. Psychiatric aspects of shiftwork. *Occup Med* (1990) **5**:301–14.
96. Scott AJ, Monk TH, Brink LL. Shiftwork as a risk factor for depression: a pilot study. *Int J Occup Environ Health* (1997) **3**:S2–9.

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Neuroinflammation and Behavior in HIV-1 Transgenic Rats Exposed to Chronic Adolescent Stress

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Highly active antiretroviral therapy (HAART) has improved prognosis for people living with HIV (PLWH) and dramatically reduced the incidence of AIDS. However, even when viral load is controlled, PLWH develop psychiatric and neurological disorders more frequently than those living without HIV. Adolescents with HIV are particularly susceptible to the development of psychiatric illnesses and neurocognitive impairments. While both psychiatric and neurocognitive disorders have been found to be exacerbated by stress, the extent to which chronic stress and HIV-1 viral proteins interact to impact behavior and relevant neuroinflammatory processes is unknown. Determination of the individual contributions of stress and HIV to neuropsychiatric disorders is heavily confounded in humans. In order to isolate the influence of HIV-1 proteins and chronic stress on behavior and neuroinflammation, we employed the HIV-1 transgenic (Tg) rat model, which expresses HIV-1 proteins with a *gag* and *pol* deletion, allowing for viral protein expression without viral replication. This Tg line has been characterized as a model of HAART-controlled HIV-1 infection due to the lack of viral replication but continued presence of HIV-1 proteins. We exposed male and female adolescent HIV-1 Tg rats to a mixed-modality chronic stress paradigm consisting of isolation, social defeat and restraint, and assessed behavior, cerebral vascularization, and neuroinflammatory endpoints. Stress, sex, and presence of the HIV-1 transgene impacted weight gain in adolescent rats. Female HIV-1 Tg rats showed decreases in central tendency during the light cycle in the open field regardless of stress exposure. Both male and female HIV-1 Tg rats exhibited decreased investigative behavior in the novel object recognition task, but no memory impairments. Adolescent stress had no effect on the tested behaviors. Microglia in female HIV-1 Tg rats exhibited a hyper-ramified structure, and gene expression of complement factor B was increased in the hippocampus. In addition, adolescent stress exposure increased microglial branching and junctions in female wild-type rats without causing any additional increase in HIV-1 rats. These data suggest that the presence of HIV-1 proteins during development leads to alterations in behavioral and neuroinflammatory endpoints that are not further impacted by concurrent chronic adolescent stress.

Keywords: sex differences, stress, HIV, neuroinflammation, anxiety, microglia

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INTRODUCTION

Following the advent of highly active antiretroviral therapy (HAART) in the treatment of HIV-1 infection, the life expectancy for people living with HIV (PLWH) has greatly increased (1). However, with increasing life expectancy among PLWH, complications not traditionally associated with HIV infection have emerged (1). These comorbidities include increased incidence of affective disorders, HIV-1-associated neurocognitive disorders (HAND), and non-AIDS-associated dementia and can occur even in individuals with HAART-controlled viral load. In fact, as many as 52% of PLWH experience some form of neuropsychological impairment (2). PLWH are at a greater risk of developing a mood disorder (3), and individuals with HAND are less likely to adhere to life-saving treatment regimens (4), increasing the risk of death (5). As many as 60% of the adolescents living with HIV develop psychiatric illnesses (6, 7) or neurocognitive impairments (8). These adolescents are at high risk of unsafe behaviors (9), but the mechanisms underlying this sensitivity to neuropsychiatric disease are unknown. Furthermore, the risks may be particularly high for HIV-infected females, given the established increased risk for affective disorders in women over men even in the absence of HIV (10, 11).

While stigma or distress associated with HIV-positive status has been suggested to contribute to neurocognitive impairment and the increased prevalence of mood disorders among PLWH, recent studies suggest that these behavioral pathologies are also mediated by biological mechanisms that extend beyond the direct impact of psychosocial stress associated with being HIV-positive (12–16). These studies have examined HIV-associated alterations in behavior and their underlying biological mechanisms, many of which involve neuroinflammatory processes. However, due to the difficulty of disentangling the impact of HIV-1 viral proteins and psychosocial stress in PLWH, the potential interaction between chronic stress and HIV-1 viral proteins remains incompletely understood. Using the HIV-1 transgenic (Tg) rat model, we sought to examine the contributions of developmental exposure to HIV-1 proteins and chronic adolescent stress in order to better understand the extent to which HIV and stress interact in adolescents with HIV.

The HIV-1 Tg rat expresses all but two of the genes contained in the HIV-1 virus, which allows expression of functional viral proteins without active viral replication (17). This non-replicating HIV-1 Tg line in the rat has been characterized as a useful tool to model comorbidities experienced by PLWH receiving HAART (18) as well as a model for other childhood HIV-1-associated disorders (19).

Here, we used the HIV-1 Tg rat to address the hypothesis that chronic adolescent stress exacerbates behavioral and neuroinflammatory impairments in HIV-1 Tg rats compared to wild-type (WT) controls in a sex-specific manner. We focused on inflammatory endpoints due to their capacity to modulate affective and cognitive behavior as well as the profound immune alterations seen in HIV-1 infection. We assessed microglial morphology in females as well as gene expression of complement factor B and lipocalin-2, two inflammatory proteins that may also be involved in neuronal structure and the impairments observed in PLWH, in non-stressed female rats. Collectively, the data presented suggest that females are more susceptible to the behavioral effects of HIV-1 protein expression during development than males and that HIV-1 proteins mirror, but do not interact with, the neuroinflammatory impact of chronic adolescent stress in females.

MATERIALS AND METHODS

Animals

Wild-type and HIV-1 Tg male and female rats were bred on-site from Tg male breeders and WT female Fisher 344/NHsd dams purchased from Harlan Laboratories (Indianapolis, IN, USA). The HIV-1 Tg rat, originally described by Reid et al. (17), was derived from the Fisher 344/NHsd Sprague–Dawley line. HIV-1 Tg rats show clinical manifestations and pathology resembling HIV, including respiratory problems, neurological changes, cataracts, nephropathy, muscle atrophy, and altered affective-type behavior (15, 17, 19, 20). The studies presented here were completed by postnatal day (PND) 55 and thus occurred prior to the onset of previously documented pathology. Offspring were group-housed with siblings (2–3 rats per cage) after weaning. Male and female rats were used for all behavioral analyses, while female rats were used for histological and gene expression assessments. Animals were kept in an AAALAC-approved temperature- and humidity-controlled vivarium and maintained on a reverse 14:10 light:dark cycle. Food and water were available *ad libitum*. For these studies, litters were split among all groups such that no more than two pups from a litter were in any one group. This measure was taken in order to avoid litter effects. Chronic mixed-modality adolescent stress took place from PND37 to PND48; behavioral assessments were performed from PND49 to PND53, and rats were euthanized between PND54 and PND55. All experiments were performed in accordance with the Institutional Animal Care and Use Committee of Emory University and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Chronic Mixed-Modality Stress

A subset of each cohort was submitted to chronic mixed-modality adolescent stress (WT-male-control $n = 8$, WT-male-stressed $n = 9$, WT-female-control $n = 11$, WT-female-stressed $n = 10$, Tg-male-control $n = 11$, Tg-male-stressed $n = 11$, Tg-female-control $n = 10$, and Tg-female-stressed $n = 9$). This mixed-modality stress paradigm has been previously described and shown to elicit both short-term and lasting behavioral and physiological changes in Wistar rats (21–24). Consistent with the previously established paradigm, animals receiving stress were individually housed at PND36 and randomly exposed to interspersed days of social defeat and restraint stress for 12 days (PND37–PND48). Stressed animals were individually housed throughout the duration of behavioral testing. Non-stressed animals remained pair-housed throughout the entire study and encountered daily handling and cage movement to control for the stress paradigm. Rats were weighed on isolation day and twice throughout stress.

The 6 days of social defeat stress were performed during the light phase of the light:dark cycle and took place in the home cage of a mature, territorial, Long–Evans rat. All experimental animals were paired with a same-sex resident Long–Evans rat, and female resident Long–Evans rats were ovariectomized to control for estrous cycle-dependent variations in aggressive behavior. Each experimental animal was placed in the home cage of the resident and separated by a barrier permeable to sound and scent; after 2 min, the barrier was lifted. After the experimental animal was aggressed by the resident up to five times on the first day, three times on the second day, and once each day thereafter, or after 5 min of interaction, the barrier was replaced. This separation continued for 25 min, after which point the experimental animal was returned to its home cage. Pairings were randomly assigned daily to prevent stabilization of a dominance hierarchy.

For the 6 days of restraint stress, animals were placed in a clear acrylic rat restraint (Braintree Scientific, Inc., Braintree, MA, USA) for 60 min during the light phase. These restraints prevented head-to-tail turns but did not compress the rat.

Behavioral Testing

Behavioral testing took place from PND49 to PND53, after the completion of the stress paradigm, and consisted of open-field testing and novel object testing. The behavioral testing was completed in six cohorts across a 4-month span of time with all groups represented equally in each cohort. The length of testing for any one cohort was 5 days. Sample sizes are detailed in **Table 1**.

Open-field Testing

Measurement of locomotor activity and spatial preference during the open-field test can be used as an assessment of both physiological capacity as well as anxiety-like behavior (25). Animals were assessed in the open field during the light phase under white light (between 8:00 and 11:00 a.m.) on PND49 and during the dark phase under red lighting (between 3:00 and 6:00 p.m.) on PND50. For both tests, each rat was placed into a 90 cm × 90 cm box and allowed to explore freely for 10 min, after which time the rat was placed back in its home cage, and the open-field arena was cleaned thoroughly with 70% ethanol. One rat was tested at a time. All behaviors in both the light and dark phase testing were recorded by a video camera that was connected to an automated behavior analysis system and analyzed by research assistant blind to experimental group (CleverSys, Inc., Reston, VA, USA). Four animals (Tg-male-stressed $n = 2$, WT-male-control $n = 1$, and WT-male-stressed $n = 1$) were excluded from open-field analysis due to a procedural error.

TABLE 1 | Sample sizes for each of the behavioral tests (open-field and novel object recognition task), animal weight data, and molecular analyses are detailed.

Test	F WT NS	F Tg NS	M WT NS	M Tg NS	F WT S	F Tg S	M WT S	M Tg S
Novel object	9	7	8	9	10	4	8	11
Open field	11	10	7	11	10	9	8	9
Body weight over stress	11	10	8	11	10	9	8	11
Terminal weight	11	10	8	9	9	9	6	11
Microglial analysis	3	3	N/A	N/A	3	3	N/A	N/A
Lcn2 gene expression	7	10	N/A	N/A	N/A	N/A	N/A	N/A
Cfb gene expression	10	11	N/A	N/A	N/A	N/A	N/A	N/A

Animals used in behavior analysis were included in weight analysis. M, male; F, female; NS, no stress; S, stress; WT, wild type; Tg, transgenic.

Novel Object Testing

The novel object test is used to assess object recognition memory, a task that is dependent on hippocampal function (26). Training and testing occurred during the light phase between 8:00 a.m. and 12:00 p.m., and two animals were tested simultaneously, individually in separate chambers. The chamber used for the open-field test was subdivided into two 90 cm × 45 cm arenas. On PND51, rats were habituated to the smaller arena with a 10-min exploration period. On PND52 and 53, rats were exposed to the no-delay and the hour-delay tasks in a counterbalanced order. In the no-delay task, the rat was placed into the arena with two identical objects ("familiar objects") and allowed to explore for 15 min. After this period, the rat was briefly returned to its home cage; the objects and arena were quickly cleaned with 70% ethanol, and one of the objects was replaced with a novel object. The rat was returned to the arena and allowed to explore the objects for 5 min. All behaviors during this period were recorded by a video camera that was connected to an automated behavior analysis system and analyzed by a research assistant blind to experimental group (CleverSys, Inc., Reston, VA, USA). The hour-delay task was identical to the no-delay task but used different novel and familiar objects, and the rat was returned to its home cage for a 1-h delay before undergoing the 5-min recorded encounter. Several rats were excluded from novel object behavioral analysis due to a procedural error (WT-female-control $n = 2$, WT-male-stressed $n = 1$, Tg-male-control $n = 2$, Tg-female-control $n = 2$, and Tg-female-stressed $n = 5$). Difference in time spent sniffing novel vs. familiar object was calculated by taking the absolute value of the difference in time spent sniffing the novel and familiar objects. One animal (Tg-female-control) was identified as an outlier *via* Grubbs' test ($\alpha = 0.05$) and was removed from novel object analysis.

Tissue Collection

On PND54–55, all animals used for behavioral tests and stereology were euthanized with Euthasol® (a combination of pentobarbital sodium and phenytoin sodium), perfused for 2 min with ice cold saline, and then perfused with 4% paraformaldehyde for 10 min. Estrous cycle was not monitored in females. Brains were removed and postfixed in 4% paraformaldehyde and cryoprotected in 30% sucrose for 24 h before freezing. Female WT ($n = 11$) and HIV-1 Tg ($n = 11$) non-stressed animals that did not undergo behavioral testing were euthanized *via* rapid decapitation for quantitative real-time polymerase chain reaction (qPCR) analysis. Brains were flash frozen on dry-ice and stored at −80°C until later analysis. In addition, adrenal glands, uteri, and testes were collected, weighed, and normalized to terminal weights. Percentage

weight change over stress was calculated by dividing the difference between day 10 stress weight and isolation weight by the isolation weight. Normalized adrenal and reproductive weights were calculated by dividing each rat's adrenal or reproductive weight (milligrams) by its terminal body weight (grams). All weight analyses were performed with animals that underwent behavioral testing.

Immunohistochemistry and Stereology

Brains from female rats were sectioned at 40 or 50 μm on a cryostat.

IBA-1 Immunohistochemistry

Sections from female WT-control, WT-stress, Tg-control, and Tg-stress ($n = 3$ per group) rats encompassing the entire rostro–caudal axis of the brain were stained for ionized calcium-binding adaptor molecule-1 (IBA-1) [four sections per animal, section sampling fraction (ssf) = 1/12]. After washing in TBS, sections were blocked in a 10 μg/ml avidin solution for 1 h at 4°C. Sections were washed again and then incubated in rabbit IBA-1 (1:1000, 019-19741, WAKO, Richmond, VA, USA) in a 50 μg/ml biotin solution overnight at 4°C. Sections were then washed and incubated in biotinylated goat anti-rabbit IgG (BA-1000, Vector Labs, Burlingame, CA, USA) for 1 h at 4°C before washing and incubating with the Vectastain Elite ABC kit (Vector Labs, Burlingame, CA, USA). The stain was then visualized with diaminobenzidine (SigmaFast 3,3′-diaminobenzidine tablets, Sigma–Aldrich, St. Louis, MO, USA), and sections were counterstained in cresyl violet. The optical fractionator probe was used to estimate the population of microglia in a single hemisphere of the hippocampus.

Microglial Morphology Analysis

Right hippocampal regions from four sections (three from one animal due to tissue integrity) from each animal were stitched together at 40× magnification (StereoInvestigator). Thirty cells were chosen at random per section, converted to 8-bit, adjusted for brightness, and cleaned with a Gaussian filter. Images were then converted to binary and skeletonized in ImageJ (National Institute of Health, version 1.49). The AnalyzeSkeleton plug-in was used to assess the number of junctions and average branch length for each microglia. Microglial properties (number of junctions, number of branches, maximum and average branch length) were averaged for each animal.

Cerebrovascular Immunohistochemistry

Sections from female rats were incubated with 1% H_2O_2 for 20 min at 4°C followed by blocking with normal goat serum (NGS) (3%) (Vector Labs, Burlingame, CA, USA), Triton X-100 (0.5%) (Sigma–Aldrich, St. Louis, MO, USA), and phosphate-buffered saline (PBS). Sections were then incubated with primary antibody mouse anti-rat endothelial cell antigen-1 [RECA-1 (0.5%) (Bio-Rad Labs, Berkeley, CA, USA)] overnight at 4°C. The following day, sections were incubated with biotinylated anti-mouse IgG secondary antibody (0.5%) (Vector Labs, Burlingame, CA, USA). Sections were then incubated with streptavidin–peroxidase–HRP. The stain was visualized with diaminobenzidine. Sections were mounted on slides, dried overnight, and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA, USA).

Blood Vessel Length and Density

To minimize bias, stereology was conducted by a single experimenter blind to experimental conditions. To determine the location of the prefrontal cortex (PFC) (WT $n = 3$, Tg $n = 5$), amygdala (WT $n = 5$, Tg $n = 6$), and hippocampus (WT $n = 5$, Tg $n = 6$) in each section, a rat atlas (27) was used as a guide to trace contours under 2× bright-field illumination. Three sections were used per animal for analysis. After tracing contours, sections were sampled under a 60× oil immersion objective. Estimates were taken using the Stereo Investigator system with the Spaceballs probe (MicroBrightField, Inc., Colchester, VT, USA). The following parameters were used in the Spaceballs probe for 40 and 50 μm sections analyzing the PFC: grid size $X = 230$ μm, grid size $Y = 315$ μm, guard zone space = fixed distance, Spaceball radius = 12 μm, and use hemisphere = true. Additionally, the following parameters were used for sections analyzing the hippocampus and amygdala: grid size $X = 275$ μm, grid size $Y = 360$ μm, guard zone space = fixed distance, Spaceball radius = 12 μm, and use hemisphere = true. These parameters resulted in a coefficient of error of ≤0.1.

Quantitative Real-Time Polymerase Chain Reaction

The PFC and hippocampus were dissected from female WT ($n = 11$) and HIV-1 Tg ($n = 11$) rats, and RNA was extracted with the TRIzol method (Invitrogen) using the RNeasy Mini Kit (Qiagen). RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNA was standardized using a Pico Green Assay (Invitrogen). TaqMan Gene Expression Mastermix and TaqMan Gene Expression Assays for *Hprt1* (Rn01527840_m1) and *Cfb* (Rn01526084_g1) were used. qPCR was performed on the Applied Biosystems 7900HT Sequence Detection System. Fold change in gene expression was calculated according to the $2^{-\Delta\Delta Ct}$ method using the *Hprt1* housekeeping gene. The targets were run in triplicate, and the cutoff for coefficient of variance (CV) was 4%. One WT animal was excluded from *Cfb* analysis because its CV value exceeded the 4% cutoff.

Gene expression of *Lcn2* (female WT $n = 7$, Tg $n = 10$) was assessed using primers from Integrated DNA Technologies (San Diego, CA, USA). Forward: GATTCGTCAGCTTTGCCAAGT and reverse: CATTGGTCGGTGGGAACAG. Absolute Blue qPCR SYBR Green ROX Mix (Thermo Scientific, Wilmington, DE, USA) was used to perform qPCR. Hippocampal gene expression of *Lcn2* was normalized to the geometric mean of *Hprt* and *B2m*, whereas PFC expression of *Lcn2* was normalized to the geometric mean of *B-actin*, *Hprt1*, *B2m*, and *Ldha*.

Statistical Analysis

Three-way analysis of variance (ANOVA) in R (version 3.2.1) was used to assess statistical significance in behavioral tests, percentage weight gain, and normalized adrenal measures. The analysis factors in three-way analysis of percentage weight gain

and normalized adrenal weight were all between-subjects and included sex, stress (control, adolescent stress), and genotype (WT, HIV-1 Tg). The analysis factors in three-way analyses of behavior were the between-subjects factors of stress (control, adolescent stress) and genotype (WT, HIV-1 Tg) with the repeated factor of time (light and dark in the open-field test or no delay and hour delay in the novel object test). Because of the added factor of time, sexes were assessed separately. If no main effect was present, the non-significant factor was removed from *post hoc* assessment leading to a *t*-test assessment. Two-way ANOVAs in GraphPad Prism (version 6) were used to assess group differences for reproductive weight, microglial population, and microglial morphology. The factors in two-way analyses were stress and genotype. Student's *t*-test was performed to assess statistical significance of number of microglial branches and number of microglial junctions between the WT control group and the Tg control group. A student's *t*-test was used to assess statistical significance for gene expression data. Each gene and region was compared to its WT control. All tests were performed with $\alpha = 0.05$, and all significant main effects and interactions are reported in the section "Results."

RESULTS

Both Chronic Adolescent Stress and HIV-1 Proteins Reduced Weight Gain

Exposure to chronic adolescent stress reduced weight gain in stressed animals [$F_{(1,70)} = 70.91$, $p < 0.01$] (**Figure 1A**). Females [$F_{(1,70)} = 79.21$, $p < 0.01$] and HIV-1 Tg rats [$F_{(1,70)} = 5.80$, $p = 0.02$] also gained less weight during stress as compared to males and WT rats. However, terminal weight was unchanged by stress [$F_{(1,65)} = 1.72$, $p > 0.05$]. HIV-1 Tg rats weighed less at collection than WT rats [$F_{(1,65)} = 150.88$, $p = 0.01$]. Female rats weighed less than males [$F_{(1,65)} = 199.81$, $p < 0.01$] (**Table 2**). Normalized adrenal weight was higher in females [$F_{(1,65)} = 44.60$, $p < 0.01$] and HIV-1 Tg rats [$F_{(1,65)} = 6.53$, $p = 0.01$] (**Figure 1B**). Normalized

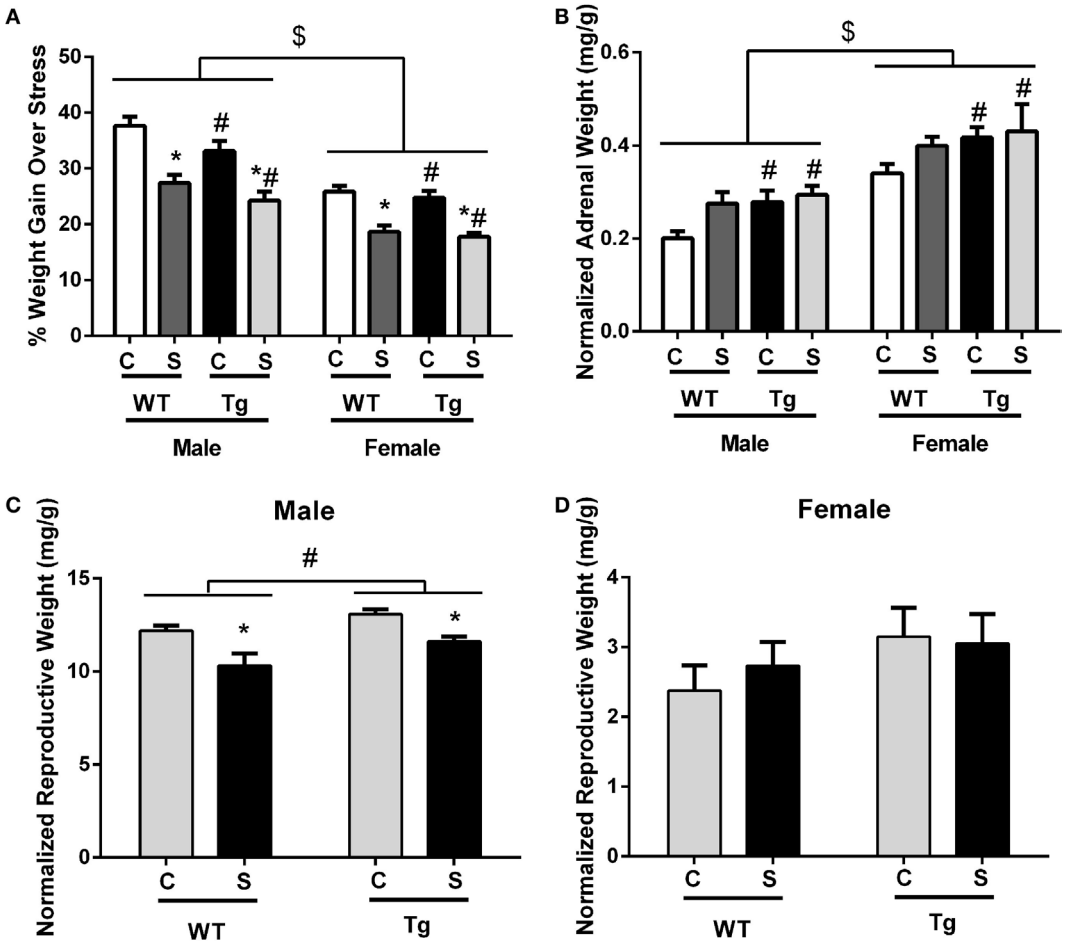

FIGURE 1 | Chronic adolescent stress reduced weight gain from PND36 to PND46. Females and HIV-1 Tg rats also gained less weight during stress **(A)**. Terminal adrenal weight was normalized to terminal body weight, and normalized adrenal weight was increased in HIV-1 Tg rats and in females **(B)**. Normalized reproductive weight was decreased in males exposed to adolescent stress but increased with HIV-1 transgene **(C)**. Normalized reproductive weight in females was unchanged by HIV-1 transgene or stress **(D)**. Data are presented as mean ± SEM. # denotes a main effect of HIV-1 Tg genotype, *Denotes a main effect of stress, and $ denotes a main effect of sex. $\alpha = 0.05$.

reproductive weight was analyzed separately between the sexes due to the distinct differences in sex organs. Stress [$F_{(1,31)} = 6.87$, $p = 0.01$] and HIV-1 transgene [$F_{(1,31)} = 16.09$, $p < 0.01$] increased male reproductive weight (**Figure 1C**). Neither stress [$F_{(1,34)} = 2.05$, $p > 0.05$] nor HIV-1 genotype [$F_{(1,34)} = 0.12$, $p > 0.05$] impacted normalized reproductive weight in females (**Figure 1D**). Details of statistical analyses are detailed in **Table 3**.

Female HIV-1 Tg Rats had Reduced Central Tendency in the Open-field Maze

As expected for a nocturnal species, male [$F_{(1,31)} = 56.86$, $p < 0.01$] and female [$F_{(1,36)} = 52.55$, $p < 0.01$] rats, regardless of stress exposure or genotype, traveled greater distance in the open-field maze during the dark cycle than during the light cycle. Stress did not independently impact locomotor activity [$F_{(1,36)} = 1.78$, $p > 0.05$] or percentage time in center [$F_{(1,36)} = 0.38$, $p > 0.05$] for female rats of either genotype. *Post hoc* assessment demonstrated that female HIV-1 Tg rats exhibited decreased locomotor activity compared to female WT rats in the light cycle ($t_{38} = 3.62$, $p < 0.001$) (**Figure 2A**) and that female HIV-1 rats spent a decreased time in the center of the open-field maze compared to female WT rats during the light cycle [$t_{(1,38)} = 2.35$, $p = 0.024$] (**Figure 2B**). Male HIV-1 rats also exhibited decreased locomotor activity compared to male WT rats in the light cycle ($t_{33} = 2.93$, $p < 0.01$). Female [$F_{(1,36)} = 29.35$, $p < 0.001$] and male [$F_{(1,31)} = 24.46$, $p < 0.001$] rats also spent an increased percentage of time in the center of the open-field maze during the dark cycle, as compared to percentage time in center during the light cycle (**Figures 2B,D**). HIV-1 transgene and time of day interacted in males [$F_{(1,31)} = 13.23$, $p < 0.001$] and females [$F_{(1,36)} = 8.68$, $p < 0.01$] to impact the locomotor activity of males in the open-field maze. HIV-1 Tg genotype, stress, and sex [$F_{(1,31)} = 5.11$, $p = 0.03$] also interacted to impact the locomotor activity of males (**Figure 2C**) in the open-field maze. However, *post hoc* assessment did not attribute these main effects or interactions to differences between any two specific groups. All main effects and interactions are detailed in **Table 4**.

TABLE 2 | Terminal weight (in grams) of rats that were exposed to chronic adolescent stress and behavioral testing.

	Wild type		HIV-1 transgenic	
	No stress	**Stress**	**No stress**	**Stress**
Male	176.0 ± 4.56 g	168.0 ± 7.32 g	135.7 ± 4.59 g[b]	138.5 ± 2.81 g[b]
Female	135.2 ± 2.34 g[a]	129.9 ± 3.12 g[a]	107.3 ± 1.56 g[a,b]	103.1 ± 3.04 g[a,b]

Mean ± SEM are shown. HIV-1 Tg rats weighed less than WT rats [$F_{(1,65)} = 150.88$, $p < 0.01$]. Females weighed less than males [$F_{(1,65)} = 199.81$, $p < 0.01$].

[a]Denotes a main effect of sex.

[b]denotes a main effect of HIV-1 genotype.

Female HIV-1 Tg Rats Exhibit Decreased Performance in Novel Object Recognition Task

The novel object recognition task was performed with no delay or 1-h delay from exposure to familiar objects. Stress did not impact total time sniffing object in males [$F_{(1,32)} = 0.31$, $p > 0.05$] or females [$F_{(1,26)} = 0.02$, $p > 0.05$]. Time (no delay vs. hour delay) [$F_{(1,26)} = 1.53$, $p > 0.05$] had no impact on total time sniffing objects in females (**Figure 3A**). Female HIV-1 Tg rats spent less time sniffing all objects compared to WT females at each time point [$F_{(1,26)} = 6.78$, $p = 0.02$] (**Figure 2A**), and HIV-1 Tg females exhibited a reduced difference in time sniffing the novel vs. the familiar object compared to female WT rats [$F_{(1,26)} = 5.39$, $p = 0.03$] (**Figure 3B**). Male HIV-1 Tg rats spent a reduced total time sniffing all objects [$F_{(1,32)} = 7.5$, $p < 0.01$] (**Figure 3C**). Stress, genotype, and time interacted [$F_{(1,32)} = 5.17$, $p = 0.03$] to impact the difference in time spent sniffing the novel vs. familiar object in males (**Figure 3D**). This difference was not attributable to a particular group difference.

HIV-1 Tg Rats Exhibit Enhanced Ramification in Hippocampal Microglia

The estimated population of microglia in the hippocampus in females was unchanged by stress [$F_{(1,8)} = 3.26$, $p > 0.05$] or genotype [$F_{(1,8)} = 0.32$, $p > 0.05$, **Table 5**]. Hippocampal microglia in HIV-1 Tg rats exhibited an increased number of branches [$F_{(1,8)} = 14.62$, $p < 0.01$], number of junctions [$F_{(1,8)} = 13.94$, $p < 0.01$], and maximum branch length [$F_{(1,8)} = 14.31$, $p < 0.01$], but average branch length was unchanged [$F_{(1,8)} = 1.45$, $p > 0.05$] compared to

TABLE 3 | *F*-statistics and *p*-values for all main effects and interactions are shown for weight analysis using a three-way ANOVA, between factors (sex, HIV-1 genotype, time), or a two-way ANOVA, between factors (HIV-1 genotype and stress).

	Sex	**HIV-1 genotype**	**Stress**	**Sex × HIV-1 genotype**	**Sex × stress**	**Stress × HIV-1 genotype**	**Sex × stress × HIV-1 genotype**
Weight over stress (1A)	*$F_{(1,70)} = 79.2$, $p < 0.01$	*$F_{(1,70)} = 5.80$, $p = 0.02$	*$F_{(1,70)} = 70.91$, $p < 0.01$	$F_{(1,70)} = 2.11$, $p > 0.05$	$F_{(1,70)} = 1.54$, $p > 0.05$	$F_{(1,70)} = 0.16$, $p > 0.05$	$F_{(1,70)} = 0.09$, $p > 0.05$
Adrenal weight (1B)	*$F_{(1,65)} = 44.60$, $p < 0.01$	*$F_{(1,65)} = 6.53$, $p = 0.01$	$F_{(1,65)} = 3.66$, $p > 0.05$	$F_{(1,65)} = 0.02$, $p > 0.05$	$F_{(1,65)} = 0.04$, $p > 0.05$	$F_{(1,65)} = 1.66$, $p > 0.05$	$F_{(1,65)} = 0.03$, $p > 0.05$
Male reproductive weight (1C)		*$F_{(1,31)} = 16.09$, $p < 0.01$	*$F_{(1,31)} = 6.87$, $p = 0.01$			$F_{(1,31)} = 0.25$, $p > 0.05$	
Female reproductive weight (1D)		$F_{(1,34)} = 0.12$, $p > 0.05$	$F_{(1,34)} = 2.05$, $p > 0.05$			$F_{(1,34)} = 0.35$, $p > 0.05$	
Terminal weight (**Table 2**)	*$F_{(1,65)} = 199.8$, $p < 0.01$	*$F_{(1,65)} = 150.9$, $p < 0.01$	$F_{(1,65)} = 1.72$, $p > 0.05$	$F_{(1,65)} = 2.41$, $p > 0.05$	$F_{(1,65)} = 0.27$, $p > 0.05$	$F_{(1,65)} = 1.22$, $p > 0.05$	$F_{(1,65)} = 0.93$, $p > 0.05$

**Indicates a significant main effect or interaction. Blank boxes indicate an absence of a particular factor from analysis. The figure or table number is included in parentheses following the description of each metric. $\alpha = 0.05$.*

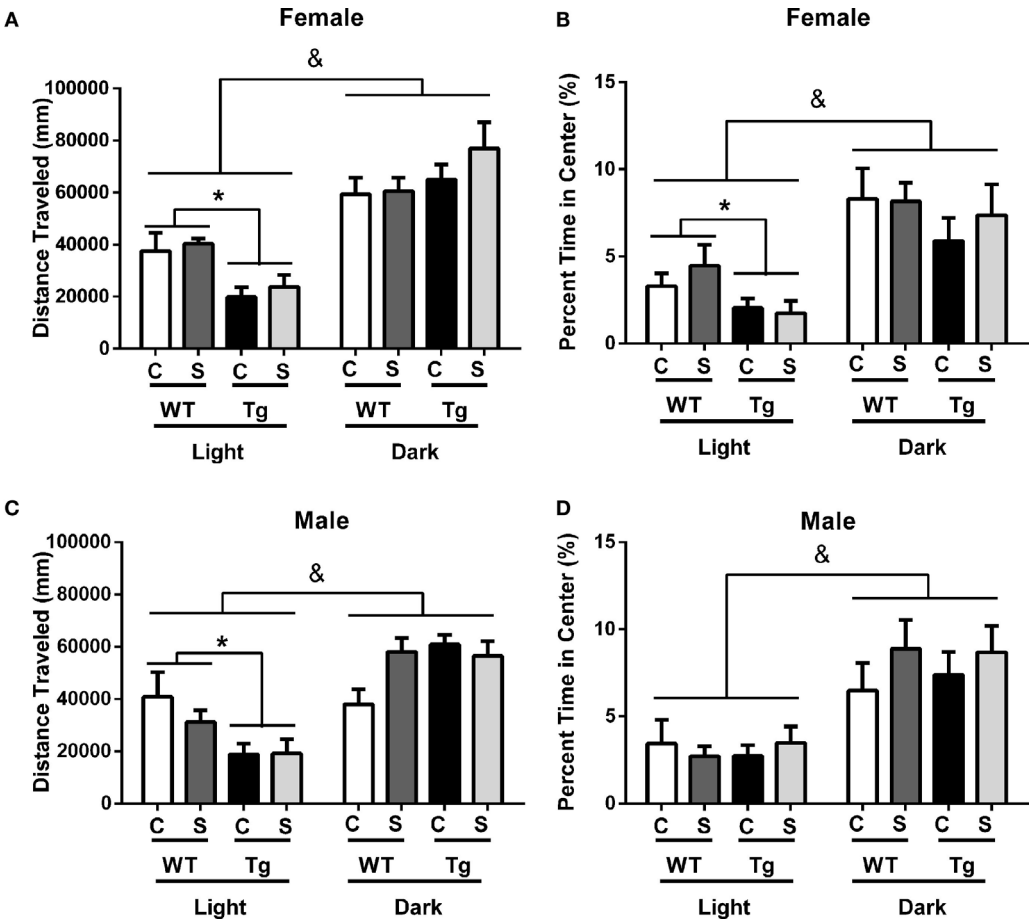

FIGURE 2 | HIV-1 Tg and WT rats were placed in an open-field maze during the rats' light and dark cycles. The distance traveled and the percentage of time the rats spent in the center of the maze were measured. HIV-1 Tg rats traveled a decreased distance compared to WT controls in the light cycle **(A)**. Male **(C)** and female **(A)** rats traveled an increased distance in the dark cycle compared to the light cycle. The percentage of time rats spent in the center was unaffected by stress or transgene in males **(D)**. Female Tg rats spent a decreased percentage of time in the center compared to WT female rats during the light cycle **(B)**. Male **(D)** and female **(B)** rats spent an increased percentage of the time in the center during the dark cycle. Data are presented as mean ± SEM. & denotes the main effect of time. *Denotes a main effect in a *t*-test collapsed across stress. $\alpha = 0.05$.

TABLE 4 | *F*-statistics and *p*-values for all main effects and interactions are shown for behavior analysis using a three-way ANOVA between factors (HIV-1 genotype, stress) and within factors (time).

	HIV-1 genotype	Stress	Time	Stress × time	HIV-1 genotype × time	Stress × HIV-1 genotype	Stress × HIV-1 genotype × time
Female distance traveled (2A)	$F_{(1,36)} = 0.78$, $p > 0.05$	$F_{(1,36)} = 1.78$, $p > 0.05$	$^{*}F_{(1,36)} = 52.5$, $p < 0.01$	$F_{(1,36)} = 0.10$, $p > 0.05$	$^{*}F_{(1,36)} = 8.68$, $p < 0.01$	$F_{(1,36)} = 0.67$, $p > 0.05$	$F_{(1,36)} = 0.26$, $p > 0.05$
Female percent time in center (2B)	$F_{(1,36)} = 4.06$, $p > 0.05$	$F_{(1,36)} = 0.38$, $p > 0.05$	$^{*}F_{(1,36)} = 29.3$, $p < 0.01$	$F_{(1,36)} < 0.01$, $p > 0.05$	$F_{(1,36)} = 0.03$, $p > 0.05$	$F_{(1,36)} < 0.01$, $p > 0.05$	$F_{(1,36)} = 0.86$, $p > 0.05$
Male distance traveled (2C)	$F_{(1,31)} = 0.66$, $p > 0.05$	$F_{(1,31)} = 0.08$, $p > 0.05$	$^{*}F_{(1,31)} = 56.9$, $p < 0.01$	$F_{(1,31)} = 1.78$, $p > 0.05$	$^{*}F_{(1,31)} = 13.2$, $p < 0.01$	$F_{(1,31)} = 0.87$, $p > 0.05$	$^{*}F_{(1,31)} = 5.11$, $p = 0.03$
Male percent time in center (2D)	$F_{(1,31)} = 0.06$, $p > 0.05$	$F_{(1,31)} = 1.53$, $p > 0.05$	$^{*}F_{(1,31)} = 24.5$, $p < 0.01$	$F_{(1,31)} = 0.72$, $p > 0.05$	$F_{(1,31)} = 0.03$, $p > 0.05$	$F_{(1,31)} = 0.01$, $p > 0.05$	$F_{(1,31)} = 0.42$, $p > 0.05$
Female total time sniffing objects (3A)	$^{*}F_{(1,26)} = 6.78$, $p = 0.02$	$F_{(1,26)} = 0.02$, $p > 0.05$	$F_{(1,26)} = 1.53$, $p > 0.05$	$F_{(1,26)} = 0.06$, $p > 0.05$	$F_{(1,26)} = 0.61$, $p > 0.05$	$F_{(1,26)} = 2.07$, $p > 0.05$	$F_{(1,26)} = 0.13$, $p > 0.05$
Female difference in sniffing time (3B)	$^{*}F_{(1,26)} = 5.39$, $p = 0.03$	$F_{(1,26)} = 0.90$, $p > 0.05$	$F_{(1,26)} = 1.25$, $p > 0.05$	$F_{(1,26)} = 0.82$, $p > 0.05$	$F_{(1,26)} = 0.45$, $p > 0.05$	$F_{(1,26)} = 3.28$, $p > 0.05$	$F_{(1,26)} = 0.45$, $p > 0.05$
Male total time sniffing objects (3C)	$^{*}F_{(1,32)} = 7.50$, $p < 0.01$	$F_{(1,32)} = 0.31$, $p > 0.05$	$F_{(1,32)} < 0.01$, $p > 0.05$	$F_{(1,32)} = 0.05$, $p > 0.05$	$F_{(1,32)} = 0.45$, $p > 0.05$	$F_{(1,32)} = 0.02$, $p > 0.05$	$F_{(1,32)} = 1.04$, $p > 0.05$
Male difference in sniffing time (3D)	$F_{(1,32)} = 0.53$, $p > 0.05$	$F_{(1,32)} = 0.64$, $p > 0.05$	$F_{(1,32)} = 0.03$, $p > 0.05$	$F_{(1,32)} = 1.53$, $p > 0.05$	$F_{(1,32)} < 0.01$, $p > 0.05$	$F_{(1,32)} = 0.55$, $p > 0.05$	$^{*}F_{(1,32)} = 5.17$, $p = 0.03$

**Indicates a significant main effect or interaction. The figure or table number is included in parentheses following the description of each metric. $\alpha = 0.05$.*

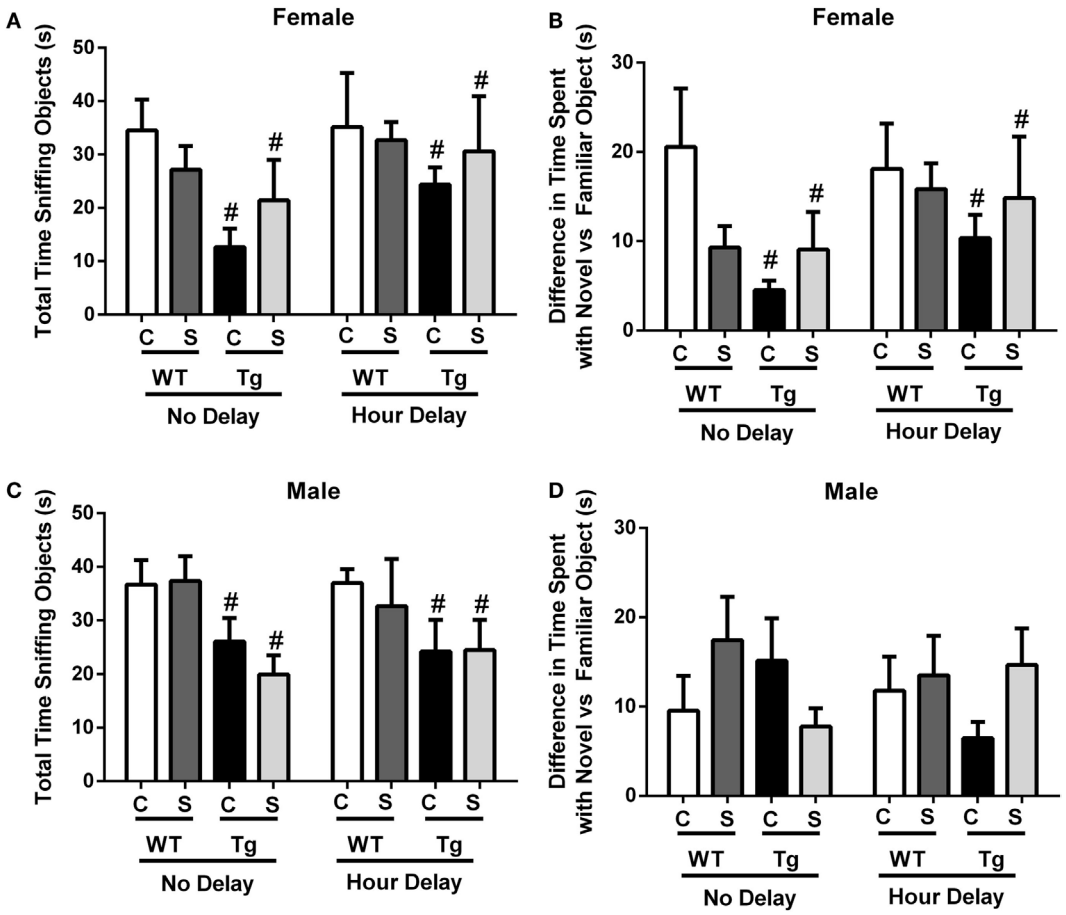

FIGURE 3 | Total time sniffing novel and familiar objects were measured in the novel object recognition task. This task was performed with no delay or 1-h delay following exposure to familiar objects. Female HIV-1 Tg rats spent less time sniffing all objects **(A)**. Female Tg rats spent a reduced difference in time sniffing the novel vs. familiar object compared to female WT control rats **(B)**. Male HIV-1 Tg rats spent less time sniffing all objects **(C)**, and genotype, stress, and time interacted to impact the difference in time spent sniffing the novel vs. familiar object in males **(D)**. Data are presented as mean ± SEM. # denotes a main effect of HIV-1 Tg genotype. $\alpha = 0.05$.

TABLE 5 | The estimated population of IBA-1-stained microglia was determined using the optical fractionator probe (Stereo Investigator).

		Mean (estimated number of cells)	SEM
Wild type	No stress	4.28×10^{10}	5.29×10^{9}
	Stress	3.95×10^{10}	1.91×10^{9}
HIV-1 transgenic	No stress	4.34×10^{10}	5.72×10^{8}
	Stress	3.53×10^{10}	2.8×10^{9}

A two-way ANOVA was used to determine the statistical significance. There were no significant main effects of stress or HIV-1 genotype nor interactions to impact the estimated population of microglia. Mean and SEM are shown. $\alpha = 0.05$.

WT rats (**Figure 4**). An *a priori t*-test showed that stress increased the number of branches ($t_4 = 4.64$, $p = 0.01$) and number of junctions ($t_4 = 4.46$, $p = 0.01$) in WT-stressed rats compared to WT controls. Representative images and skeletonized microglia are shown in **Figure 5**.

Estimated blood vessel length in WT and HIV-1 Tg rats was not significantly different in the PFC ($t_6 = 0.2436$, $p = 0.8157$), hippocampus ($t_9 = 1.667$, $p = 0.1298$), or amygdala ($t_9 = 0.2770$, $p = 0.7880$). Blood vessel density was also unchanged in the PFC ($t_6 = 1.064$, $p > 0.05$), hippocampus ($t_9 = 1.007$, $p > 0.05$), and amygdala ($t_9 = 1.044$, $p > 0.05$) (**Table 6**).

Expression of Inflammatory Markers is Increased in Female HIV-1 Tg Rats

Gene expression of complement factor B (*Cfb*) in the hippocampus was higher in female HIV-1 Tg rats compared to WT rats ($t_{19} = 2.87$, $p = 0.01$) (**Figure 6B**). Gene expression of lipocalin-2 (*Lcn2*) was unchanged in the hippocampus ($t_{15} = 1.18$, $p > 0.05$) but decreased in the PFC ($t_{15} = 2.40$, $p = 0.03$) in female HIV-1 Tg rats compared to WT controls (**Figure 6A**). Statistical analysis information is detailed in **Table 7**.

DISCUSSION

Collectively, the data presented here demonstrate that development in the presence of HIV-1 proteins leads to anxiety-like

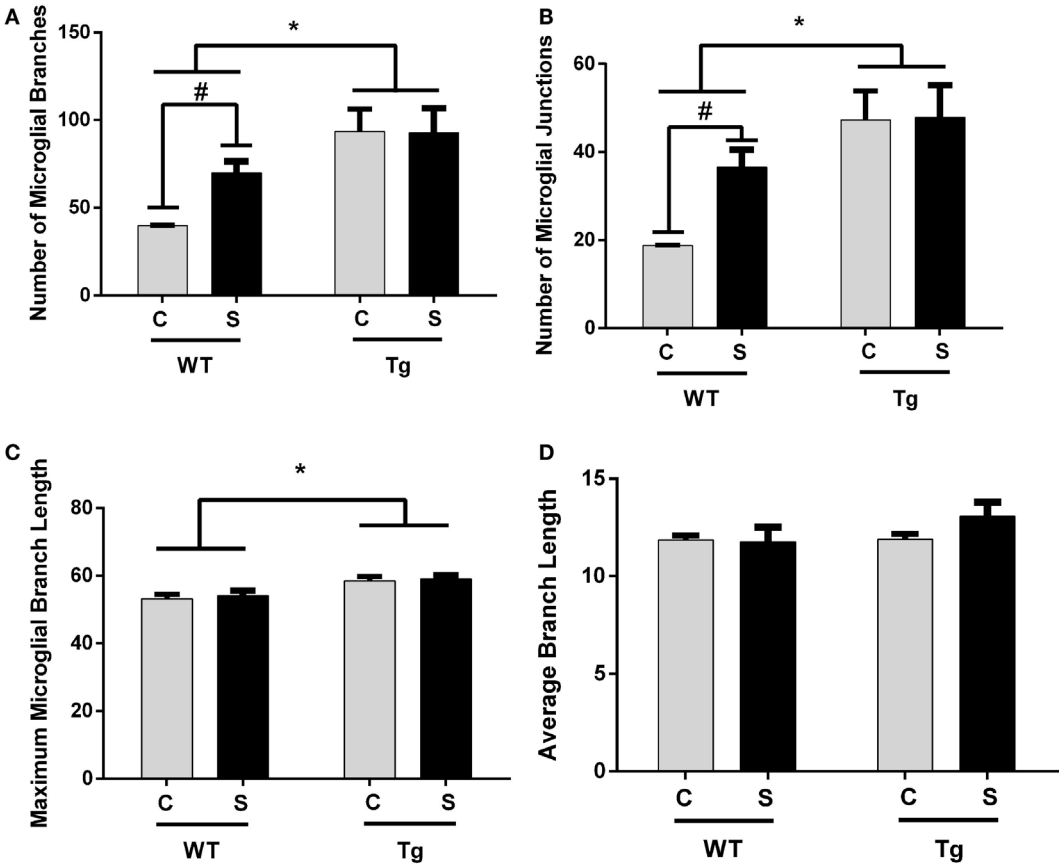

FIGURE 4 | Morphology of IBA-1-stained microglia was assessed using the AnalyzeSkeleton function in ImageJ. Microglia in female HIV-1 transgenic rats exhibited enhanced ramification characterized by an increased number of branches **(A)**, junctions **(B)**, and maximum branch length **(C)**. Stress did not impact microglial morphology, and the average branch length was unchanged in any group **(D)**. Data are presented as mean ± SEM. *Denotes a main effect of HIV-1 transgene. # denotes significant effect of stress in an *a priori t*-test. $\alpha = 0.05$.

behavior and ramification of microglia. The combination of chronic adolescent stress with HIV-1 protein expression did not further exacerbate the impact of HIV-1 proteins in females. While the presence of HIV-1 proteins can alter male body morphology and behavior, the impact on these metrics appears to be more salient in adolescent females.

Although HIV-1 Tg female rats exhibited normal locomotor behavior during the dark cycle, during the more anxiety-provoking light cycle, female HIV-1 Tg rats exhibited decreased locomotor activity in the open field and reduced central tendency as compared to WT females. This context-specific change in locomotor activity suggests that the alterations in distance traveled are not due to a physical impairment. This is consistent with a previous study in adolescent female HIV-1 Tg rats in which a trend toward decreased time in the center of an open-field maze was observed (15). All rats, regardless of genotype, sex, or stress, showed reduced central tendency in the light cycle compared to the dark cycle, suggesting that all rats responded to the anxiety-provoking stimulus of bright light.

Unlike previous reports from our group that demonstrated an increase in anxiety-like behavior in female rats following chronic adolescent stress (21, 22, 28), the current study did not find a main effect of stress on central tendency in the open field. This may be a reflection of strain-specific sensitivities to chronic adolescent stress, as the previous reports from our group were conducted in Wistar or Sprague–Dawley rats (21, 22, 28), and the current study was conducted in Fisher rats as these are the background strain of the HIV-1 Tg rat. While the timing of the adolescent stress paradigm was consistent with previous studies from our group that documented behavioral effects of chronic adolescent stress (21), it is possible that, had the adolescent stress paradigm continued throughout the behavioral testing days, the behavioral effects could have been more robust. And while the adolescent stress paradigm takes place during the average age of pubertal onset, timing that may be particularly disruptive to female behavior, no behavioral effects of adolescent stress were observed in females or males. However, both chronic adolescent stress and the presence of HIV-1 viral proteins reduced adolescent weight gain (PND36–46). The fact that chronic stress was able to alter physiological endpoints suggests that although Fisher rats may not be as behaviorally sensitive to chronic adolescent stress as Wistar and Sprague–Dawley rats,

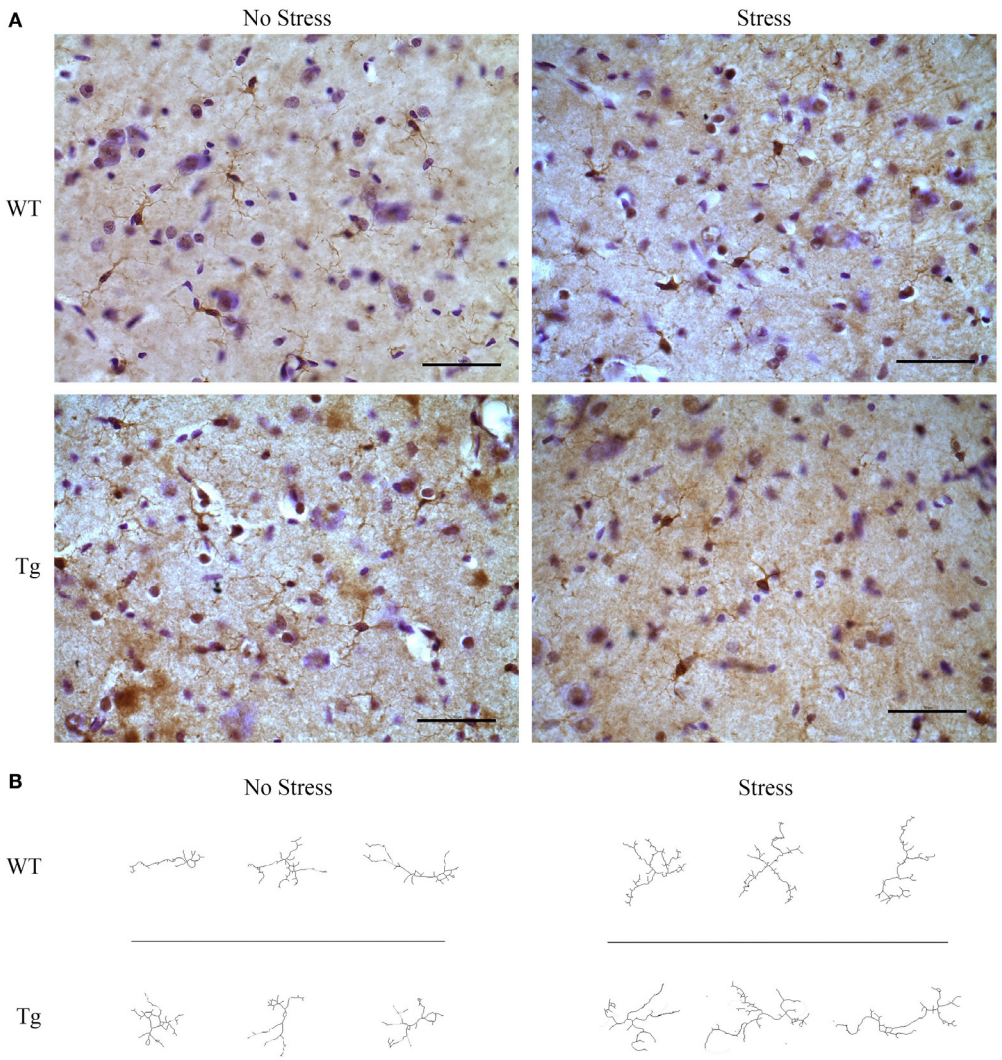

FIGURE 5 | HIV-1 transgenic and wild-type rats were exposed to chronic adolescent stress or left non-stressed during adolescence. Brains were sectioned at 40 μm and stained for IBA-1 and visualized with diaminobenzidine. Representative images of the hippocampus for HIV-1 Tg, WT, stressed, and non-stressed rats are shown **(A)**. Images were adjusted for brightness and contrast. Scale bar = 50 μm. IBA-1-stained microglia were then converted to 8-bit, adjusted for brightness, and cleaned with a Gaussian filter. Images were converted to binary and skeletonized in ImageJ. Representative images of non-stressed WT, stressed WT, non-stressed Tg, and stressed Tg rats are shown **(B)**.

TABLE 6 | Blood vessel length and density were estimated using the Spaceballs probe (Stereo Investigator).

Region	Measure	Genotype	*n*	Mean ± SEM	*T* statistic	*p*-value
Hippocampus	Length (μm)	WT	5	$5.653 \times 10^6 \pm 439454$	$t_9 = 1.667$	0.1298
		Tg	6	$4.743 \times 10^6 \pm 339171$		
	Density (μm/μm³)	WT	5	2.025 ± 0.2465	$t_9 = 1.007$	0.3401
		Tg	6	1.774 ± 0.1019		
PFC	Length (μm)	WT	3	$4.380 \times 10^6 \pm 920262$	$t_6 = 0.2436$	0.8157
		Tg	5	$4.633 \times 10^6 \pm 595337$		
	Density (μm/μm³)	WT	3	2.551 ± 0.08156	$t_6 = 1.064$	0.3281
		Tg	5	3.164 ± 0.4294		
Amygdala	Length (μm)	WT	5	$3.591 \times 10^6 \pm 189420$	$t_9 = 0.2770$	0.788
		Tg	6	$3.700 \times 10^6 \pm 320519$		
	Density (μm/μm³)	WT	5	1.280 ± 0.07705	$t_9 = 1.044$	0.3237
		Tg	6	1.402 ± 0.08481		

A student's t-test was used to compare vessel length and density between WT and HIV-1 Tg rats in the hippocampus, PFC, and amygdala. Mean, SEM, and test statistics are shown. HIV-1 Tg rats did not exhibit significantly different blood vessel length or density in any region. $\alpha = 0.05$.

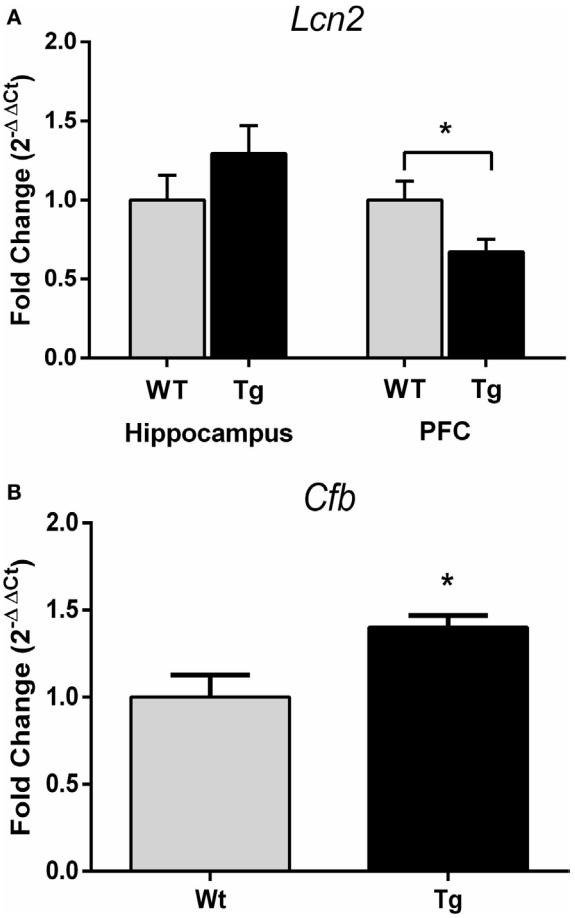

FIGURE 6 | Quantitative real-time PCR was performed to assess gene expression of complement factor B and lipocalin-2. *Lcn2* expression was decreased in the prefrontal cortex but not hippocampus of HIV-1 Tg rats **(A)**. *Cfb* gene expression was increased in the hippocampus of HIV-1 Tg rats **(B)**. Data are presented as mean ± SEM. *Denotes a main effect of HIV-1 transgene. $\alpha = 0.05$.

TABLE 7 | *F*-statistics and *p*-values for all main effects and interactions are shown for microglia and gene expression analysis.

	HIV-1 genotype	Stress	Stress × HIV-1 genotype
Microglial branches (4A)	$*F_{(1,8)} = 14.62$, $p < 0.01$	$F_{(1,8)} = 2.17$, $p > 0.05$	$F_{(1,8)} = 2.38$, $p > 0.05$
Microglial junctions (4B)	$*F_{(1,8)} = 13.94$, $p < 0.01$	$F_{(1,8)} = 2.98$, $p > 0.05$	$F_{(1,8)} = 2.59$, $p > 0.05$
Microglial max branch length (4C)	$*F_{(1,8)} = 14.31$, $p < 0.01$	$F_{(1,8)} = 0.29$, $p > 0.05$	$F_{(1,8)} = 0.02$, $p > 0.05$
Average branch length (4D)	$F_{(1,8)} = 1.45$, $p > 0.05$	$F_{(1,8)} = 0.97$, $p > 0.05$	$F_{(1,8)} = 1.32$, $p > 0.05$
Lcn2 hippocampus (4A)	$t_{15} = 1.18$, $p > 0.05$		
Lcn2 PFC (4A)	$*t_{15} = 2.40$, $p = 0.03$		
Cfb (4B)	$*t_{19} = 2.87$, $p = 0.01$		
IBA-1-estimated population (**Table 5**)	$F_{(1,8)} = 0.32$, $p > 0.05$	$F_{(1,8)} = 3.26$, $p > 0.05$	$F_{(1,8)} = 0.54$, $p > 0.05$

*A two-way ANOVA was used for microglial morphology analysis (between-factors HIV-1 genotype and stress). A student's t-test was used to assess significance of gene expression data.*Indicates a significant main effect or interaction. Blank boxes indicate an absence of a particular factor from analysis. The figure or table number is included in parentheses following the description of each metric. $\alpha = 0.05$.*

chronic adolescent stress is a salient environmental stimuli in the Fisher strain.

The novel object recognition task was performed to assess object recognition memory. The test was performed with either no delay or 1-h delay following exposure to the familiar objects. The no-delay condition serves as a control for confounds of differences in investigative behavior that could be driven by an underlying difference in anxiety-like behavior. A non-zero difference in novel vs. familiar object investigation may be interpreted as lack of memory impairment; that is, the animal differentiates between the two objects and displays a preference for one. Both female and male HIV-1 Tg rats spent a decreased amount of time sniffing all objects in the no-delay paradigm as compared to WT controls. Female HIV-1 Tg rats displayed a reduction in investigation of novel and familiar objects compared to WT females. Though this reduction is present in both the no delay and 1-h delay conditions, rats persisted in displaying a difference in time spent exploring the novel vs. familiar objects, suggesting object recognition despite reduced overall exploration. Although a cognitive deficit cannot be completely ruled out, the combination of reduced investigative behavior and no difference between no-delay and hour-delay conditions suggests that these animals do not suffer from overt memory impairments, but rather a decrease in investigation. The current testing is not designed to determine if this decreased investigation is a reflection of decreased motivation or increased anxiety-like behavior; however, an interpretation of increased anxiety-like behavior is consistent with the anxiety-like behavior observed in the open-field test. Previous studies have reported mixed results regarding memory impairment in HIV-1 Tg rats. In the Morris Water Maze, HIV-1 Tg rats exhibited decreased learning but no change in memory of the hidden platform (29). Conversely, a study by Repunte-Canonigo et al. observed impaired working memory in the T-maze (16). Here, we had hypothesized that stress and HIV-1 proteins would interact to impair memory, but these data suggest that neither condition alone nor the combined impact of these factors is sufficient to cause memory impairment in adolescent rats.

In order to collect tissue at the same age and without the stress of estrous cycle tracking, samples from female rats could not be collected at every stage of the estrous cycle. Both the behavioral metrics (PND49–53) and the weight data were collected across multiple days and therefore could not be tied to any one stage of the estrous cycle. As we have reported previously (21, 22, 28), we collected uterine weights at the completion of the study to determine whether systematic group differences in estrous cycle might be present. **Figure 1D** demonstrates that uterine weights were not systematically different among groups, and therefore, there likely was not a group difference in estrous cycle stage. These data confirm that reported differences in behavior and inflammatory endpoints are likely not caused by different estrous cycle stages

among groups; however, variations in estrous cycle stage may contribute to variability within groups, though a previous report suggests that differences in estrous cycle do not increase group variability over what is observed in males (30).

Our current findings of anxiety-like behavioral alterations in females are consistent with our previous demonstration of altered affective-like behavior and stress responses in female HIV-1 Tg rats (15, 31). In order to elucidate the mechanisms potentially underlying these behavioral changes, we evaluated microglial and vascular morphology in female HIV-1 Tg rats. The hippocampus has been previously investigated in HIV-1 Tg rats due to its established susceptibility to neuroinflammation and damage by HIV-1 viral protein exposure (32, 33), and we have previously reported HIV-1 induced deficits in hippocampal neurogenesis (15). In the present study, we first examined whether microglial activation, indicative of neuroinflammation, was altered with stress or in female HIV-1 Tg rats. The estimated population of IBA-1 positive microglia was unchanged with stress or exposure to HIV-1 transgene. While we did not observe differences in the number of IBA-1 positive microglia, a previous study found increased gene expression of *Iba-1* in adult HIV-1 Tg rats, though they did not specify the sex of the animals tested (16). Thus, there may be potential sex- or age-dependent variations in microglial profiles in the HIV-1 Tg rats.

For more in-depth assessment of microglia, we examined microglial morphology and branching. Microglia in HIV-1 Tg female rats exhibited a hyper-ramified structure, characterized by increased number of junctions and branches. Hyper-ramification has been hypothesized to be an intermediate stage in microglial activation (34), and hyper-ramified microglia have been associated with impaired working memory following stress (35); however, detailed behavioral consequences of enhanced microglial ramification are not well established (35). The enhanced microglial ramification observed in the hippocampus of the adolescent female HIV-1 Tg rats is likely a consequence of the presence of HIV-1 viral proteins because this morphology is present even in the absence of chronic stress exposure. However, it is premature to conclude that these changes in microglial morphology are behaviorally salient in this particular case. We observed similar ramification following chronic adolescent stress in female adolescent WT rats that did not co-occur with behavioral alterations. Collectively, these data suggest that both development with HIV-1 proteins and chronic adolescent stress are independently capable of inducing ramified microglia in the female adolescent hippocampus, but these two stimuli do not appear to interact at the level of microglial ramification.

In addition to microglial morphology, we assessed vascularization of the hippocampus based on previous demonstrations of altered vascularization following stress (36). There was no effect of HIV-1 on vascularization of the hippocampus (**Table 1**). Because of previous reports of alterations in the PFC of HIV-1 Tg rats (31) and the potential for stress to impact vascularization in other brain regions (36), we also assessed vascular endpoints in the PFC and amygdala. Similar to the hippocampus, vascularization of neither the PFC nor the amygdala was impacted by development in the presence of HIV-1 proteins. When these vascularization data are viewed in light of the alterations in microglial morphology reported above as well as previously published demonstration of reduced neurogenesis (15), they suggest that glial and neuronal alterations are not secondary to a primary change in vascular structure.

In order to determine if the inflammatory profile suggested by microglial ramification extended to other metrics, we assessed the expression of two inflammatory markers linked to HIV-1. We have previously reported that *Mcp-1* expression is elevated in the hippocampus of HIV-1 Tg female rats, but assessments of other portions of the innate immune system have not been reported (15). We assessed expression of complement factor B, a component of the alternative complement pathway in the innate immune system. Activity in the complement cascade in individuals with HIV-1 has previously been studied in context of HIV-associated cognitive impairments; previous studies found elevated protein expression of complement component 3 (C3) in patients diagnosed with HIV-associated dementia (37). Complement activation has also been investigated as a mechanism of neurodegeneration in patients with HIV-1 (38) as well as in other central nervous system disorders (39). As demonstrated in **Figure 6**, expression of *Cfb* was increased in the presence of HIV-1 proteins in adolescent female rats. An increase in activation of both the alternate and classical complement pathway in HIV patients has been reported (40), and it has been hypothesized that factor B is protective against HIV-1 infection (41). Interestingly, here, we saw increased complement factor B in the absence of a true infection as only the viral proteins are expressed, suggesting that the proteins themselves are an inflammatory stimulus.

The complement system has effects on neuronal architecture beyond the commonly appreciated inflammatory effects of this system (42), and these non-traditional impacts of innate immune system activation could contribute to impairments observed in PLWH. With this in mind, we also examined gene expression of lipocalin-2 (Lcn2), an acute phase protein that plays an important role in dendritic spine remodeling and anxiety-like behavior (43, 44), as dendritic damage has been cited as a contributor to development of HAND (45, 46). Although *Lcn2* expression was unaltered in the hippocampus, it was reduced in the PFC of HIV-1 Tg female rats. Region-specific alterations in gene expression have been reported previously in the HIV-1 Tg rat (31, 47). Further investigating inflammatory markers for their potential to effect stress- and HIV-1-induced changes in structural plasticity may help elucidate the mechanisms underlying the behavioral changes reported here.

Collectively, the current findings suggest that HIV-1 proteins are a potent physiologic and behavioral modifier. These findings reinforce the hypothesis that the biological impacts of HIV-1 viral protein exposure, rather than the psychosocial impacts of diagnosis and disease management, may be responsible for HIV-1-associated behavioral alterations. The use of a stress-based animal model provided the tools necessary to isolate the impact of psychosocial variables from biological influences of HIV-1 to gain insight into the mechanisms potentially underlying psychiatric and cognitive dysfunction in PLWH. Future studies should further elucidate specific mechanisms involved in HIV-1-related behavioral changes. The data presented here, in combination with previous reports (15), suggest that HIV-1 proteins disrupt

behavior and lead to neuroinflammatory effects. However, the behavioral and neuroinflammatory effects are not directly linked, at least not by traditional mechanisms. Although HIV-1 is an immune disorder and has profound inflammatory implications, other systems may be more directly influential in behavioral alterations, such as the hypothalamic–pituitary–adrenal axis (31), neuronal morphology (42), or dopaminergic systems (13, 14).

AUTHOR CONTRIBUTIONS

GN generated the hypotheses and secured funding for the studies performed. CH designed the behavioral experiments in collaboration with GN and then performed the work. MB and SR designed the qPCR experiments in collaboration with GN and then performed the work. AG designed the microglial analysis plan under the supervision of GN and then performed the work. SK, MW, and RR designed the vascular analysis plan in consultation with GN and then performed the immunohistochemistry and vascular analysis. SK oversaw Tg rat care and performed adolescent stress. GN oversaw all analyses of the collected data. GN and SR wrote and edited the manuscript. All authors revised the manuscript.

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REFERENCES

1. Smith CJ, Ryom L, Weber R, Morlat P, Pradier C, Reiss P, et al. Trends in underlying causes of death in people with HIV from 1999 to 2011 (D:A:D): a multicohort collaboration. *Lancet* (2014) 384(9939):241–8. doi:10.1016/S0140-6736(14)60604-8
2. Heaton RK, Clifford DB, Franklin DR Jr, Woods SP, Ake C, Vaida F, et al. HIV-associated neurocognitive disorders persist in the era of potent antiretroviral therapy: CHARTER Study. *Neurology* (2010) 75(23):2087–96. doi:10.1212/WNL.0b013e318200d727
3. Lopes M, Olfson M, Rabkin J, Hasin DS, Alegria AA, Lin KH, et al. Gender, HIV status, and psychiatric disorders: results from the National Epidemiologic Survey on alcohol and related conditions. *J Clin Psychiatry* (2012) 73(3):384–91. doi:10.4088/JCP.10m06304
4. Hinkin CH, Castellon SA, Durvasula RS, Hardy DJ, Lam MN, Mason KI, et al. Medication adherence among HIV+ adults: effects of cognitive dysfunction and regimen complexity. *Neurology* (2002) 59(12):1944–50. doi:10.1212/01.WNL.0000038347.48137.67
5. Ellis RJ, Deutsch R, Heaton RK, Marcotte TD, McCutchan JA, Nelson JA, et al. Neurocognitive impairment is an independent risk factor for death in HIV infection. San Diego HIV Neurobehavioral Research Center Group. *Arch Neurol* (1997) 54(4):416–24. doi:10.1001/archneur.1997.00550160054016
6. Mellins CA, Brackis-Cott E, Leu CS, Elkington KS, Dolezal C, Wiznia A, et al. Rates and types of psychiatric disorders in perinatally human immunodeficiency virus-infected youth and seroreverters. *J Child Psychol Psychiatry* (2009) 50(9):1131–8. doi:10.1111/j.1469-7610.2009.02069.x
7. Wood SM, Shah SS, Steenhoff AP, Rutstein RM. The impact of AIDS diagnoses on long-term neurocognitive and psychiatric outcomes of surviving adolescents with perinatally acquired HIV. *AIDS* (2009) 23(14):1859–65. doi:10.1097/QAD.0b013e32832d924f
8. Paramesparan Y, Garvey LJ, Ashby J, Foster CJ, Fidler S, Winston A. High rates of asymptomatic neurocognitive impairment in vertically acquired HIV-1-infected adolescents surviving to adulthood. *J Acquir Immune Defic Syndr* (2010) 55(1):134–6. doi:10.1097/QAI.0b013e3181d90e8c
9. Wilson PA, Kahana SY, Fernandez MI, Harper GW, Mayer K, Wilson CM, et al. Sexual risk behavior among virologically detectable human immunodeficiency virus-infected young men who have sex with men. *JAMA Pediatr* (2016) 167(2):125–131. doi:10.1001/jamapediatrics.2015.3333
10. Kessler RC. Epidemiology of women and depression. *J Affect Disord* (2003) 74(1):5–13. doi:10.1016/S0165-0327(02)00426-3
11. Vesga-Lopez O, Schneier FR, Wang S, Heimberg RG, Liu SM, Hasin DS, et al. Gender differences in generalized anxiety disorder: results from the National Epidemiologic Survey on Alcohol and Related Conditions (NESARC). *J Clin Psychiatry* (2008) 69(10):1606–16. doi:10.4088/JCP.v69n1011
12. June HL, Tzeng Yang AR, Bryant JL, Jones O, Royal W III. Vitamin A deficiency and behavioral and motor deficits in the human immunodeficiency virus type 1 transgenic rat. *J Neurovirol* (2009) 15(5–6):380–9. doi:10.3109/13550280903350200
13. Moran LM, Aksenov MY, Booze RM, Webb KM, Mactutus CF. Adolescent HIV-1 transgenic rats: evidence for dopaminergic alterations in behavior and neurochemistry revealed by methamphetamine challenge. *Curr HIV Res* (2012) 10(5):415–24. doi:10.2174/157016212802138788
14. Moran LM, Booze RM, Webb KM, Mactutus CF. Neurobehavioral alterations in HIV-1 transgenic rats: evidence for dopaminergic dysfunction. *Exp Neurol* (2013) 239:139–47. doi:10.1016/j.expneurol.2012.10.008
15. Nemeth CL, Glasper ER, Harrell CS, Malviya SA, Otis JS, Neigh GN. Meloxicam blocks neuroinflammation, but not depressive-like behaviors, in HIV-1 transgenic female rats. *PLoS One* (2014) 9(10):e108399. doi:10.1371/journal.pone.0108399
16. Repunte-Canonigo V, Lefebvre C, George O, Kawamura T, Morales M, Koob GF, et al. Gene expression changes consistent with neuroAIDS and impaired working memory in HIV-1 transgenic rats. *Mol Neurodegener* (2014) 9:26. doi:10.1186/1750-1326-9-26
17. Reid W, Sadowska M, Denaro F, Rao S, Foulke J Jr, Hayes N, et al. An HIV-1 transgenic rat that develops HIV-related pathology and immunologic dysfunction. *Proc Natl Acad Sci U S A* (2001) 98(16):9271–6. doi:10.1073/pnas.161290298
18. Peng J, Vigorito M, Liu X, Zhou D, Wu X, Chang SL. The HIV-1 transgenic rat as a model for HIV-1 infected individuals on HAART. *J Neuroimmunol* (2010) 218(1–2):94–101. doi:10.1016/j.jneuroim.2009.09.014
19. Ray PE, Liu XH, Robinson LR, Reid W, Xu L, Owens JW, et al. A novel HIV-1 transgenic rat model of childhood HIV-1-associated nephropathy. *Kidney Int* (2003) 63(6):2242–53. doi:10.1046/j.1523-1755.2003.00028.x
20. Otis JS, Ashikhmin YI, Brown LA, Guidot DM. Effect of HIV-1-related protein expression on cardiac and skeletal muscles from transgenic rats. *AIDS Res Ther* (2008) 5:8. doi:10.1186/1742-6405-5-8
21. Bourke CH, Neigh GN. Behavioral effects of chronic adolescent stress are sustained and sexually dimorphic. *Horm Behav* (2011) 60(1):112–20. doi:10.1016/j.yhbeh.2011.03.011
22. Bourke CH, Raees MQ, Malviya S, Bradburn CA, Binder EB, Neigh GN. Glucocorticoid sensitizers Bag1 and Ppid are regulated by adolescent stress in a sex-dependent manner. *Psychoneuroendocrinology* (2013) 38(1):84–93. doi:10.1016/j.psyneuen.2012.05.001
23. Pyter LM, Kelly SD, Harrell CS, Neigh GN. Sex differences in the effects of adolescent stress on adult brain inflammatory markers in rats. *Brain Behav Immun* (2013) 30:88–94. doi:10.1016/j.bbi.2013.01.075
24. Kelly SD, Harrell CS, Neigh GN. Chronic stress modulates regional cerebral glucose transporter expression in an age-specific and sexually-dimorphic manner. *Physiol Behav* (2014) 126:39–49. doi:10.1016/j.physbeh.2013.12.002

25. Whishaw IQ, Kolb B. *The Behavior of the Laboratory Rat: A Handbook with Tests*. New York: Oxford University Press (2005).
26. Broadbent NJ, Squire LR, Clark RE. Spatial memory, recognition memory, and the hippocampus. *Proc Natl Acad Sci U S A* (2004) 101(40):14515–20. doi:10.1073/pnas.0406344101
27. Paxinos G, Watson C. *The Rat Brain in Stereotaxic Coordinates*. San Diego: Academic Press (1998).
28. Harrell CS, Hardy E, Boss-Williams K, Weiss JM, Neigh GN. Sex and lineage interact to predict behavioral effects of chronic adolescent stress in rats. *Behav Brain Res* (2013) 248:57–61. doi:10.1016/j.bbr.2013.04.003
29. Vigorito M, LaShomb AL, Chang SL. Spatial learning and memory in HIV-1 transgenic rats. *J Neuroimmune Pharmacol* (2007) 2(4):319–28. doi:10.1007/s11481-007-9078-y
30. Prendergast BJ, Onishi KG, Zucker I. Female mice liberated for inclusion in neuroscience and biomedical research. *Neurosci Biobehav Rev* (2014) 40:1–5. doi:10.1016/j.neubiorev.2014.01.001
31. Panagiotakopoulos L, Kelly S, Neigh GN. HIV-1 proteins accelerate HPA axis habituation in female rats. *Physiol Behav* (2015) 150:8–15. doi:10.1016/j.physbeh.2015.02.011
32. Krucker T, Toggas SM, Mucke L, Siggins GR. Transgenic mice with cerebral expression of human immunodeficiency virus type-1 coat protein gp120 show divergent changes in short- and long-term potentiation in CA1 hippocampus. *Neuroscience* (1998) 83(3):691–700. doi:10.1016/S0306-4522(97)00413-2
33. Zink WE, Anderson E, Boyle J, Hock L, Rodriguez-Sierra J, Xiong H, et al. Impaired spatial cognition and synaptic potentiation in a murine model of human immunodeficiency virus type 1 encephalitis. *J Neurosci* (2002) 22(6):2096–105.
34. Streit WJ, Walter SA, Pennell NA. Reactive microgliosis. *Prog Neurobiol* (1999) 57(6):563–81. doi:10.1016/S0301-0082(98)00069-0
35. Hinwood M, Tynan RJ, Charnley JL, Beynon SB, Day TA, Walker FR. Chronic stress induced remodeling of the prefrontal cortex: structural re-organization of microglia and the inhibitory effect of minocycline. *Cereb Cortex* (2013) 23(8):1784–97. doi:10.1093/cercor/bhs151
36. Neigh GN, Owens MJ, Taylor WR, Nemeroff CB. Changes in the vascular area fraction of the hippocampus and amygdala are induced by prenatal dexamethasone and/or adult stress. *J Cereb Blood Flow Metab* (2010) 30(6):1100–4. doi:10.1038/jcbfm.2010.46
37. Rozek W, Ricardo-Dukelow M, Holloway S, Gendelman HE, Wojna V, Melendez LM, et al. Cerebrospinal fluid proteomic profiling of HIV-1-infected patients with cognitive impairment. *J Proteome Res* (2007) 6(11):4189–99. doi:10.1021/pr070220c
38. Speth C, Schabetsberger T, Mohsenipour I, Stockl G, Wurzner R, Stoiber H, et al. Mechanism of human immunodeficiency virus-induced complement expression in astrocytes and neurons. *J Virol* (2002) 76(7):3179–88. doi:10.1128/JVI.76.7.3179-3188.2002
39. Jongen PJ, Doesburg WH, Ibrahim-Stappers JL, Lemmens WA, Hommes OR, Lamers KJ. Cerebrospinal fluid C3 and C4 indexes in immunological disorders of the central nervous system. *Acta Neurol Scand* (2000) 101(2):116–21. doi:10.1034/j.1600-0404.2000.101002116.x
40. Fust G, Ujhelyi E, Hidvegi T, Paloczi K, Mihalik R, Hollan S, et al. The complement system in HIV disease. *Immunol Invest* (1991) 20(2):231–41. doi:10.3109/08820139109050792
41. Nozaki-Renard J, Kim T, Iino T, Tsukamoto T, Furutani-Seiki M, Tada T. Human complement component factor B rescues HIV-1-infected leukemic T cells from cytopathic death. *Int Immunol* (1991) 3(4):403–6. doi:10.1093/intimm/3.4.403
42. Lian H, Yang L, Cole A, Sun L, Chiang AC, Fowler SW, et al. NFkappaB-activated astroglial release of complement C3 compromises neuronal morphology and function associated with Alzheimer's disease. *Neuron* (2015) 85(1):101–15. doi:10.1016/j.neuron.2014.11.018
43. Mucha M, Skrzypiec AE, Schiavon E, Attwood BK, Kucerova E, Pawlak R. Lipocalin-2 controls neuronal excitability and anxiety by regulating dendritic spine formation and maturation. *Proc Natl Acad Sci U S A* (2011) 108(45):18436–41. doi:10.1073/pnas.1107936108
44. Skrzypiec AE, Shah RS, Schiavon E, Baker E, Skene N, Pawlak R, et al. Stress-induced lipocalin-2 controls dendritic spine formation and neuronal activity in the amygdala. *PLoS One* (2013) 8(4):e61046. doi:10.1371/journal.pone.0061046
45. Zheng J, Zhuang W, Yan N, Kou G, Peng H, McNally C, et al. Classification of HIV-1-mediated neuronal dendritic and synaptic damage using multiple criteria linear programming. *Neuroinformatics* (2004) 2(3):303–26. doi:10.1385/NI:2:3:303
46. Tremblay ME, Marker DF, Puccini JM, Muly EC, Lu SM, Gelbard HA. Ultrastructure of microglia-synapse interactions in the HIV-1 Tat-injected murine central nervous system. *Commun Integr Biol* (2013) 6(6):e27670. doi:10.4161/cib.27670
47. Li MD, Cao J, Wang S, Wang J, Sarkar S, Vigorito M, et al. Transcriptome sequencing of gene expression in the brain of the HIV-1 transgenic rat. *PLoS One* (2013) 8(3):e59582. doi:10.1371/journal.pone.0059582

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REVIEW
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Acute Stress Decreases but Chronic Stress Increases Myocardial Sensitivity to Ischemic Injury in Rodents

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Cardiovascular disease (CVD) is the largest cause of mortality worldwide, and stress is a significant contributor to the development of CVD. The relationship between acute and chronic stress and CVD is well evidenced. Acute stress can lead to arrhythmias and ischemic injury. However, recent evidence in rodent models suggests that acute stress can decrease sensitivity to myocardial ischemia–reperfusion injury (IRI). Conversely, chronic stress is arrhythmogenic and increases sensitivity to myocardial IRI. Few studies have examined the impact of validated animal models of stress-related psychological disorders on the ischemic heart. This review examines the work that has been completed using rat models to study the effects of stress on myocardial sensitivity to ischemic injury. Utilization of animal models of stress-related psychological disorders is critical in the prevention and treatment of cardiovascular disorders in patients experiencing stress-related psychiatric conditions.

Keywords: stress, cardiovascular, ischemia, anxiety, PTSD, rodent

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INTRODUCTION

The goal of this review is to analyze recent literature utilizing rodent models to examine the impact of psychological stress on sensitivity to myocardial ischemia–reperfusion injury (IRI) in the context of the well-established relationship between stress, myocardial ischemic injury, and cardiovascular disease (CVD). Stress is a general adaptive response provoked by stimuli that disrupt homeostasis (1, 2). The stress response activates systems responsible for mobilizing the energy and resources necessary to overcome this homeostatic disturbance. The main systems activated include the hypothalamic–pituitary–adrenal (HPA) axis and the sympathetic adrenomedullary (SAM) system (3, 4). Stress results in the release of corticotropin-releasing hormone (CRH) from the paraventricular nucleus, which then causes the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH acts on the adrenal cortex to synthesize and secrete the glucocorticoid (GC) hormone cortisol (in humans) or corticosterone (in rodents) (3, 5). The hypothalamus also activates the adrenal medulla *via* the sympathetic nervous system (SNS), which results in the release of the catecholamines epinephrine and norepinephrine. ACTH, CRH, and GCs provide the negative feedback necessary to dampen the stress response and return the body to homeostasis (4, 6). Cessation of the stress response is important to prevent damage associated with a prolonged stress response (3, 4, 7). Acute stress generally results in an adaptive response to homeostatic changes; the stress response becomes

harmful if it persists chronically (8–11). Thus, stress research can be roughly divided into research examining the effects of acute or chronic stress (3, 4, 7, 9–11).

Physical or psychological stressors can result in the stress response. Physical stressors disrupt the internal or external environment of an organism and include stimuli such as anoxia, heat, cold, or physical strain (exercise or injury). Psychological stressors are stimuli that affect emotion and result in fear, anxiety, or frustration (8–11). As previously discussed, anything disrupting homeostasis can be a stressor; however, this review focuses on stressors with a psychological component.

Chronic stress can have damaging effects on the whole organism (4). Stress precipitates psychiatric disease, such as depression and post-traumatic stress disorder (PTSD), and worsens physical health outcomes, such as CVD (12, 13). Furthermore, patients with psychiatric disorders have a higher incidence of CVD and cardiovascular risk factors, such as atherosclerosis, hypertension, and myocardial infarction (MI) (14–16). Patients with psychiatric disorders experience worse outcomes in response to cardiovascular disorders (e.g., higher mortality). It is suggested that appropriate monitoring for psychiatric disorders could improve outcomes in patients with ischemic heart disease (8, 14, 17–21). Thus, research directed at minimizing the negative impact of stress is important (19, 21–25).

Stress and Cardiovascular Disease

Cardiovascular disease is the leading cause of mortality worldwide (26, 27), and stress is a well-established contributor to the development of CVD (3, 8, 20). Stress is relevant at all stages of CVD; stress can increase exposure to risk factors for CVD (e.g., smoking), the long-term development of atherosclerosis, and the triggering of cardiac events in people with CVD (28).

The most common form of CVD is ischemic heart disease (also known as coronary artery disease), which includes disease states such as angina, MI, and sudden cardiac death (SCD) (29, 30). MI occurs when blood flow to a region of the heart stops. The heart is an electromechanical pump; SCD most commonly occurs in response to ventricular fibrillation, a disturbance in electrical activity, as a result of acute coronary ischemia (31, 32). MI and SCD can lead to cardiac arrest and death. Stress may acutely trigger MI or SCD or worsen underling CVD leading to one of these events (3). Thus, stress is closely related to ischemic heart disease. Research investigating the relationship between stress and the cardiovascular system is critical to improve patient outcomes in CVD (20, 25, 28).

Myocardial Ischemia–Reperfusion Injury

Myocardial IRI refers to the damage created by the stoppage of and the subsequent restoration of blood flow to the heart. Without blood flow, an imbalance between oxygen supply and demand is created which results directly in irreversible damage to cardiac tissue, eventually resulting in apoptosis or necrosis; this oxygen imbalance is referred to as ischemia. The duration of ischemia and amount of tissue exposed to ischemia are well established as the primary determinants of infarct size (IS), or the amount of non-viable tissue following ischemia. The mechanisms by which damage and protection occur in response to myocardial IRI has been described in detail previously (33–39). Thus, myocardial IRI is the primary mechanism by which cardiac tissue is damaged in MI, SCD, cardiac bypass surgery, and organ transplantation (40). Acute and chronic stress has an impact on myocardial IRI (3, 41, 42). Because myocardial IRI plays a major role in the morbidity and mortality associated with ischemic heart disease and MI, direct study of this pathology is desirable (35, 43–46). To better elucidate the mechanisms underlying CVD and ischemic injury, researchers have utilized animal models.

The Utility of Animal Models in Stress Biology and Cardiovascular Disease

Animal models are used extensively to study the relationship between stress and CVD. Animal models are especially important in studying stress biology, as they allow researchers to standardize the conditions of stress. Furthermore, a high level of experimental control and the potential to study causal neurobiological and behavioral mechanisms (with easier access to tissue samples and physiological manipulation) makes animal models advantageous for studying cardiovascular function and stress (22, 47, 48). By using validated methodology with translational relevance to human patients, researchers can use animal models effectively to examine underlying mechanisms and potential treatment options in CVD and stress (22, 49).

The Langendorff Isolated Heart – An Experimental Model of Ischemic Injury

Animal models have been developed to experimentally induce and study acute ischemia both *in vivo* (50, 51) and *ex vivo* (44, 52, 53). The Langendorff isolated heart preparation is one of the most extensively used animal models for the study of heart physiology and ischemia (53). In this model, crystalloid perfusates (or blood) is delivered through a cannula inserted in the ascending aorta. Retrograde flow closes the leaflets in the aortic valve, leading to perfusion of the coronary vasculature (52, 53). This model is commonly used to study myocardial IRI. This is accomplished by occlusion of a coronary artery (typically the left anterior descending artery), leading to regional ischemia, or by turning off flow, leading to global ischemia. This model allows the generation of data including IS, the recovery of contractile function, and electrical activity in response to induced ischemia. In regional ischemia, researchers use the IS relative to the area at risk (AAR), or the area normally perfused by the clamped artery, whereas global ischemia allows measurement of the total amount of non-viable tissue [for a complete methodological review of the Langendorff isolated heart, see Ref. (52)].

Notably, the Langendorff isolated heart system studies ischemic injury in the absence of normal humoral or neuronal stimulation, potentially limiting the translation of experimental findings to the clinical setting (52, 53). Furthermore, this model has additional disadvantages, including a high coronary flow rate, limited supply of high-energy phosphate, a reduced oxygen requirement, and a degree of technical skill required to perform successfully (53–55). These disadvantages have led to the development of alternative methods to study cardiovascular injury; other potentially more clinically relevant methods include altering the Langendorff

procedure (54) or using *in vivo* models of cardiovascular injury (56). Despite its disadvantages, the Langendorff isolated heart system has proven invaluable to the study of myocardial IRI (52, 53). This model has been used effectively to identify potential strategies and pharmacological agents to decrease the amount of damage caused to the heart following MI (43, 53).

The Langendorff Isolated Heart Preparation in Rats

The Langendorff heart preparation is appropriate in mammalian species. Although this preparation has been used rarely in large animals or man (57–61), the most frequently used isolated heart model is that of the rat. The rat model allows for relatively low costs, easy handling, and uncomplicated equipment (53). Furthermore, the consistency of limited collateral circulation allows the study of regional ischemia in the rat. This provides an advantage over models with significant collateralization such as dog (62), guinea-pig (62, 63), and hamster models (63). Furthermore, the rat's consistent coronary structure makes it a better model than, for example, rabbits, whose coronary structure varies significantly between animals (64). However, it is important to recognize that the rat suffers distinct disadvantages in cardiovascular study because of its short action potential duration, which lacks a plateau phase. This makes this animal a poor choice for study of arrhythmogenesis and antiarrhythmic drugs (60, 65–68). Similarly, dogs have been shown to have elevated levels of troponin and creatine kinase, markers of cardiac damage, in response to cardiac injury (69). However, rats have only shown elevations in troponin, making them relatively poor candidates to study drug-induced injury using these markers (69, 70). Thus, one must remain mindful of the potential clinical relevance of studies in the context of the species being utilized (52).

Both myocardial ischemic injury and cardiovascular responses to stress have been described in detail in both human patients and animal models; however, only several recent studies have focused directly on the sensitivity to myocardial ischemic injury in response following acute or chronic psychological stress exposure.

ACUTE STRESS AND CARDIOVASCULAR DISEASE

The association between acute stress and cardiac rhythm, acute MI, SCD, and stress cardiomyopathy has been supported by epidemiological studies (71–75). Cardiac rhythm changes in response to acute stress has been evidenced by a marked increase in tachyarrhythmia among patients with implanted cardioverter defibrillators in the New York area of the USA during the attacks on the World Trade Center on September 11, 2001 (71). An association between intense emotional stress or anger and the triggering of acute cardiac events, such as acute MI or SCD, has been demonstrated by multiple studies demonstrating a significant number of patients experiencing an emotional episode roughly 2 h before cardiac arrest (72–75). This increased incidence of MI has been evidenced in individual patients following a significant acute stressor, such as the loss of a loved one. Moreover, acute cardiac event incidence is increased in geographical areas where a major trauma, such as an earthquake, serves as an acute stressor (8, 20, 76). SCD and MIs are rare in patients with no underlying coronary heart disease, whereas stress cardiomyopathy can occur with no underlying disorder (77–79).

Acute Stress and Myocardial Ischemic Injury

The association between intense emotional stress and ischemic heart disease, specifically the incidence of SCD, has been researched for over 50 years (80, 81). Acute psychological stress in human patients leads to ischemia, stress cardiomyopathy, MI, and SCD (8). Stress cardiomyopathy is induced by intense stress that results in heart weakness without underlying pathology. Thus, stress cardiomyopathy is a recently identified disease state mirroring MI with symptoms, such as chest pain and ECG abnormalities, but without concomitant coronary spasm or ischemia-induced enzymatic release (82, 83). Mental stress elicits regional ischemic damage due to epicardial or microvascular constriction, as evidenced by changes in regional perfusion. Interestingly, this ischemia is not associated with the angina and ECG changes that are associated with exercise-induced stress (84–89). This transient myocardial ischemia and coronary artery constriction have been shown to occur in patients with advanced coronary artery disease in response to mental stress (89–91). Furthermore, mental stress has been shown to lead to ECG alternans, a predictor of ventricular arrhythmias and SCD (92–94).

Acute mental stress has been shown to alter the action potential duration of cardiac tissue in humans. Adrenergic stimulation with isoprenaline and adrenaline increases the steepness of the slope of action potential duration restitution; this suggests that adrenergic stimulation can lead to electrical instability, which could lead to ventricular fibrillation or arrhythmias (95). In an elegant study, Child et al. showed that a mental challenge was able to elicit this effect on action potential duration independent of the respiration or heart rate changes that occur in response to mental stress (96). Ventricular fibrillation has been shown to occur in response to both regional myocardial ischemia and electrical instability. Ventricular fibrillation leads to global cardiac ischemia, which can lead to cardiac death (97, 98). The ability of mental stress to cause cardiac ischemia and electrical instability in the heart is supported by epidemiological studies. The underlying risk factors inherent in clinical study complicate cardiovascular research. As previously discussed, the standardization of stress conditions makes animal models advantageous for investigating the underlying pathology of disease, including CVD.

Experimental Acute Stress and Cardiovascular Disease

Experimental work using animal models supports the effects of acute psychological stress on the cardiovascular system seen in human patients. Psychological stress has been shown to reduce the ventricular fibrillation threshold in dog (42, 99–103) and porcine models (104). Verrier and colleagues have demonstrated the ability of acute stress to precipitate ventricular arrhythmias in dogs exposed to anger and fear in both healthy hearts and hearts exposed to coronary artery occlusion (99–103, 105–108). Acute

stress was able to precipitate ventricular fibrillation and cardiac arrest; albeit, these studies did not utilize dogs exposed to a single acute stressor but rather an acute stress session following aversive conditioning (99–101, 103). These researchers found that behaviorally induced changes in vulnerability to fibrillation are mediated by the direct effects of catecholamines on beta receptors (109, 110). Further supporting the centrally mediated nature of cardiac arrhythmias generated by acute stress, Skinner and Reed were able to prevent an increase in ventricular fibrillation by cryogenic blockage of the forebrain, posterior hypothalamus, or fields of Forel (104). Thus, acute psychological stress has the ability to generate and exacerbate ischemia and ventricular arrhythmia.

Stress-limiting endogenous systems have been identified with the ability to abolish or reduce cardiac arrhythmias in response to sympathetic stimulation, acute stress, or ischemic injury (4, 7). The endogenous hormones utilized by these systems with protective effects on the cardiovascular system include GABA (111, 112), opioids (113), or vagal stimulation with cholinergic agonists (114, 115). Furthermore, it has been suggested that electrical instability does not necessarily disturb cardiac contractility (4, 116). Supporting the role of stress-limiting systems in cardiovascular injury, recent work in rodents demonstrates that acute stress may decrease damage in response to induced regional ischemia, possibly as a compensatory mechanism.

Experimental Acute Stress and Myocardial Ischemic Injury

Recent rodent studies looking at the effect of acute psychological stress on the impact of myocardial ischemic injury have found acute stress to be cardioprotective and reduce IS [see **Table 1** (45, 117)]. The identified relevant studies utilized cold-restraint stress (117) and forced swim stress (45) before using the Langendorff method to induce regional ischemia. Acute swim stress and acute restraint stress are validated psychological stressors that have been used in combination with other stressors to model PTSD and depression (118–121). These stressors, individually or in combination, have resulted in anxiety-like and fear-related behavior in rodents as assessed by tests such as the elevated plus maze (EPM) and contextual fear conditioning (CF) (119, 122, 123). The decreased sensitivity to myocardial IRI provided by acute psychological stress is supported by similar findings in studies utilizing acute physiologic stressors, such as exercise or hyperthermia (124–128). The existence of endogenous signaling pathways that protect the heart from ischemic injury is well evidenced (46, 129–131).

Research has previously shown that short-term stress is accompanied by enhanced contractile function and resistance to hypoxia in hearts isolated from stressed animals, while long-term stress resulted in the opposite effect (4, 7). Additionally, acute stressors seem to result in the redistribution of the immune system to the site of inflammation, which could provide an adaptive response to stress (137–139). Interestingly, opioid antagonists were able to eliminate the cardioprotection afforded by cold-restraint stress, supporting this stress-limiting system's role in decreased sensitivity to ischemic damage (113, 117, 140).

Though acute psychological stress decreases the sensitivity of ischemic damage in response to myocardial IRI, the work does not necessarily contradict the previously discussed, well-established effects of acute stress in both animal models and clinical research, including triggering MI or independently leading to ischemic damage (72–75, 100–103). While electrical instability of the heart occurs in response to acute stress, it is possible that protective pathways exist to reduce the sensitivity to ischemic damage (4, 7, 116, 140). Additionally, it is important to recognize that while removing the additional stressors and underlying pathology found in humans adds experimental control, it does diminish the clinical translatability of this work (33, 52, 53). Furthermore, while investigators look at the myocardial ischemic injury of all rodents exposed to acute psychological stress, MI data in humans in response to acute stressors typically only represent patients who experienced an MI or symptoms of an MI (72–75). As a final potential limitation, rodent models look at the same ischemic injury in all subjects, whereas human patients can present with very different ischemic damage due to underlying disease and the possible collateralization of vessels over many years (135).

Contrasting the protective effects of acute stress, chronic stress in rodent models has impacted sensitivity to myocardial ischemic injury in rodent models by decreasing recovery of cardiac contractility and increasing ischemic injury (10, 132, 133, 134). The effect of chronic psychological stress is especially relevant because of the numerous stressors facing human patients, which have effects on cardiovascular outcomes (8, 14, 17–22, 141, 142). Thus, diminishing the negative effects of chronic stress on the heart has the ability to reduce cardiovascular morbidity and mortality. Therefore, the effect of chronic stress on the cardiovascular system has been an emerging area of research with several recent studies looking directly at myocardial ischemic injury.

CHRONIC STRESS AND CARDIOVASCULAR DISEASE

Chronic stress has been implicated to cause or worsen CVD in human patients (20, 141–145). Chronic stress has been linked to increased risk of ischemic heart disease (20, 28). The INTERHEART case–control study showed that significant long-term stress over the course of 12 months more than doubled the risk of acute MI, even after adjusting for conventional risk factors such as diabetes mellitus, hypertension, and smoking (146). Prospective cohort studies have supported the effect of long-term stress on risk of coronary heart disease. Studies have linked coronary heart disease risk with work-related stressors, specifically when an imbalance between effort and reward is experienced (147–151). Furthermore, the effects of long-term stress may persist long after the cessation of the chronic stressors. Survivors of the siege of Leningrad were found to have increased blood pressure and increased mortality from CVD, relative to Russians who were not in the besieged city, over 50 years after the event (152).

Chronic Stress and Cardiovascular Disease

Psychological conditions related to chronic stress and CVD include depression, anxiety, and PTSD (3). As previously discussed, psychiatric disorders can worsen outcomes in CVD.

TABLE 1 | Studies examining myocardial ischemic injury in rodent models of psychological stress.

Subjects	Stress protocol	Reperfusion injury (RI) protocol	Primary finding	Reference
Acute psychological stress				
Adult male Wistar rats	Forced swim for 10 min RI 10 min after	30 min ischemia 60 min reperfusion	Decreased infarct size (IS)/area at risk (AAR)%	Moghimian et al. (45)
Adult male Sprague-Dawley rats	Individual immobilization, placed in a cold room for 3 h at 4 ± 0.3°C RI immediately after	30 min ischemia 120 min reperfusion	Decreased IS/AAR%	Wu et al. (117)
Chronic psychological stress				
Adult male Sprague-Dawley rats	1–1.5 h daily restraint stress for 8–14 days RI 24 h later	30 min ischemia 180 min reperfusion	Increased IS/AAR% Increased # of fatal arrhythmias	Scheuer and Mifflin (132)
Adult male Sprague-Dawley rats	2 h daily restraint stress for 11–12 days RI 24 h later	30 min ischemia 180 min reperfusion	Increased IS/AAR% Increased # of fatal arrhythmias	Scheuer and Mifflin (132)
Adult male Wistar-Kyoto (WKY) rats	Crowding stress (living space 200 cm^2/rat) for 8 weeks RI unspecified	30 min ischemia 120 min reperfusion (reperfusion-induced tachyarrhythmias and contractile function measured 40 min after reperfusion initiation)	Decreased LVDP recovery Increased duration of ventricular tachycardia (VT)	Ravingerova et al. (133)
Adult male spontaneously hypertensive (SHR) rats	Crowding stress (living space 200 cm^2/rat) for 8 weeks RI unspecified	30 min ischemia 120 min reperfusion (reperfusion-induced tachyarrhythmias and contractile function measured 40 min after reperfusion initiation)	Increased LVDP recovery Decreased duration of VT	Ravingerova et al. (133)
Adult male Wistar rats	10 s electrical shock, 50 s rest for 1 h daily for 7 days RI 24 h later	30 min ischemia 120 min reperfusion	Increased IS/AAR%	Rakhshan et al. (10)
Adult male Wistar rats	Witnessed rats receive but did not receive 10 s electrical shock, 50 s rest for 1 h daily for 7 days (psychological shock) RI 24 h later	30 min ischemia 120 min reperfusion	Increased IS/AAR%	Rakhshan et al. (10)
5-week-old male Wistar-Kyoto (WKY) rats	Crowding stress (~70 cm^2 living space per 100g body mass) for 14 days RI unspecified	30 min ischemia 120 min reperfusion (reperfusion-induced tachyarrhythmias and contractile function measured 40 min after reperfusion initiation)	No significant difference between stress and no stress groups	Ledvenyiova-Farkasova et al. (134)
5-week-old female Wistar-Kyoto (WKY) rats	Crowding stress (~70 cm^2 living space per 100 g body mass) for 14 days RI unspecified	30 min ischemia 120 min reperfusion (reperfusion-induced tachyarrhythmias and contractile function measured 40 min after reperfusion initiation)	Decreased VT duration	Ledvenyiova-Farkasova et al. (134)
5-week-old female spontaneously hypertensive (SHR) rats	Crowding stress (~70 cm^2 living space per 100 g body mass) for 14 days RI unspecified	30 min ischemia 120 min reperfusion (reperfusion-induced tachyarrhythmias and contractile function measured 40 min after reperfusion initiation)	Increased VT duration	Ledvenyiova-Farkasova et al. (134)
5-week-old male spontaneously hypertensive (SHR) rats	Crowding stress (~70 cm^2 living space per 100 g body mass) for 14 days RI unspecified	30 min ischemia 120 min reperfusion (reperfusion-induced tachyarrhythmias and contractile function measured 40 min after reperfusion initiation)	Increased VT duration	Ledvenyiova-Farkasova et al. (134)
Adult male Sprague-Dawley rats	31 days chronic social instability (randomized paired housing) 1 h immobilized predator exposure on days 1 and 11 See Zoladz et al. (136) for complete PTSD paradigm RI 48 h later	20 min ischemia 120 min reperfusion	Increased IS/AAR% Decreased RPP Decreased + dP/dT	Rorabaugh et al. (135)
Adult female Sprague-Dawley rats	31 days chronic social instability (randomized paired housing) 1 h immobilized predator exposure on days 1 and 11 See Zoladz et al. (136) for complete PTSD paradigm RI 48 h after	20 min ischemia 120 min reperfusion	No significant effect	Rorabaugh et al. (135)

However, this relationship may be bidirectional. For example, it has been shown that coronary heart disease leads to a higher incidence of depression, and depression leads to worse outcomes in coronary heart disease (14, 15, 17, 49, 153). Furthermore, the association between depression and coronary heart disease occurs independent of comorbid risk factors such as high cholesterol, hypertension, or obesity (13, 49, 154, 155). PTSD also increases a patient's risk for developing coronary heart disease. This association is independent of comorbid depression, genetic influences, and other confounding factors (156–158). The negative cardiovascular outcomes exhibited in both depression and PTSD have been attributed to underlying dysfunction in the autonomic nervous system and HPA axis (13, 22, 48, 49, 135). However, precisely defining the contribution of long-term stress to CVD is difficult due to potential confounding factors including the aforementioned psychological disorders (28). Thus, animal models provide an acceptable means to study chronic stress in the controlled experimental setting (22).

Experimental Chronic Stress and Cardiovascular Disease

Animal models support the negative effects of chronic stress on the cardiovascular system evidenced by epidemiological studies. Experimental studies have found exposure to chronic stress results in enhanced development of atherosclerosis and plaque destabilization (3, 159, 160). Chronic stress has also been shown to lower the threshold for ventricular arrhythmias (103, 107–109, 161, 162). In a landmark study, Verrier and Lown conditioned dogs to associate a sling with an aversive shock for 3 days. On days 4 and 5, these researchers found that coronary occlusion in dogs re-exposed to the sling environment (in the absence of shock) led to ventricular fibrillation, whereas dogs in a non-aversive cage environment did not experience ventricular fibrillation. Research has continued to focus on this ability of chronic psychological stress to result in cardiac instability (101, 102, 107).

Researchers have used validated models of psychological disorders to study the relationship between psychological disorders and the cardiovascular system. For example, the relationship between depression and CVD has been studied using chronic stress models [e.g., chronic mild stress (CMS) and social isolation] of depression in rodents. The CMS model of depression involves exposure to mild and unpredictable stressors, including changing cage mates, cage tilt, and periods of water or food deprivation, for a period greater than 2 weeks (49, 153, 163). These models of depression decrease rodent intake of a sweet solution, suggestive of anhedonia. Rodents exposed to these well-established animal models display depressive-like behavior, and have a decreased threshold for arrhythmias and tissue fibrosis (22, 49, 153, 163–167). Although animal models have been used to study stress biology and cardiovascular outcomes, few studies exist using validated models of psychological disorders to study the effect of stress on sensitivity to myocardial ischemic injury.

Experimental Chronic Stress and Myocardial Ischemic Injury

In several recent rodent studies, researchers have found greater ISs, decreased cardiac output, and decreased recovery of contractile function in response to chronic psychological stress [see **Table 1** (10, 132, 133, 134, 135)]. Chronic physiologic stress has previously shown mixed results; both decreased (168) and increased (169) sensitivity to myocardial ischemic injury have been reported. Evidencing only negative effects of chronic stress on myocardial ischemic injury, the impact of chronic psychological stress represents an emerging area of research to minimize the detrimental effect of chronic stress (135, 170). The disruptive effect of chronic psychological stress exposure on myocardial ischemic injury has been demonstrated using several different chronic stressors, including chronic restraint stress (132), daily foot shocks or witnessing rats receiving those foot shocks (10), or crowding stress (133, 134).

These stressors are frequently utilized in modeling psychological disorders that result from stress. Restraint stress has been used as a psychological stressor in rats and has been utilized in combination with other stressors to model PTSD and depression (119, 122, 123, 136). Inescapable footshock is used to model depressive symptoms in rodents. Rats exposed to inescapable footshock have demonstrated anxiety-like behavior on an EPM, impaired growth rates, decreased rearing in an open field, and decreased locomotion (50, 171–173). Crowding stress is a well-known and ethologically valid model of psychological stress in rats which causes social competition for resources, such as space, food, and water. Crowding stress results in behavioral and physiologic data reflecting psychological stress (174–178). These chronic psychological stressors resulted in disruption to the cardiovascular system following induced myocardial ischemic injury, either by causing increased IS and decreased contractile function recovery (10, 132) or only decreased contractile function recovery (133, 134). These studies suggest that chronic stress not only increases the likelihood of a MI or SCD but also exacerbates the damage in response to ischemic injury.

A potential limitation of these studies is that researchers did not take behavioral measures of stress prior to myocardial ischemic injury. Although the methods of stress used to stress these animals are validated as methods of inducing psychological stress, individual susceptibility may play a role in the response of the animal to a psychological stressor (10, 132, 133, 134). Stress exposure may affect animals differently, and thus, measurement of the stress response at the behavioral level is important. The only known published study utilizing a model of a chronic psychological disorder where animals' response to stress was validated prior to myocardial ischemic injury is utilizing a predator-based psychosocial model of PTSD (135).

A Predator-Based Psychosocial Model of PTSD and Myocardial Ischemia–Reperfusion Injury

A predator-based psychosocial model of PTSD has been utilized to study sensitivity to myocardial ischemic injury. This model involves two 1-h cat exposures, during which rats are restrained while they can see, smell, and hear a cat but cannot be physically harmed. The two exposures are separated by a period of 10 days. Starting on the day of the first cat exposure, rodents experience chronic social instability by having their housing partner changed daily for 31 days. After the 31-day paradigm,

rats exhibit a fear memory associated with the cat exposures (evidenced by freezing in response to conditioned context and cues), heightened anxiety-like behavior on the EPM, an exaggerated startle response, and impaired memory for newly learned information. Furthermore, rats exposed to this paradigm have demonstrated physiological changes reflecting elevated SNS activity and HPA axis abnormalities, including elevated heart rate and blood pressure, decreased baseline corticosterone levels, and enhanced negative feedback of the HPA axis (135, 136, 179–181). Replicating and expanding on these results, researchers utilizing this model have shown stressed rats exhibit decreased serotonin, increased norepinephrine, and increased measures of oxidative stress and inflammation in the brain, adrenal glands, and systemic circulation (182, 183).

Recently, we found that, subsequent to this chronic psychological stress paradigm, male rats exposed to myocardial ischemic injury exhibited greater ISs and decreased recovery of contractile function [**Figure 1** (135)]. The disruptive effect of this PTSD paradigm on the heart is further strengthened by anxiety-like behavior in rats on the EPM prior to myocardial ischemic injury. These data suggest that the psychological stress induced by the PTSD paradigm is having an effect directly on the heart, causing the heart to be more susceptible to damage following a MI (135). The ability of chronic stress to worsen the extent of ischemic injury and decrease the recovery of cardiac contractility further exacerbates the supported negative effects of stress in CVD, which make rodents exposed to chronic stress more susceptible to ventricular fibrillation and MI (13, 22, 48, 49, 135).

THE IMPORTANCE OF THE EFFECT OF PSYCHOLOGICAL STRESS ON MYOCARDIAL ISCHEMIA–REPERFUSION INJURY

Shown presently, acute and chronic psychological stress affects sensitivity to myocardial ischemic injury in opposite directions; acute psychological stress decreases, whereas chronic psychological stress increases sensitivity to myocardial ischemic injury (45, 117). It is possible that protective mechanisms exist in response to an optimal level of acute stress, but these mechanisms are eventually overcome by more intense levels of stress (4).

Physiologically, a possible explanation for this differential effect is that acute psychological stress causes norepinephrine release and acute alpha stimulation, which results in ischemic preconditioning (184, 185). Chronic psychological stress may result in chronic beta stimulation, worsening the ischemic injury (186–190). The previously discussed advantages of the isolated rat heart (66), the wide variety of validated psychological stressors in rodents (119, 122, 123, 136, 174–178), and the existence of rodent models of psychiatric disorders (49, 153, 181) add weight to the presently discussed findings. However, it is important to qualify

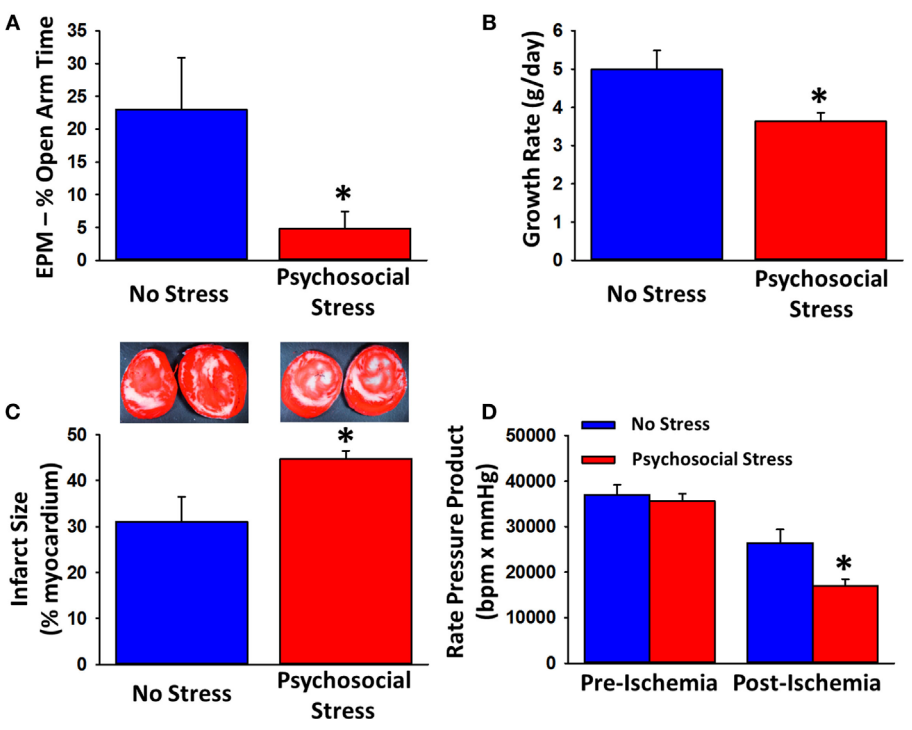

FIGURE 1 | Effects of a predator-based psychosocial model of PTSD on anxiety-like behavior, growth rate, and myocardial sensitivity to ischemic injury. Rats exposed to the 31-day psychosocial stress paradigm spend less time in the open arms on the EPM **(A)** and exhibit reduced growth rats **(B)**. Following 20-min ischemia, hearts from psychosocially stressed animals exhibit larger infarcts **(C)**, white regions of representative tissue (samples in the insets) and impaired recovery of contractile function **(D)**. Data are means ± SEM. *$p < 0.05$ relative to no stress. Adapted from Rorabaugh et al. (135).

these findings by recognizing the methodological differences in a limited amount of studies and the previously discussed weaknesses of translating the isolated rodent heart to humans.

Utilizing ethologically valid models of stress to further study the effect of psychological stress on myocardial ischemic injury will best translate to improving patient outcomes in the clinical setting (22, 49). Additionally, further research investigating the effects of stress on the cardiovascular system in females will be important in translating findings to the clinical setting, as the current literature is currently dominated by studies in male subjects (135).

CONCLUSION

The relationship between stress and CVD continues to receive a substantial amount of attention. Here, we reviewed research studying the sensitivity of the rodent heart to ischemic injury in response acute and chronic psychological stress in the context of clinical and experimental studies on the effects of stress on the cardiovascular system. Elucidation of stress-limiting systems will help identify novel therapeutic options to decrease cardiovascular mortality. Further research investigating the relationship between acute and chronic stress and ischemic injury will improve patient care with implications that extend beyond cardiovascular disease.

AUTHOR CONTRIBUTIONS

EE wrote the first draft of the manuscript and revised it following peer review. BR provided comments on each draft. PZ helped EE prepare the manuscript, provided comments on each draft, and prepared the figures.

REFERENCES

1. Cannon WB. Stresses and strains of homeostasis. *Am J Med Sci* (1935) **189**(1):13–4. doi:10.1097/00000441-193501000-00001
2. Selye H. The story of the adaptation syndrome. *Am J Med Sci* (1952) **224**(6):711. doi:10.1097/00000441-195212000-00039
3. Lagraauw HM, Kuiper J, Bot I. Acute and chronic psychological stress as risk factors for cardiovascular disease: insights gained from epidemiological, clinical and experimental studies. *Brain Behav Immun* (2015) **50**:18–30. doi:10.1016/j.bbi.2015.08.007
4. Meerson FZ. Stress-induced arrhythmic disease of the heart – part I. *Clin Cardiol* (1994) **17**(7):362–71. doi:10.1002/clc.4960170705
5. McEwen BS. Protective and damaging effects of stress mediators. *N Engl J Med* (1998) **338**(3):171–9. doi:10.1056/NEJM199801153380307
6. Meijer OC. Understanding stress through the genome. *Stress* (2006) **9**(2):61–7. doi:10.1080/10253890600799669
7. Meerson FZ. *Adaptive Protection of the Heart*. Boca Raton, FL: CRC Press (1990).
8. Dimsdale JE. Psychological stress and cardiovascular disease. *J Am Coll Cardiol* (2008) **51**(13):1237–46. doi:10.1016/j.jacc.2007.12.024
9. Johnson EO, Kamilaris TC, Chrousos GP, Gold PW. Mechanisms of stress: a dynamic overview of hormonal and behavioral homeostasis. *Neurosci Biobehav Rev* (1992) **16**(2):115–30. doi:10.1016/S0149-7634(05)80175-7
10. Rakhshan K, Imani A, Faghihi M, Nabavizadeh F, Golnazari M, Karimian S. Evaluation of chronic physical and psychological stress induction on cardiac ischemia/reperfusion injuries in isolated male rat heart: the role of sympathetic nervous system. *Acta Med Iran* (2015) **53**(8):482–90.
11. Tennant C, Langeluddecke P, Byrne D. The concept of stress. *Aust N Z J Psychiatry* (1985) **19**(2):113–8. doi:10.3109/00048678509161308
12. Parker GB, Owen CA, Brotchie HL, Hyett MP. The impact of differing anxiety disorders on outcome following an acute coronary syndrome: time to start worrying? *Depress Anxiety* (2010) **27**(3):302–9. doi:10.1002/da.20602
13. Penninx BW, Beekman AT, Honig A, Deeg DJ, Schoevers RA, van Eijk JT, et al. Depression and cardiac mortality: results from a community-based longitudinal study. *Arch Gen Psychiatry* (2001) **58**(3):221–7. doi:10.1001/archpsyc.58.3.221
14. Anda R, Williamson D, Jones D, Macera C, Eaker E, Glassman A, et al. Depressed affect, hopelessness, and the risk of ischemic heart disease in a cohort of U.S. adults. *Epidemiology* (1993) **4**(4):285–94. doi:10.1097/00001648-199307000-00003
15. Mavrides N, Nemeroff C. Treatment of depression in cardiovascular disease. *Depress Anxiety* (2013) **30**(4):328–41. doi:10.1002/da.22051
16. Mavrides N, Nemeroff CB. Treatment of affective disorders in cardiac disease. *Dialogues Clin Neurosci* (2015) **17**(2):127–40.
17. Barefoot JC, Helms MJ, Mark DB, Blumenthal JA, Califf RM, Haney TL, et al. Depression and long-term mortality risk in patients with coronary artery disease. *Am J Cardiol* (1996) **78**(6):613–7. doi:10.1016/S0002-9149(96)00380-3
18. Ketterer MW. Secondary prevention of ischemic heart disease. The case for aggressive behavioral monitoring and intervention. *Psychosomatics* (1993) **34**(6):478–84. doi:10.1016/S0033-3182(93)71821-6
19. Monroe SM, Harkness KL. Life stress, the "kindling" hypothesis, and the recurrence of depression: considerations from a life stress perspective. *Psychol Rev* (2005) **112**(2):417–45. doi:10.1037/0033-295X.112.2.417
20. Steptoe A, Kivimaki M. Stress and cardiovascular disease: an update on current knowledge. *Annu Rev Public Health* (2013) **34**:337–54. doi:10.1146/annurev-publhealth-031912-114452
21. Swaab DF, Bao AM, Lucassen PJ. The stress system in the human brain in depression and neurodegeneration. *Ageing Res Rev* (2005) **4**(2):141–94. doi:10.1016/j.arr.2005.03.003
22. Grippo AJ. The utility of animal models in understanding links between psychosocial processes and cardiovascular health. *Soc Personal Psychol Compass* (2011) **5**(4):164–79. doi:10.1111/j.1751-9004.2011.00342.x
23. Gianaros PJ, Wager TD. Brain-body pathways linking psychological stress and physical health. *Curr Dir Psychol Sci* (2015) **24**(4):313–21. doi:10.1177/0963721415581476
24. Richtig E, Trapp EM, Avian A, Brezinsek HP, Trapp M, Egger JW, et al. Psychological stress and immunological modulations in early-stage melanoma patients. *Acta Derm Venereol* (2015) **95**(6):691–5. doi:10.2340/00015555-2045
25. Davey A, Shukla A, Sharma P, Srivastava K, Davey S, Vyas S. Are the adverse psychiatric outcomes reflection of occupational stress among nurses: an exploratory study. *Asian J Med Sci* (2016) **7**(1):96–100. doi:10.3126/ajms.v7i1.12869
26. Mathers CD, Loncar D. Projections of global mortality and burden of disease from 2002 to 2030. *PLoS Med* (2006) **3**(11):e442. doi:10.1371/journal.pmed.0030442
27. Quam L, Smith R, Yach D. Rising to the global challenge of the chronic disease epidemic. *Lancet* (2006) **368**(9543):1221–3. doi:10.1016/S0140-6736(06)69422-1
28. Steptoe A, Kivimaki M. Stress and cardiovascular disease. *Nat Rev Cardiol* (2012) **9**(6):360–70. doi:10.1038/nrcardio.2012.45
29. GBD 2013 Mortality and Causes of Death Collaborators. Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet* (2015) **385**(9963):117–71. doi:10.1016/S0140-6736(14)61682-2
30. Mendis S, Puska P, Norrving B. *Global Atlas on Cardiovascular Disease Prevention and Control*. Geneva: World Health Organization (2011).
31. Janse MJ. Electrophysiological changes in heart failure and their relationship to arrhythmogenesis. *Cardiovasc Res* (2004) **61**(2):208–17. doi:10.1016/j.cardiores.2003.11.018
32. Janse MJ, Wit AL. Electrophysiological mechanisms of ventricular arrhythmias resulting from myocardial ischemia and infarction. *Physiol Rev* (1989) **69**(4):1049–169.
33. Braunwald E, Kloner RA. Myocardial reperfusion: a double-edged sword? *J Clin Invest* (1985) **76**(5):1713–9. doi:10.1172/JCI112160

34. Eefting F, Rensing B, Wigman J, Pannekoek WJ, Liu WM, Cramer MJ, et al. Role of apoptosis in reperfusion injury. *Cardiovasc Res* (2004) **61**(3):414–26. doi:10.1016/j.cardiores.2003.12.023
35. Frank A, Bonney M, Bonney S, Weitzel L, Koeppen M, Eckle T. Myocardial ischemia reperfusion injury: from basic science to clinical bedside. *Semin Cardiothorac Vasc Anesth* (2012) **16**(3):123–32. doi:10.1177/1089253211436350
36. Gottlieb RA, Burleson KO, Kloner RA, Babior BM, Engler RL. Reperfusion injury induces apoptosis in rabbit cardiomyocytes. *J Clin Invest* (1994) **94**(4):1621–8. doi:10.1172/JCI117504
37. Rao PR, Kumar VK, Viswanath RK, Subbaraju GV. Cardioprotective activity of alcoholic extract of *Tinospora cordifolia* in ischemia-reperfusion induced myocardial infarction in rats. *Biol Pharm Bull* (2005) **28**(12):2319–22. doi:10.1248/bpb.28.2319
38. Wang QD, Swardh A, Sjoquist PO. Relationship between ischaemic time and ischaemia/reperfusion injury in isolated Langendorff-perfused mouse hearts. *Acta Physiol Scand* (2001) **171**(2):123–8. doi:10.1046/j.1365-201x.2001.00788.x
39. Jennings RB, Sommers HM, Smyth GA, Flack HA, Linn H. Myocardial necrosis induced by temporary occlusion of a coronary artery in the dog. *Arch Pathol* (1960) **70**:68–78.
40. McMichael M, Moore RM. Ischemia-reperfusion injury pathophysiology, part I. *J Vet Emerg Crit Care* (2004) **14**(4):231–41. doi:10.1111/j.1476-4431.2004.04004.x
41. Taggart P, Critchley H, Lambiase PD. Heart-brain interactions in cardiac arrhythmia. *Heart* (2011) **97**(9):698–708. doi:10.1136/hrt.2010.209304
42. Verrier RL. Behavioral stress, myocardial ischemia, and arrhythmias. In: Zipes DP, Jalife J, editors. *Cardiac Electrophysiology: From Cell to Bedside*. Toronto: WB Saunders (1990). p. 343–52.
43. Kocoglu H, Karaaslan K, Gonca E, Bozdogan O, Gulcu N. Preconditioning effects of dexmedetomidine on myocardial ischemia/reperfusion injury in rats. *Curr Ther Res Clin Exp* (2008) **69**(2):150–8. doi:10.1016/j.curtheres.2008.04.003
44. Mirica SN, Ordodi V, Apostol A, Ana D, Răducan A, Duicu O, et al. Langendorff perfused heart – the 110 years old experimental model that gets better with age. *Studia Univ Vasile Goldis Seria Stiintele Vietii* (2009) **19**(1):81–6.
45. Moghimian M, Faghihi M, Karimian SM, Imani A. The effect of acute stress exposure on ischemia and reperfusion injury in rat heart: role of oxytocin. *Stress* (2012) **15**(4):385–92. doi:10.3109/10253890.2011.630436
46. Murphy E, Steenbergen C. Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury. *Physiol Rev* (2008) **88**(2):581–609. doi:10.1152/physrev.00024.2007
47. Neumann ID, Wegener G, Homberg JR, Cohen H, Slattery DA, Zohar J, et al. Animal models of depression and anxiety: what do they tell us about human condition? *Prog Neuropsychopharmacol Biol Psychiatry* (2011) **35**(6):1357–75. doi:10.1016/j.pnpbp.2010.11.028
48. Zoladz PR, Diamond DM. Current status on behavioral and biological markers of PTSD: a search for clarity in a conflicting literature. *Neurosci Biobehav Rev* (2013) **37**(5):860–95. doi:10.1016/j.neubiorev.2013.03.024
49. Grippo AJ. Mechanisms underlying altered mood and cardiovascular dysfunction: the value of neurobiological and behavioral research with animal models. *Neurosci Biobehav Rev* (2009) **33**(2):171–80. doi:10.1016/j.neubiorev.2008.07.004
50. Li H, Zhou C, Chen D, Fang N, Yao Y, Li L. Failure to protect against myocardial ischemia-reperfusion injury with sevoflurane postconditioning in old rats *in vivo*. *Acta Anaesthesiol Scand* (2013) **57**(8):1024–31. doi:10.1111/aas.12156
51. Nagata T, Yasukawa H, Kyogoku S, Oba T, Takahashi J, Nohara S, et al. Cardiac-specific SOCS3 deletion prevents *in vivo* myocardial ischemia reperfusion injury through sustained activation of cardioprotective signaling molecules. *PLoS One* (2015) **10**(5):e0127942. doi:10.1371/journal.pone.0127942
52. Bell RM, Mocanu MM, Yellon DM. Retrograde heart perfusion: the Langendorff technique of isolated heart perfusion. *J Mol Cell Cardiol* (2011) **50**(6):940–50. doi:10.1016/j.yjmcc.2011.02.018
53. Skrzypiec-Spring M, Grotthus B, Szelag A, Schulz R. Isolated heart perfusion according to Langendorff – still viable in the new millennium. *J Pharmacol Toxicol Methods* (2007) **55**(2):113–26. doi:10.1016/j.vascn.2006.05.006
54. Ye JX, Chen DZ. Novel cardioprotective strategy combining three different preconditioning methods to prevent ischemia/reperfusion injury in aged hearts in an improved rabbit model. *Exp Ther Med* (2015) **10**(4):1339–47. doi:10.3892/etm.2015.2680
55. Suzuki Y, Yeung AC, Ikeno F. The pre-clinical animal model in the translational research of interventional cardiology. *JACC Cardiovasc Interv* (2009) **2**(5):373–83. doi:10.1016/j.jcin.2009.03.004
56. Vidavalur R, Swarnakar S, Thirunavukkarasu M, Samuel SM, Maulik N. *Ex vivo* and *in vivo* approaches to study mechanisms of cardioprotection targeting ischemia/reperfusion (i/r) injury: useful techniques for cardiovascular drug discovery. *Curr Drug Discov Technol* (2008) **5**(4):269–78. doi:10.2174/157016308786733555
57. Ytrehus K. The ischemic heart – experimental models. *Pharmacol Res* (2000) **42**(3):193–203. doi:10.1006/phrs.2000.0669
58. Hill AJ, Laske TG, Coles JA Jr, Sigg DC, Skadsberg ND, Vincent SA, et al. *In vitro* studies of human hearts. *Ann Thorac Surg* (2005) **79**(1):168–77. doi:10.1016/j.athoracsur.2004.06.080
59. Sutherland FJ, Hearse DJ. The isolated blood and perfusion fluid perfused heart. *Pharmacol Res* (2000) **41**(6):613–27. doi:10.1006/phrs.1999.0653
60. Hearse DJ, Sutherland FJ. Experimental models for the study of cardiovascular function and disease. *Pharmacol Res* (2000) **41**(6):597–603. doi:10.1006/phrs.1999.0651
61. Verdouw PD, van den Doel MA, de Zeeuw S, Duncker DJ. Animal models in the study of myocardial ischaemia and ischaemic syndromes. *Cardiovasc Res* (1998) **39**(1):121–35. doi:10.1016/S0008-6363(98)00069-8
62. Maxwell MP, Hearse DJ, Yellon DM. Species variation in the coronary collateral circulation during regional myocardial ischaemia: a critical determinant of the rate of evolution and extent of myocardial infarction. *Cardiovasc Res* (1987) **21**(10):737–46. doi:10.1093/cvr/21.10.737
63. Johns TN, Olson BJ. Experimental myocardial infarction. I. A method of coronary occlusion in small animals. *Ann Surg* (1954) **140**(5):675–82. doi:10.1097/00000658-195411000-00006
64. Day SB, Johnson JA. The distribution of the coronary arteries of the rabbit. *Anat Rec* (1958) **132**(4):633–43. doi:10.1002/ar.1091320411
65. Rees SA, Curtis MJ. Selective IK blockade as an antiarrhythmic mechanism: effects of UK66, 914 on ischaemia and reperfusion arrhythmias in rat and rabbit hearts. *Br J Pharmacol* (1993) **108**(1):139–45. doi:10.1111/j.1476-5381.1993.tb13453.x
66. Curtis MJ. Characterisation, utilisation and clinical relevance of isolated perfused heart models of ischaemia-induced ventricular fibrillation. *Cardiovasc Res* (1998) **39**(1):194–215. doi:10.1016/S0008-6363(98)00083-2
67. Rees SA, Curtis MJ. Specific IK1 blockade: a new antiarrhythmic mechanism? Effect of RP58866 on ventricular arrhythmias in rat, rabbit, and primate. *Circulation* (1993) **87**(6):1979–89. doi:10.1161/01.CIR.87.6.1979
68. Curtis MJ, Macleod BA, Walker MJ. Models for the study of arrhythmias in myocardial ischaemia and infarction: the use of the rat. *J Mol Cell Cardiol* (1987) **19**(4):399–419. doi:10.1016/S0022-2828(87)80585-0
69. Feng X, Taggart P, Hall L, Bryant S, Sansone J, Kemmerer M, et al. Limited additional release of cardiac troponin I and T in isoproterenol-treated beagle dogs with cardiac injury. *Clin Chem* (2005) **51**(7):1305–7. doi:10.1373/clinchem.2005.049643
70. Bleuel H, Deschl U, Bertsch T, Bolz G, Rebel W. Diagnostic efficiency of troponin T measurements in rats with experimental myocardial cell damage. *Exp Toxicol Pathol* (1995) **47**(2–3):121–7. doi:10.1016/S0940-2993(11)80297-6
71. Steinberg JS, Arshad A, Kowalski M, Kukar A, Suma V, Vloka M, et al. Increased incidence of life-threatening ventricular arrhythmias in implantable defibrillator patients after the World Trade Center attack. *J Am Coll Cardiol* (2004) **44**(6):1261–4. doi:10.1016/j.jacc.2004.06.032
72. Steptoe A, Brydon L. Emotional triggering of cardiac events. *Neurosci Biobehav Rev* (2009) **33**(2):63–70. doi:10.1016/j.neubiorev.2008.04.010
73. Chan C, Elliott J, Troughton R, Frampton C, Smyth D, Crozier I, et al. Acute myocardial infarction and stress cardiomyopathy following the Christchurch earthquakes. *PLoS One* (2013) **8**(7):e68504. doi:10.1371/journal.pone.0068504
74. Moller J, Theorell T, de Faire U, Ahlbom A, Hallqvist J. Work related stressful life events and the risk of myocardial infarction. Case-control and case-crossover analyses within the Stockholm heart epidemiology programme (SHEEP). *J Epidemiol Community Health* (2005) **59**(1):23–30. doi:10.1136/jech.2003.019349

75. Mittleman MA, Maclure M, Sherwood JB, Mulry RP, Tofler GH, Jacobs SC, et al. Triggering of acute myocardial infarction onset by episodes of anger. Determinants of Myocardial Infarction Onset Study Investigators. *Circulation* (1995) **92**(7):1720–5. doi:10.1161/01.CIR.92.7.1720
76. Krantz DS, Kop WJ, Santiago HT, Gottdiener JS. Mental stress as a trigger of myocardial ischemia and infarction. *Cardiol Clin* (1996) **14**(2):271–87. doi:10.1016/S0733-8651(05)70280-0
77. Brotman DJ, Golden SH, Wittstein IS. The cardiovascular toll of stress. *Lancet* (2007) **370**(9592):1089–100. doi:10.1016/S0140-6736(07)61305-1
78. Wittstein IS, Thiemann DR, Lima JA, Baughman KL, Schulman SP, Gerstenblith G, et al. Neurohumoral features of myocardial stunning due to sudden emotional stress. *N Engl J Med* (2005) **352**(6):539–48. doi:10.1056/NEJMoa043046
79. Sharkey SW, Windenburg DC, Lesser JR, Maron MS, Hauser RG, Lesser JN, et al. Natural history and expansive clinical profile of stress (tako-tsubo) cardiomyopathy. *J Am Coll Cardiol* (2010) **55**(4):333–41. doi:10.1016/j.jacc.2009.08.057
80. Engel GL. Sudden and rapid death during psychological stress. Folklore or folk wisdom? *Ann Intern Med* (1971) **74**(5):771–82. doi:10.7326/0003-4819-74-5-771
81. Engel GL. Psychologic stress, vasodepressor (vasovagal) syncope, and sudden death. *Ann Intern Med* (1978) **89**(3):403–12. doi:10.7326/0003-4819-89-3-403
82. Akashi YJ, Nef HM, Mollmann H, Ueyama T. Stress cardiomyopathy. *Annu Rev Med* (2010) **61**:271–86. doi:10.1146/annurev.med.041908.191750
83. Tsuchihashi K, Ueshima K, Uchida T, Oh-mura N, Kimura K, Owa M, et al. Transient left ventricular apical ballooning without coronary artery stenosis: a novel heart syndrome mimicking acute myocardial infarction. Angina Pectoris-Myocardial Infarction Investigations in Japan. *J Am Coll Cardiol* (2001) **38**(1):11–8. doi:10.1016/S0735-1097(01)01316-X
84. Rozanski A, Bairey CN, Krantz DS, Friedman J, Resser KJ, Morell M, et al. Mental stress and the induction of silent myocardial ischemia in patients with coronary artery disease. *N Engl J Med* (1988) **318**(16):1005–12. doi:10.1056/NEJM198804213181601
85. Deanfield JE, Shea M, Kensett M, Horlock P, Wilson RA, de Landsheere CM, et al. Silent myocardial ischaemia due to mental stress. *Lancet* (1984) **2**(8410):1001–5. doi:10.1016/S0140-6736(84)91106-1
86. Pimple P, Shah A, Rooks C, Bremner JD, Nye J, Ibeanu I, et al. Association between anger and mental stress-induced myocardial ischemia. *Am Heart J* (2015) **169**(1):115.e–21.e. doi:10.1016/j.ahj.2014.07.031
87. Burg MM, Jain D, Soufer R, Kerns RD, Zaret BL. Role of behavioral and psychological factors in mental stress-induced silent left ventricular dysfunction in coronary artery disease. *J Am Coll Cardiol* (1993) **22**(2):440–8. doi:10.1016/0735-1097(93)90048-6
88. Gottdiener JS, Krantz DS, Howell RH, Hecht GM, Klein J, Falconer JJ, et al. Induction of silent myocardial ischemia with mental stress testing: relation to the triggers of ischemia during daily life activities and to ischemic functional severity. *J Am Coll Cardiol* (1994) **24**(7):1645–51. doi:10.1016/0735-1097(94)90169-4
89. Becker LC, Pepine CJ, Bonsall R, Cohen JD, Goldberg AD, Coghlan C, et al. Left ventricular, peripheral vascular, and neurohumoral responses to mental stress in normal middle-aged men and women. Reference Group for the Psychophysiological Investigations of Myocardial Ischemia (PIMI) study. *Circulation* (1996) **94**(11):2768–77. doi:10.1161/01.CIR.94.11.2768
90. Strike PC, Steptoe A. Systematic review of mental stress-induced myocardial ischaemia. *Eur Heart J* (2003) **24**(8):690–703. doi:10.1016/S0195-668X(02)00615-2
91. Yeung AC, Vekshtein VI, Krantz DS, Vita JA, Ryan TJ Jr, Ganz P, et al. The effect of atherosclerosis on the vasomotor response of coronary arteries to mental stress. *N Engl J Med* (1991) **325**(22):1551–6. doi:10.1056/NEJM199111283252205
92. Rosenbaum DS, Jackson LE, Smith JM, Garan H, Ruskin JN, Cohen RJ. Electrical alternans and vulnerability to ventricular arrhythmias. *N Engl J Med* (1994) **330**(4):235–41. doi:10.1056/NEJM199401273300402
93. Kop WJ, Krantz DS, Nearing BD, Gottdiener JS, Quigley JF, O'Callahan M, et al. Effects of acute mental stress and exercise on T-wave alternans in patients with implantable cardioverter defibrillators and controls. *Circulation* (2004) **109**(15):1864–9. doi:10.1161/01.CIR.0000124726.72615.60
94. Kovach JA, Nearing BD, Verrier RL. Angerlike behavioral state potentiates myocardial ischemia-induced T-wave alternans in canines. *J Am Coll Cardiol* (2001) **37**(6):1719–25. doi:10.1016/S0735-1097(01)01196-2
95. Taggart P, Sutton P, Chalabi Z, Boyett MR, Simon R, Elliott D, et al. Effect of adrenergic stimulation on action potential duration restitution in humans. *Circulation* (2003) **107**(2):285–9. doi:10.1161/01.CIR.0000044941.13346.74
96. Child N, Hanson B, Bishop M, Rinaldi CA, Bostock J, Western D, et al. Effect of mental challenge induced by movie clips on action potential duration in normal human subjects independent of heart rate. *Circ Arrhythm Electrophysiol* (2014) **7**(3):518–23. doi:10.1161/CIRCEP.113.000909
97. Bradley CP, Clayton RH, Nash MP, Mourad A, Hayward M, Paterson DJ, et al. Human ventricular fibrillation during global ischemia and reperfusion: paradoxical changes in activation rate and wavefront complexity. *Circ Arrhythm Electrophysiol* (2011) **4**(5):684–91. doi:10.1161/CIRCEP.110.961284
98. Kazbanov IV, Clayton RH, Nash MP, Bradley CP, Paterson DJ, Hayward MP, et al. Effect of global cardiac ischemia on human ventricular fibrillation: insights from a multi-scale mechanistic model of the human heart. *PLoS Comput Biol* (2014) **10**(11):e1003891. doi:10.1371/journal.pcbi.1003891
99. Lown B. Sudden cardiac death: the major challenge confronting contemporary cardiology. *Am J Cardiol* (1979) **43**(2):313–28. doi:10.1016/S0002-9149(79)80021-1
100. Lown B. Sudden cardiac death: biobehavioral perspective. *Circulation* (1987) **76**(1 Pt 2):I186–96.
101. Lown B, Temte JV, Reich P, Gaughan C, Regestein Q, Hal H. Basis for recurring ventricular fibrillation in the absence of coronary heart disease and its management. *N Engl J Med* (1976) **294**(12):623–9. doi:10.1056/NEJM197603182941201
102. Lown B, Verrier RL, Rabinowitz SH. Neural and psychologic mechanisms and the problem of sudden cardiac death. *Am J Cardiol* (1977) **39**(6):890–902. doi:10.1016/S0002-9149(77)80044-1
103. Verrier RL, Hagestad EL, Lown B. Delayed myocardial ischemia induced by anger. *Circulation* (1987) **75**(1):249–54. doi:10.1161/01.CIR.75.1.249
104. Skinner JE, Reed JC. Blockade of frontocortical-brain stem pathway prevents ventricular fibrillation of ischemic heart. *Am J Physiol* (1981) **240**(2):H156–63.
105. Corbalan R, Verrier R, Lown B. Psychological stress and ventricular arrhythmias during myocardial infarction in the conscious dog. *Am J Cardiol* (1974) **34**(6):692–6. doi:10.1016/0002-9149(74)90159-3
106. Lown B, Verrier R, Corbalan R. Psychologic stress and threshold for repetitive ventricular response. *Science* (1973) **182**(4114):834–6. doi:10.1126/science.182.4114.834
107. Verrier RL, Lown B. Influence of neural activity on ventricular electrical instability during acute myocardial ischemia and infarction. In: Sandoe E, Julian DG, Bell JW, editors. *Management of Ventricular Tachycardia-Role of Mexiletine*. Amsterdam: Excerpta Medica (International Congress Series No. 458) (1978). p. 133–50.
108. Verrier RL, Lown B. Behavioral stress and cardiac arrhythmias. *Annu Rev Physiol* (1984) **46**:155–76. doi:10.1146/annurev.ph.46.030184.001103
109. Verrier R, Lombardi F, Lown B, editors. Restraint of myocardial blood-flow during behavioral stress. *Circulation*. Dallas: American Heart Association (1982).
110. Adameova A, Abdellatif Y, Dhalla NS. Role of the excessive amounts of circulating catecholamines and glucocorticoids in stress-induced heart disease. *Can J Physiol Pharmacol* (2009) **87**(7):493–514. doi:10.1139/y09-042
111. Gillis R. Neurotransmitters involved in the central nervous-system control of cardiovascular function. *Dev Neurosci* (1982) **15**:41–53.
112. Segal SA, Jacob T, Gillis RA. Blockade of central nervous system GABAergic tone causes sympathetic-mediated increases in coronary vascular resistance in cats. *Circ Res* (1984) **55**(3):404–15. doi:10.1161/01.RES.55.3.404
113. Pasyk S, Walton J, Pitt B, editors. Central opioid mediated coronary and systemic vasoconstriction in the conscious dog. *Circulation*. Dallas: American Heart Association (1981).
114. Kent KM, Smith ER, Redwood DR, Epstein SE. Electrical stability of acutely ischemic myocardium. Influences of heart rate and vagal stimulation. *Circulation* (1973) **47**(2):291–8. doi:10.1161/01.CIR.47.2.291
115. DeSilva RA, Verrier RL, Lown B. The effects of psychological stress and vagal stimulation with morphine on vulnerability to ventricular fibrillation (VF) in the conscious dog. *Am Heart J* (1978) **95**(2):197–203. doi:10.1016/0002-8703(78)90463-5
116. Meerson FZ, Ustinova EE, Manukhina EB. Prevention of cardiac arrhythmias by adaptation to hypoxia: regulatory mechanisms and cardiotropic effect. *Biomed Biochim Acta* (1989) **48**(2–3):S83–8.

117. Wu S, Wong MC, Chen M, Cho CH, Wong TM. Role of opioid receptors in cardioprotection of cold-restraint stress and morphine. *J Biomed Sci* (2004) **11**(6):726–31. doi:10.1007/BF02254356
118. Havoundjian H, Paul SM, Skolnick P. Acute, stress-induced changes in the benzodiazepine/gamma-aminobutyric acid receptor complex are confined to the chloride ionophore. *J Pharmacol Exp Ther* (1986) **237**(3):787–93.
119. Liberzon I, Krstov M, Young EA. Stress-restress: effects on ACTH and fast feedback. *Psychoneuroendocrinology* (1997) **22**(6):443–53. doi:10.1016/S0306-4530(97)00044-9
120. Purdy RH, Morrow AL, Moore PH Jr, Paul SM. Stress-induced elevations of gamma-aminobutyric acid type A receptor-active steroids in the rat brain. *Proc Natl Acad Sci U S A* (1991) **88**(10):4553–7. doi:10.1073/pnas.88.10.4553
121. Schwartz RD, Wess MJ, Labarca R, Skolnick P, Paul SM. Acute stress enhances the activity of the GABA receptor-gated chloride ion channel in brain. *Brain Res* (1987) **411**(1):151–5. doi:10.1016/0006-8993(87)90692-5
122. Miao YL, Guo WZ, Shi WZ, Fang WW, Liu Y, Liu J, et al. Midazolam ameliorates the behavior deficits of a rat posttraumatic stress disorder model through dual 18 kDa translocator protein and central benzodiazepine receptor and neurosteroidogenesis. *PLoS One* (2014) **9**(7):e101450. doi:10.1371/journal.pone.0101450
123. Resstel LB, Tavares RF, Lisboa SF, Joca SR, Correa FM, Guimaraes FS. 5-HT1A receptors are involved in the cannabidiol-induced attenuation of behavioural and cardiovascular responses to acute restraint stress in rats. *Br J Pharmacol* (2009) **156**(1):181–8. doi:10.1111/j.1476-5381.2008.00046.x
124. Qian YZ, Shipley JB, Levasseur JE, Kukreja RC. Dissociation of heat shock proteins expression with ischemic tolerance by whole body hyperthermia in rat heart. *J Mol Cell Cardiol* (1998) **30**(6):1163–72. doi:10.1006/jmcc.1998.0680
125. Taylor RP, Harris MB, Starnes JW. Acute exercise can improve cardioprotection without increasing heat shock protein content. *Am J Physiol* (1999) **276**(3 Pt 2):H1098–102.
126. Hoshida S, Yamashita N, Otsu K, Hori M. Repeated physiologic stresses provide persistent cardioprotection against ischemia-reperfusion injury in rats. *J Am Coll Cardiol* (2002) **40**(4):826–31. doi:10.1016/S0735-1097(02)02001-6
127. Joyeux M, Godin-Ribuot D, Patel A, Demenge P, Yellon DM, Ribuot C. Infarct size-reducing effect of heat stress and alpha1 adrenoceptors in rats. *Br J Pharmacol* (1998) **125**(4):645–50. doi:10.1038/sj.bjp.0702137
128. Locke M, Tanguay RM, Klabunde RE, Ianuzzo CD. Enhanced postischemic myocardial recovery following exercise induction of HSP 72. *Am J Physiol* (1995) **269**(1 Pt 2):H320–5.
129. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* (1986) **74**(5):1124–36. doi:10.1161/01.CIR.74.5.1124
130. Das M, Das DK. Molecular mechanism of preconditioning. *IUBMB Life* (2008) **60**(4):199–203. doi:10.1002/iub.31
131. Wang GY, Wu S, Pei JM, Yu XC, Wong TM. Kappa- but not delta-opioid receptors mediate effects of ischemic preconditioning on both infarct and arrhythmia in rats. *Am J Physiol Heart Circ Physiol* (2001) **280**(1):H384–91.
132. Scheuer DA, Mifflin SW. Repeated intermittent stress exacerbates myocardial ischemia-reperfusion injury. *Am J Physiol* (1998) **274**(2 Pt 2):R470–5.
133. Ravingerova T, Bernatova I, Matejikova J, Ledvenyiova V, Nemcekova M, Pechanova O, et al. Impaired cardiac ischemic tolerance in spontaneously hypertensive rats is attenuated by adaptation to chronic and acute stress. *Exp Clin Cardiol* (2011) **16**(3):e23–9.
134. Ledvenyiova-Farkasova V, Bernatova I, Balis P, Puzserova A, Bartekova M, Gablovsky I, et al. Effect of crowding stress on tolerance to ischemia-reperfusion injury in young male and female hypertensive rats: molecular mechanisms. *Can J Physiol Pharmacol* (2015) **93**(9):793–802. doi:10.1139/cjpp-2015-0026
135. Rorabaugh BR, Krivenko A, Eisenmann ED, Bui AD, Seeley S, Fry ME, et al. Sex-dependent effects of chronic psychosocial stress on myocardial sensitivity to ischemic injury. *Stress* (2015) **18**(6):645–53. doi:10.3109/10253890.2015.1087505
136. Zoladz PR, Conrad CD, Fleshner M, Diamond DM. Acute episodes of predator exposure in conjunction with chronic social instability as an animal model of post-traumatic stress disorder. *Stress* (2008) **11**(4):259–81. doi:10.1080/10253890701768613
137. Dhabhar FS, Malarkey WB, Neri E, McEwen BS. Stress-induced redistribution of immune cells – from barracks to boulevards to battlefields: a tale of three hormones – Curt Richter Award winner. *Psychoneuroendocrinology* (2012) **37**(9):1345–68. doi:10.1016/j.psyneuen.2012.05.008
138. Viswanathan K, Dhabhar FS. Stress-induced enhancement of leukocyte trafficking into sites of surgery or immune activation. *Proc Natl Acad Sci U S A* (2005) **102**(16):5808–13. doi:10.1073/pnas.0501650102
139. Huang M, Pang X, Letourneau R, Boucher W, Theoharides TC. Acute stress induces cardiac mast cell activation and histamine release, effects that are increased in Apolipoprotein E knockout mice. *Cardiovasc Res* (2002) **55**(1):150–60. doi:10.1016/S0008-6363(02)00336-X
140. Meerson F, Ustinova E. Effect of long-term and short-term stress actions on the heart resistance to anoxia. *Bulleten Eksperimentalnoyi Biologii i Meditziny* (1983) **1**:21–3.
141. Kiecolt-Glaser JK, Dura JR, Speicher CE, Trask OJ, Glaser R. Spousal caregivers of dementia victims: longitudinal changes in immunity and health. *Psychosom Med* (1991) **53**(4):345–62. doi:10.1097/00006842-199107000-00001
142. Vrshek-Schallhorn S, Stroud CB, Mineka S, Hammen C, Zinbarg RE, Wolitzky-Taylor K, et al. Chronic and episodic interpersonal stress as statistically unique predictors of depression in two samples of emerging adults. *J Abnorm Psychol* (2015) **124**(4):918–32. doi:10.1037/abn0000088
143. Davey A, Sharma P, Davey S, Shukla A, Srivastava K, Vyas S. Are the adverse psychiatric outcomes reflection of occupational stress among nurses: an exploratory study. *Asian J Med Sci* (2015) **7**(1):96–100. doi:10.3126/ajms.v7i1.12869
144. Seib C, Whiteside E, Humphreys J, Lee K, Thomas P, Chopin L, et al. A longitudinal study of the impact of chronic psychological stress on health-related quality of life and clinical biomarkers: protocol for the Australian Healthy Aging of Women Study. *BMC Public Health* (2014) **14**:9. doi:10.1186/1471-2458-14-9
145. Sans S, Kesteloot H, Kromhout D. The burden of cardiovascular diseases mortality in Europe. Task Force of the European Society of Cardiology on Cardiovascular Mortality and Morbidity Statistics in Europe. *Eur Heart J* (1997) **18**(8):1231–48. doi:10.1093/oxfordjournals.eurheartj.a015434
146. Rosengren A, Subramanian SV, Islam S, Chow CK, Avezum A, Kazmi K, et al. Education and risk for acute myocardial infarction in 52 high, middle and low-income countries: INTERHEART case-control study. *Heart* (2009) **95**(24):2014–22. doi:10.1136/hrt.2009.182436
147. Ferrie JE, Kivimaki M, Shipley MJ, Davey Smith G, Virtanen M. Job insecurity and incident coronary heart disease: the Whitehall II prospective cohort study. *Atherosclerosis* (2013) **227**(1):178–81. doi:10.1016/j.atherosclerosis.2012.12.027
148. Netterstrom B, Kristensen TS, Jensen G, Schnor P. Is the demand-control model still a useful tool to assess work-related psychosocial risk for ischemic heart disease? Results from 14 year follow up in the Copenhagen City Heart study. *Int J Occup Med Environ Health* (2010) **23**(3):217–24. doi:10.2478/v10001-010-0031-6
149. Toren K, Schioler L, Giang WK, Novak M, Soderberg M, Rosengren A. A longitudinal general population-based study of job strain and risk for coronary heart disease and stroke in Swedish men. *BMJ Open* (2014) **4**(3):e004355. doi:10.1136/bmjopen-2013-004355
150. Kivimaki M, Ferrie JE, Brunner E, Head J, Shipley MJ, Vahtera J, et al. Justice at work and reduced risk of coronary heart disease among employees: the Whitehall II study. *Arch Intern Med* (2005) **165**(19):2245–51. doi:10.1001/archinte.165.19.2245
151. Kivimaki M, Jokela M, Nyberg ST, Singh-Manoux A, Fransson EI, Alfredsson L, et al. Long working hours and risk of coronary heart disease and stroke: a systematic review and meta-analysis of published and unpublished data for 603,838 individuals. *Lancet* (2015) **386**(10005):1739–46. doi:10.1016/S0140-6736(15)60295-1
152. Sparen P, Vagero D, Shestov DB, Plavinskaja S, Parfenova N, Hoptiar V, et al. Long term mortality after severe starvation during the siege of Leningrad: prospective cohort study. *BMJ* (2004) **328**(7430):11. doi:10.1136/bmj.37942.603970.9A
153. Grippo AJ, Beltz TG, Johnson AK. Behavioral and cardiovascular changes in the chronic mild stress model of depression. *Physiol Behav* (2003) **78**(4–5):703–10. doi:10.1016/S0031-9384(03)00050-7
154. Carney RM, Freedland KE. Depression, mortality, and medical morbidity in patients with coronary heart disease. *Biol Psychiatry* (2003) **54**(3):241–7. doi:10.1016/S0006-3223(03)00111-2

155. Wulsin LR, Singal BM. Do depressive symptoms increase the risk for the onset of coronary disease? A systematic quantitative review. *Psychosom Med* (2003) **65**(2):201–10. doi:10.1097/01.PSY.0000058371.50240.E3
156. Edmondson D, Cohen BE. Posttraumatic stress disorder and cardiovascular disease. *Prog Cardiovasc Dis* (2013) **55**(6):548–56. doi:10.1016/j.pcad.2013.03.004
157. Edmondson D, Kronish IM, Shaffer JA, Falzon L, Burg MM. Posttraumatic stress disorder and risk for coronary heart disease: a meta-analytic review. *Am Heart J* (2013) **166**(5):806–14. doi:10.1016/j.ahj.2013.07.031
158. Vaccarino V, Goldberg J, Rooks C, Shah AJ, Veledar E, Faber TL, et al. Post-traumatic stress disorder and incidence of coronary heart disease: a twin study. *J Am Coll Cardiol* (2013) **62**(11):970–8. doi:10.1016/j.jacc.2013.04.085
159. Kaplan JR, Manuck SB, Clarkson TB, Lusso FM, Taub DM, Miller EW. Social stress and atherosclerosis in normocholesterolemic monkeys. *Science* (1983) **220**(4598):733–5. doi:10.1126/science.6836311
160. Ratcliffe HL, Luginbuhl H, Schnarr WR, Chacko K. Coronary arteriosclerosis in swine: evidence of a relation to behavior. *J Comp Physiol Psychol* (1969) **68**(3):385–92. doi:10.1037/h0027520
161. Liang B, Verrier RL, Melman J, Lown B. Correlation between circulating catecholamine levels and ventricular vulnerability during psychological stress in conscious dogs. *Exp Biol Med* (1979) **161**(3):266–9. doi:10.3181/00379727-161-40533
162. Rosenfeld J, Rosen MR, Hoffman BF. Pharmacologic and behavioral effects on arrhythmias that immediately follow abrupt coronary occlusion: a canine model of sudden coronary death. *Am J Cardiol* (1978) **41**(6):1075–82. doi:10.1016/0002-9149(78)90860-3
163. Grippo AJ, Johnson AK. Biological mechanisms in the relationship between depression and heart disease. *Neurosci Biobehav Rev* (2002) **26**(8):941–62. doi:10.1016/S0149-7634(03)00003-4
164. Grippo AJ, Francis J, Beltz TG, Felder RB, Johnson AK. Neuroendocrine and cytokine profile of chronic mild stress-induced anhedonia. *Physiol Behav* (2005) **84**(5):697–706. doi:10.1016/j.physbeh.2005.02.011
165. Grippo AJ, Beltz TG, Weiss RM, Johnson AK. The effects of chronic fluoxetine treatment on chronic mild stress-induced cardiovascular changes and anhedonia. *Biol Psychiatry* (2006) **59**(4):309–16. doi:10.1016/j.biopsych.2005.07.010
166. Sgoifo A, Carnevali L, Grippo AJ. The socially stressed heart. Insights from studies in rodents. *Neurosci Biobehav Rev* (2014) **39**:51–60. doi:10.1016/j.neubiorev.2013.12.005
167. Wood SK. Cardiac autonomic imbalance by social stress in rodents: understanding putative biomarkers. *Front Psychol* (2014) **5**:950. doi:10.3389/fpsyg.2014.00950
168. Mancardi D, Tullio F, Crisafulli A, Rastaldo R, Folino A, Penna C, et al. Omega 3 has a beneficial effect on ischemia/reperfusion injury, but cannot reverse the effect of stressful forced exercise. *Nutr Metab Cardiovasc Dis* (2009) **19**(1):20–6. doi:10.1016/j.numecd.2008.01.004
169. Kolar F, Jezkova J, Balkova P, Breh J, Neckar J, Novak F, et al. Role of oxidative stress in PKC-delta upregulation and cardioprotection induced by chronic intermittent hypoxia. *Am J Physiol Heart Circ Physiol* (2007) **292**(1):H224–30. doi:10.1152/ajpheart.00689.2006
170. Nalivaiko E. Animal models of psychogenic cardiovascular disorders: what we can learn from them and what we cannot. *Clin Exp Pharmacol Physiol* (2011) **38**(2):115–25. doi:10.1111/j.1440-1681.2010.05465.x
171. Jia M, Smerin SE, Zhang L, Xing G, Li X, Benedek D, et al. Corticosterone mitigates the stress response in an animal model of PTSD. *J Psychiatr Res* (2015) **60**:29–39. doi:10.1016/j.jpsychires.2014.09.020
172. Chourbaji S, Zacher C, Sanchis-Segura C, Dormann C, Vollmayr B, Gass P. Learned helplessness: validity and reliability of depressive-like states in mice. *Brain Res Brain Res Protoc* (2005) **16**(1–3):70–8. doi:10.1016/j.brainresprot.2005.09.002
173. Li B, Yang CJ, Yue N, Liu Y, Yu J, Wang YQ, et al. Clomipramine reverses hypoalgesia/hypoesthesia and improved depressive-like behaviors induced by inescapable shock in rats. *Neurosci Lett* (2013) **541**:227–32. doi:10.1016/j.neulet.2013.01.055
174. Armario A, Ortiz R, Balasch J. Effect of crowding on some physiological and behavioral variables in adult male rats. *Physiol Behav* (1984) **32**(1):35–7. doi:10.1016/0031-9384(84)90066-0
175. Bugajski J. Social stress adapts signaling pathways involved in stimulation of the hypothalamic-pituitary-adrenal axis. *J Physiol Pharmacol* (1999) **50**(3):367–79.
176. Bugajski J, Borycz J, Glod R, Bugajski AJ. Crowding stress impairs the pituitary-adrenocortical responsiveness to the vasopressin but not corticotropin-releasing hormone stimulation. *Brain Res* (1995) **681**(1–2):223–8. doi:10.1016/0006-8993(95)00297-4
177. Sgoifo A, Koolhaas J, De Boer S, Musso E, Stilli D, Buwalda B, et al. Social stress, autonomic neural activation, and cardiac activity in rats. *Neurosci Biobehav Rev* (1999) **23**(7):915–23. doi:10.1016/S0149-7634(99)00025-1
178. Vicario M, Alonso C, Guilarte M, Serra J, Martinez C, Gonzalez-Castro AM, et al. Chronic psychosocial stress induces reversible mitochondrial damage and corticotropin-releasing factor receptor type-1 upregulation in the rat intestine and IBS-like gut dysfunction. *Psychoneuroendocrinology* (2012) **37**(1):65–77. doi:10.1016/j.psyneuen.2011.05.005
179. Zoladz PR, Fleshner M, Diamond DM. Psychosocial animal model of PTSD produces a long-lasting traumatic memory, an increase in general anxiety and PTSD-like glucocorticoid abnormalities. *Psychoneuroendocrinology* (2012) **37**(9):1531–45. doi:10.1016/j.psyneuen.2012.02.007
180. Zoladz PR, Fleshner M, Diamond DM. Differential effectiveness of tianeptine, clonidine and amitriptyline in blocking traumatic memory expression, anxiety and hypertension in an animal model of PTSD. *Prog Neuropsychopharmacol Biol Psychiatry* (2013) **44**:1–16. doi:10.1016/j.pnpbp.2013.01.001
181. Zoladz PR, Park CR, Fleshner M, Diamond DM. Psychosocial predator-based animal model of PTSD produces physiological and behavioral sequelae and a traumatic memory four months following stress onset. *Physiol Behav* (2015) **147**:183–92. doi:10.1016/j.physbeh.2015.04.032
182. Wilson CB, McLaughlin LD, Nair A, Ebenezer PJ, Dange R, Francis J. Inflammation and oxidative stress are elevated in the brain, blood, and adrenal glands during the progression of post-traumatic stress disorder in a predator exposure animal model. *PLoS One* (2013) **8**(10):e76146. doi:10.1371/journal.pone.0076146
183. Wilson CB, Ebenezer PJ, McLaughlin LD, Francis J. Predator exposure/psychosocial stress animal model of post-traumatic stress disorder modulates neurotransmitters in the rat hippocampus and prefrontal cortex. *PLoS One* (2014) **9**(2):e89104. doi:10.1371/journal.pone.0089104
184. Rorabaugh BR, Ross SA, Gaivin RJ, Papay RS, McCune DF, Simpson PC, et al. Alpha1a- but not alpha1B-adrenergic receptors precondition the ischemic heart by a staurosporine-sensitive, chelerythrine-insensitive mechanism. *Cardiovasc Res* (2005) **65**(2):436–45. doi:10.1016/j.cardiores.2004.10.009
185. Banerjee A, Locke-Winter C, Rogers KB, Mitchell MB, Brew EC, Cairns CB, et al. Preconditioning against myocardial dysfunction after ischemia and reperfusion by an alpha 1-adrenergic mechanism. *Circ Res* (1993) **73**(4):656–70. doi:10.1161/01.RES.73.4.656
186. Davel AP, Kawamoto EM, Scavone C, Vassallo DV, Rossoni LV. Changes in vascular reactivity following administration of isoproterenol for 1 week: a role for endothelial modulation. *Br J Pharmacol* (2006) **148**(5):629–39. doi:10.1038/sj.bjp.0706749
187. Kim HK, Park WS, Warda M, Park SY, Ko EA, Kim MH, et al. Beta adrenergic overstimulation impaired vascular contractility via actin-cytoskeleton disorganization in rabbit cerebral artery. *PLoS One* (2012) **7**(8):e43884. doi:10.1371/journal.pone.0043884
188. Pye MP, Cobbe SM. Mechanisms of ventricular arrhythmias in cardiac failure and hypertrophy. *Cardiovasc Res* (1992) **26**(8):740–50. doi:10.1093/cvr/26.8.740
189. Lochner A, Genade S, Tromp E, Podzuweit T, Moolman JA. Ischemic preconditioning and the beta-adrenergic signal transduction pathway. *Circulation* (1999) **100**(9):958–66. doi:10.1161/01.CIR.100.9.958
190. Suematsu Y, Anttila V, Takamoto S, del Nido P. Cardioprotection afforded by ischemic preconditioning interferes with chronic beta-blocker treatment. *Scand Cardiovasc J* (2004) **38**(5):293–9. doi:10.1080/14017430410021507

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REVIEW
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Functional and structural remodeling of glutamate synapses in prefrontal and frontal cortex induced by behavioral stress

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Increasing evidence has shown that the pathophysiology of neuropsychiatric disorders, including mood disorders, is associated with abnormal function and regulation of the glutamatergic system. Consistently, preclinical studies on stress-based animal models of pathology showed that glucocorticoids and stress exert crucial effects on neuronal excitability and function, especially in cortical and limbic areas. In prefrontal and frontal cortex, acute stress was shown to induce enhancement of glutamate release/transmission dependent on activation of corticosterone receptors. Although the mechanisms whereby stress affects glutamate transmission have not yet been fully understood, it was shown that synaptic, non-genomic action of corticosterone is required to increase the readily releasable pool of glutamate vesicles, but is not sufficient to enhance transmission in prefrontal and frontal cortex. Slower, partly genomic mechanisms are probably necessary for the enhancement of glutamate transmission induced by stress. Combined evidence has suggested that the changes in glutamate release and transmission are responsible for the dendritic remodeling and morphological changes induced by stress and it has been argued that sustained alterations of glutamate transmission may play a key role in the long-term structural/functional changes associated with mood disorders in patients. Intriguingly, modifications of the glutamatergic system induced by stress in the prefrontal cortex seem to be biphasic. Indeed, while the fast response to stress suggests an enhancement in the number of excitatory synapses, synaptic transmission and working memory, long-term adaptive changes – including those consequent to chronic stress – induce opposite effects. Better knowledge of the cellular effectors involved in this biphasic effect of stress may be useful to understand the pathophysiology of stress-related disorders, and open new paths for the development of therapeutic approaches.

Keywords: mood disorder, glutamate transmission, prefrontal cortex, behavioral stress, neuronal remodeling, working memory

Introduction

Starting from the evidence of the antidepressant properties of drugs increasing the availability of monoamines, the pathophysiology of mood and anxiety disorders has been linked for many

years to alterations in monoaminergic transmission (1, 2). However, the monoaminergic hypothesis of depression is simplistic and does not explain the delayed pharmacological effect of antidepressants and the high number of non-responding patients. The more recent and well-accepted "neuroplasticity hypothesis" of depression claims that concomitant changes in intracellular signaling, neurotrophic mechanisms, neurogenesis, synaptic function and plasticity, and remodeling of neuronal cells/circuitry are involved in pathophysiology and treatment of mood disorders (3–5). These "neuroplastic" changes are hypothesized to lead to disruption of homeostatic mechanisms, resulting in destabilization and loss of synaptic connections in emotional/cognitive circuitry. The hypothesis is supported by brain-imaging studies in patients with mood and anxiety disorders, showing consistent evidence of volume and connectivity reductions in cortical and limbic brain regions, such as prefrontal cortex (PFC), hippocampus, and amygdala (6–8). Interestingly, since most of the connections between and within these brain areas are glutamatergic, the maladaptive morphological changes described in the brain of depressed subjects are accompanied by alterations in glutamate levels, metabolism, and receptors, suggesting that the dysregulation of glutamate neurotransmission plays an important role in the pathophysiology of neuropsychiatric diseases.

In the present review, we focus on the functional alterations in the glutamate system and in dendritic reorganization and neuronal connectivity in the PFC [a brain region critical for working memory, executive function, and extinction of learning, Ref. (9)], reported in patients with mood disorders and induced by stress in preclinical models. As discussed in the following sections, while acute stress was shown to enhance rapidly the function of the PFC at both cellular and behavioral levels, repeated exposure to stress, as well as long-term effects of some types of acute stressors, bring about atrophy and retraction of dendrites, loss of synapses, reduction of synaptic transmission, and consequent behavioral impairments. Understanding the mechanisms and the molecular effectors involved in this biphasic action of stress is essential to the development of new diagnostic and therapeutic strategies for stress-related neuropsychiatric disorders.

Clinical Evidence of Glutamatergic and Morphological Dysfunctions in Cortical Areas of Patients with Mood Disorders

Glutamatergic Alterations in Brain of Depressed Patients

Evidence collected from clinical studies showed alterations in the levels of glutamate and of its metabolites in plasma, cerebrospinal fluid, and selected brain areas of patients affected by mood and anxiety disorders.

In particular, increased glutamate levels were measured both in the plasma of depressed patients compared with healthy controls (10) and in post-mortem frontal cortex (FC) and dorsolateral PFC from depressed and bipolar patients, respectively (11). Moreover, region-specific abnormalities in mRNA and protein expression levels of ionotropic glutamate receptor subunits and of associated postsynaptic density proteins were measured in post-mortem studies, suggesting compromised glutamate-mediated synaptic neurotransmission in brain areas from depressed individuals (12, 13).

In vivo proton magnetic resonance spectroscopy (1H-MRS) was used to measure glutamate-related metabolites in the brain of depressed patients. Despite some inconsistencies, a large number of studies provided evidence for reduction in Glx levels, a composite measure of glutamate and glutamine, with a minor contribution from GABA and other metabolites, in FC and cingulate regions of depressed subjects in the midst of a current depressive episode, and in non-responders patients (14–23). In contrast, glutamate metabolite measures in the occipital and parietal/occipital regions have been found to be elevated in medication-free major depression patients (4, 19, 24). Apart from technical limitations of 1H-MRS studies, these findings strongly suggest that abnormalities in glutamate/glutamine/GABA cycling are involved in the pathophysiology of mood and anxiety disorders.

Volumetric and Morphological Changes in the Cortex of Depressed Patients

A large number of clinical neuroimaging studies of depressed patients have consistently shown regional volumetric changes in brain areas where glutamate neurons and synapses predominate (6–8, 25). In particular, significant volumetric reduction has been found for cortical areas, where reduced gray matter volume in the anterior cingulate cortex, PFC, and lateral and medial orbitofrontal cortices were reported (26–28). Interestingly, reduced volume of the caudal anterior cingulate cortex and altered white matter integrity in the body of the corpus callosum were also recently reported in untreated patients with first episode of major depression (29).

In a separate study, decreased PFC gray matter volume and density were measured in depressed patients compared to both healthy controls subjects and remitted major depression disorder subjects (30). Interestingly, morphometric studies on PFC of depressed subjects showed smaller size of neuronal bodies and reduced neuronal and glial densities, thus suggesting that volumetric changes are at least in part dependent on neuronal abnormalities (25, 31–33). In line with this hypothesis, decreased expression of synapse-related genes and loss of synapses have been recently described in PFC of patients with mood disorders, confirming that synaptic dysfunction contributes to the volumetric changes observed in depressed patients (34).

Indeed, although the reasons for morphological changes in brain areas of depressed subjects have not yet been fully understood, it has been proposed that atrophy and remodeling of dendrites and reduction of synapses are major factors (4–6, 35). In particular, since the brain areas where volumetric changes were reported are prevalently glutamatergic (6–8, 25), it is conceivable that glutamate synapses are particularly affected in the pathology. The evidence for this hypothesis mainly comes from preclinical stress-based models of mood and anxiety disorders. Indeed, the morphological changes induced in brain areas of chronically stressed animals were found to be accompanied by dysfunctional glutamatergic synaptic plasticity and transmission (see below).

Glutamatergic and Morphological Dysfunctions in Cortical Areas of Preclinical Stress-Based Models of Mood and Anxiety Disorders

Since behavioral stress is recognized as a major predisposing and triggering factor for mood and anxiety disorders in humans, the high majority of rodent models of depression are based on the exposure to standardized acute and chronic stress protocols (36–38). In this section, we report the structural/functional changes induced by several protocols of chronic and acute stress in PFC glutamate synapses and circuitry.

Morphological Changes and PFC-Dependent Cognitive Impairment Induced by Chronic Stress

It has been widely documented that, after prolonged stress, pyramidal neurons in medial PFC undergo dendritic atrophy, reduction of synapses number and volumetric reductions, resembling those observed in patients with mood and anxiety disorders (**Table 1**). In particular, different protocols of chronic stress were

TABLE 1 | Effect of chronic stress on neuronal remodeling in the prefrontal cortex: animal studies.

Stress	Morphological changes	Reference
Restraint stress (21 days)	Reduction in the number and length of apical dendritic branches in distal and higher-order branches in layer II/III pyramidal neurons	(39)
Social isolation (8 weeks)	Reductions in dendritic spine density in layer III pyramidal neurons	(40)
Restraint stress (21 days)	Reduction in the total length and branch numbers of apical dendrites in layer II/III pyramidal neurons of infralimbic and prelimbic cortices	(41)
Restraint stress (7 days)	Atrophy of distal branches and sparing of proximal branches in layer II–III pyramidal neurons	(42)
Restraint stress (3 and 6 weeks)	Reduction in total apical dendritic length in layer II/III pyramidal neurons	(43)
Forced swim (3 days)	Retraction of terminal branches of apical, but not basilar, dendrites in infralimbic cortex pyramidal neurons	(44)
Restraint stress (21 days)	Retraction of apical dendritic arbors in in layer II/III pyramidal neurons	(45)
Chronic noise stress (30 days)	Reduction in the number of apical dendrites in layer II/III pyramidal neurons	(46)
Restraint stress (21 days)	Reduction in the total length and branch numbers of apical dendrites and of axospinous synapses number in layer II/III pyramidal neurons of prelimbic cortices	(47)
Prenatal stress (7 days) followed by chronic mild stress (3 weeks)	Reduction in spine densities, particularly on spines of the mushroom type in medial PFC	(48)
Restraint stress (14 days)	Reduction in the total length of apical dendrites in prelimbic cortex pyramidal neurons	(49, 50)
Restraint stress (21 days)	Decrease in dendritic spine volume and surface area, mainly in the distal portion of apical dendritic fields; reduction in large spines and increase in small spines	(51)
Chronic unpredictable stress (21 days)	Volumetric and dendritic atrophy in layer II/III pyramidal neurons of infralimbic and prelimbic cortices	(52)
Restraint stress (7 days)	Reduction in the number and length of apical dendritic branches in layer II/III pyramidal neurons of infralimbic and prelimbic cortices (selectively in male and not female mice)	(53)
Restraint stress (21 days)	Reduction in apical dendritic length and in apical dendritic branch intersections in layer II/III pyramidal neurons	(54)
Restraint stress (10 days)	Retraction of apical dendrites in infralimbic cortex pyramidal neurons	(55)
Restraint stress (21 days)	Reduction in apical dendritic length and in apical dendritic branch intersections in layer II/III pyramidal neurons of prelimbic cortex	(56)
Restraint stress (7 days)	Retraction of apical dendrites in layer II/III pyramidal neurons	(57)
Restraint stress (21 days)	Reduction in the number and length of apical dendritic branches in prelimbic cortex pyramidal neurons	(58)
Prenatal stress (7 days)	Reduction in dendritic complexity in prelimbic cortex pyramidal neurons	(59)
Isolation (8–9 weeks)	Reduction in dendritic complexity, spine density, and elongated terminal branches in layer II/III pyramidal neurons	(60)
Early life stress (maternal separation, 14 days)	Atrophy of basal dendritic tree and reduced spine density on both apical and basal dendrites in layer II/III pyramidal neurons	(61)
Prenatal stress (7 days)	Decrease in the apical dendritic length of pyramidal neurons in the orbitofrontal cortex at postnatal day 14	(62)
Prenatal stress (7 days)	Apical dendrite arbor simplification in layer III pyramidal neurons	(63)
Restraint stress (3 or 7 days)	Atrophy of distal apical dendritic in layer V pyramidal neurons	(64)
Restraint stress (21 days)	Atrophy of apical dendritic tree and reduced spine density in layer V pyramidal neurons of infralimbic cortex	(65)
Chronic unpredictable stress (21 days)	Reduction in spine density in both distal and proximal dendrites in layer V pyramidal neurons	(66)

reported to induce dendritic remodeling of pyramidal neurons in layers II/III, where reductions in total apical dendritic length, arborization, and spine density were consistently reported (39–63). Recent studies also demonstrated a significant retraction after chronic stress of layer V pyramidal neurons apical dendrites within distal cortical layers (64–66).

Interestingly, a number of convergent studies reported that the reduction of spine densities, apical dendritic length, and branch points in medial PFC layer II/III pyramidal neurons, induced by chronic stress in prenatal, early life or adult life, is accompanied by behavioral impairments in tests for emotional/cognitive behavior (52, 56, 58, 60–62, 66). Chronic restraint stress (21 days) was shown to selectively impair attentional set-shifting task (a function mediated by medial PFC), together with retraction of apical dendritic arbors in anterior cingulate cortex and extension in lateral orbitofrontal cortex (45), and to worsen working memory in the spatial delayed alternation T-maze task, in concomitance with atrophy of apical dendrites in pyramidal neurons from layer II/III of prelimbic cortex (54). It was also shown that the retraction of apical dendrites of pyramidal neurons induced in PFC by repeated stress is accompanied by alterations in fear conditioning and extinction (44).

Together, all these lines of evidence are clearly in favor of a correlation between chronic stress, dendritic remodeling, and impaired PFC-dependent cognitive performance.

Although the mechanisms underlying the effects of chronic stress on PFC are far from being fully elucidated, it was consistently reported that chronic systemic injections of the stress hormone corticosterone are able to reproduce, at least in part, the structural and functional changes induced by stress in this brain area. Chronic high-dose systemic injections of corticosterone were shown to cause significant reduction of spines in PFC pyramidal neurons of layer V and deficits in memory retention (67), and to induce a significant redistribution of apical dendrites in layers II/III, with an increase in the number of proximal dendrites and a reduction of distal dendrites (68). Repeated corticosterone injections within infralimbic and prelimbic medial PFC were also found to impair working memory and to improve memory consolidation, through a glucocorticoid receptor-dependent mechanism (69). On the other hand, repeated corticotropin-releasing factor infusion directly into the medial PFC increased general anxiety, but did not affect cue-conditioned fear 10 days post infusion (70). Moreover, some studies also reported an involvement in dendritic remodeling of a number of intracellular signaling mediators and receptors, including protein kinase C (54), glucocorticoid receptor (71), NMDA receptors (57), AMPA receptors, postsynaptic density protein 95, α calcium/calmodulin-dependent protein kinase II (61), estrogen (63, 72), glutamate decarboxylase enzyme 64, NCAM, synaptophysin and GABA(A) α 1 (73), catecholamines (64, 74), and cannabinoid CB1 receptor (56).

Alterations in Synaptic Transmission and Related Molecular Mechanisms Induced by Chronic Stress in the Prefrontal Cortex

Together with morphological and behavioral changes, a number of studies analyzed the effects of chronic stress on synaptic function, i.e., presynaptic glutamate release and function/membrane insertion of postsynaptic glutamate receptors.

An early paper correlated electrophysiological and morphological changes in medial PFC layer V pyramidal neurons, using a combination of whole-cell recording and two-photon imaging in rat medial PFC slices (64). The authors clearly showed that repeated mild restraint stress, together with a decrease in distal apical dendritic branch length and spine density, induced deficits in apically targeted excitatory postsynaptic currents (EPSCs), involving corticosterone-dependent mechanisms. Similarly, the reduction of large mushroom spines of layer V pyramidal neurons measured after chronic unpredictable stress was found to be accompanied by a reduction in the amplitude and frequency of serotonin- and hypocretin-induced EPSCs (66). More recently, a reduction of both AMPA and NMDA receptor-dependent synaptic responses and spontaneous action potential firing in pyramidal PFC cells were reported after 5–7 days of restraint or unpredictable stress in young rats, in association with ubiquitin/proteasome-mediated degradation of selective glutamate receptor subunits (74, 75).

Moreover, sex differences were shown in PFC excitatory transmission, glutamate receptor surface expression, and behavioral response after repeated restraint stress (72). Indeed, chronic stress-induced memory impairment together with decreased AMPA and NMDA receptors surface expression, receptor-dependent EPSCs, and miniature EPSCs, in medial PFC of male rats selectively, with no effect in female rats. Looking for potential mechanisms underlying the contrasting effects of repeated stress on PFC functions in female vs. male animals, the authors demonstrated that estrogen both protects against the detrimental effects of repeated stress in females, and prevents the stress-induced impairments when administered to males. This suggests that the stress hormone corticosterone and estrogen interact in the modulation of excitatory synaptic transmission in PFC neurons, leading to a fine tuning of functional plasticity within this brain area. In this context, it is interesting to notice that local brain synthesis of estrogen from endogenous cholesterol, through the action of neuronal aromatases, could play a role in the modulation of neurotransmission in response to repeated stress. Indeed, it was shown that the inhibition of aromatase in female rats resulted in the loss of protection against neural and behavioral consequences of chronic stress, thus suggesting that central estrogen production is necessary for the protective action of estrogen (72).

In addition to neuronal structure, chronic stress was also reported to induce impairments in synaptic plasticity (i.e., long-term potentiation, LTP) in medial PFC (61, 65). The decrease of spine density in both apical and basal dendrites and the atrophy of the basal dendritic tree in medial PFC layer II/III pyramidal neurons, induced by chronic early life stress (maternal separation) in young rats, were found to be accompanied by attenuation of LTP and changes in the expression of proteins involved in LTP, including AMPA receptor subunits (61). Moreover, in a different study, chronic restraint stress was found to inhibit D1 receptor-dependent LTP, while post-stress recovery fully reversed the impairments in catecholaminergic-mediated synaptic plasticity, suggesting that recovery may be related with circuitry reestablishment (65).

These studies strongly suggest that the structural remodeling induced by chronic stress in PFC is accompanied by dysfunctions in neurotransmission and plasticity.

Regarding the effects of chronic stress on glutamate levels and presynaptic release, less information is available. Early evidence was provided by microdialysis studies, which found selective changes in the adaptation of extracellular glutamate in hippocampus and PFC after application of a few consecutive stressors (76). In a more recent study, it was found that glutamate release induced by BDNF in slices of the PFC was attenuated in rats subjected to chronic restraint stress, coupled with anxious/depressive phenotype and down-regulation of glucocorticoid receptors (71). Moreover, reduction in the levels of glutamate, glutamine, *N*-acetyl aspartate, and taurine was reported in the PFC of social defeated mice (77).

Together, these studies strongly suggest that dendritic atrophy and volumetric reduction induced by chronic stress in PFC are related with changes in glutamate transmission and plasticity and, ultimately, may induce severe behavioral deficits.

Acute Stress Increases Glutamate Transmission and Release in Prefrontal Cortex

Although the effects of chronic stress on glutamate release and transmission remain largely unknown, compelling preclinical studies have clearly shown that acute stress and glucocorticoids can deeply affect glutamatergic neurotransmission in the PFC, inducing changes in glutamate release, glutamate receptors, and glutamate clearance and metabolism [as a review, see Ref. (78, 79)].

A study measuring changes in glutamate release in PFC, by using enzyme-based microelectrode arrays coupled to amperometric recording techniques, showed significant transient increase of extracellular glutamate levels during tail pinch stress, which was completely blocked by local application of tetrodotoxin, thus suggesting increased exocytotic release of glutamate (80).

In rat PFC and FC, we have shown that acute stress rapidly enhances glutamate release and transmission, an effect mediated by corticosterone receptors. We applied one single 40 min session of inescapable footshock (FS)-stress to rats, and then purified synaptic terminals (synaptosomes) from PFC and FC with Percoll gradients (81). Basal and depolarization-evoked glutamate release was measured by using the technique of isolated synaptosomes in superfusion, a method allowing to measure the exocytotic release of neurotransmitters, preventing or limiting reuptake by neurotransmitter transporters, or synaptic receptors activation (78, 82). Acute FS-stress-induced rapid enhancement of depolarization-evoked glutamate (not GABA) overflow in PFC and FC, by increasing corticosterone levels, stimulation of corticosterone receptors, and rapid accumulation of presynaptic SNARE protein complexes, which mediate vesicle fusion (81). Enhancement of glutamate release was confirmed by reduction of paired-pulse facilitation and its calcium-dependence in PFC of stressed rats. It was also shown that chronic antidepressants prevent the enhancement of glutamate release, with a mechanism downstream of corticosterone rise.

More recently, the synaptic effects of acute stress and corticosterone in PFC and FC were dissected, showing that the increase of corticosterone induced by FS-stress is responsible for a rapid (non-genomic) enhancement of trafficking of glutamate synaptic vesicles into the so-called readily releasable pool (RRP), through the activation of synaptic glucocorticoid/mineralocorticoid receptors (83). Indeed, both acute stress and brief *in vitro* application of corticosterone to purified PFC and FC synaptosomes were shown to increase the RRP size of glutamate vesicles (assessed in synaptosomes superfused with hypertonic sucrose). In line with this evidence, FS-stress increased the number of synaptic vesicles docked onto presynaptic membranes of excitatory perforated synapses, measured with electron microscopy stereology in medial PFC (83, 84). Similarly, by using total internal reflection fluorescence microscopy, we showed that application *in vitro* of corticosterone to synaptosomes for up to 10 min rapidly increased trafficking of FM1-43 labeled synaptic vesicles toward the presynaptic membrane. The increase of vesicle mobilization and RRP induced by both acute stress and *in vitro* corticosterone application was demonstrated to be dependent on the activation of synaptic corticosterone receptors and downstream increase of site 1 (Ser9) synapsin I phosphorylation in presynaptic membranes (83). Indeed, despite the molecular mechanism involved in corticosterone-induced increase of vesicle mobilization is far to be fully elucidated, the phosphorylation of synaptic membrane-located synapsin I at site 1 was found to be necessary for the enhancement of RRP.

However, while corticosterone rapidly primes synapses for enhanced release, it does not likewise rapidly enhance glutamate release and transmission. Indeed, acute application of corticosterone failed to reproduce the stress-induced increase of depolarization-evoked release of glutamate in purified PFC and FC synaptosomes, and to induce any change in intracellular EPSCs amplitude or paired-pulse ratio in acute medial PFC slices (83, 85), suggesting that the synaptic non-genomic action of corticosterone is necessary but not sufficient to enhance glutamate release and transmission [see Ref. (86); **Figure 1**]. In line with this hypothesis, it was consistently reported that both acute stress and corticosterone induce delayed and long-lasting potentiation of glutamate transmission in the PFC (see below).

The Increase of Glutamate Transmission and Release in Prefrontal Cortex is Mediated by Slower, Likely Genomic, Corticosterone Effect

Acute stressors of diverse types, including acute forced swim, acute restraint, and elevated-platform stress, as well as acute corticosterone treatment of rats, were shown to induce in PFC pyramidal neurons a significant long-lasting potentiation, starting 1 h after stress and sustained for up to 24 h after cessation of stress, of glutamatergic transmission and an increase of surface NMDA and AMPA receptor subunits level, through the activation of glucocorticoid receptors (87). This was the first evidence showing that corticosterone is necessary and sufficient for increasing glutamate transmission in PFC, and also suggested that the potentiation of glutamate transmission in this brain area involves delayed mechanisms (no significant effect measured until 1 h after corticosterone elevation). Similarly, acute *in vitro* treatment of both PFC neuronal cultures and acute PFC slices was shown to induce synaptic potentiation at least 1 h and up

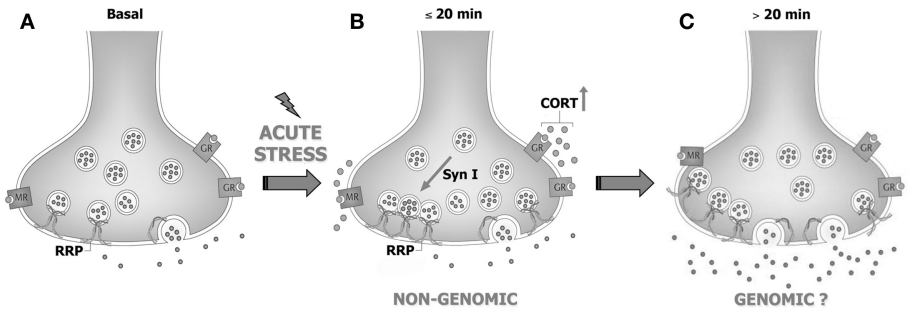

FIGURE 1 | Acute stress and corticosterone rapidly increase the readily releasable pool of glutamate vesicles in prefrontal and frontal cortex synaptic terminals through non-genomic mechanisms. **(A)** PFC glutamate synaptic terminal in basal conditions, showing the readily releasable pool (RRP) of vesicles anchored to the membrane and ready for release and membrane-located mineralocorticoid and glucocorticoid receptors (MR and GR, respectively). **(B)** The rise of corticosterone (CORT) induced by acute stress causes a rapid increase of vesicle trafficking into the RRP. This requires binding of corticosterone to synaptic MR/GR and non-genomic mechanisms involving phosphorylation of the presynaptic protein synapsin I. The increase of RRP size non-genomically induced by corticosterone primes the terminal but is not sufficient for the enhancement of glutamate release and transmission induced by acute stress. **(C)** We speculate that slower (>20 min), likely genomic effects are required to promote the enhancement of glutamate release and transmission induced by acute stress. See text for details. Adapted from Treccani et al. (86).

to 24 h after corticosterone application (88, 89). These studies also showed that the long-lasting potentiation of glutamatergic transmission in PFC pyramidal neurons is likely caused by the increase in the delivery of NMDA and AMPA receptors to the synaptic membrane, in turn dependent on activation of glucocorticoid receptors and glucocorticoid-inducible kinase/Rab4 signaling (88).

Although these studies suggest that the enhancement of glutamate release and transmission induced by acute stress in PFC is dependent on the transcriptional activation of immediate early genes, other studies also suggested non-genomic, synaptic effects of corticosterone. Indeed, it was shown that both acute stress and incubation of medial PFC slices with corticosterone induces rapid changes in neurotransmission, dependent on local synthesis of endocannabinoids, thus suggesting that some of the short-term effects of corticosterone may be partly mediated by the local release of other mediators (56). In particular, the activation of endocannabinoid signaling induced by corticosterone, inhibiting GABA release onto layer V pyramidal neurons in the prelimbic cortex, contributes to the long-negative feedback loop to inhibit corticosterone secretion following cessation of stress.

Behavioral and Morphological Changes Induced by Acute Stress

Although chronic stress has been widely shown to induce profound structural remodeling of medial PFC and related behavioral alterations (see Morphological Changes and PFC-Dependent Cognitive Impairment Induced by Chronic Stress), less is known about the effects of acute stress on PFC synaptic plasticity and memory. Neurons in cortical and limbic areas are highly plastic and undergo rapid activity-dependent morphological transformations, including modulation of neuronal excitability and connectivity (90, 91). However, only a few recent studies focused on the changes in structural remodeling and in PFC-dependent behavioral performance induced by a single stressful event. Interestingly, as described in detail below, the changes in synaptic plasticity and memory seem to be time-dependent and biphasic, inducing a general enhancement of transmission, plasticity, and performance in the first hours after stress, followed by negative effects on learning, memory, and their neural underpinnings, in the subsequent hours and days.

In a recent study, we have shown that both acute FS-stress and restraint stress induce marked effects on synaptic plasticity, increasing the total number of asymmetric (i.e., excitatory) non-perforated synapses in pyramidal neurons of prelimbic PFC layers II/III (84). FS-stress, a stress protocol inducing significantly higher levels of corticosterone compared with restraint stress, also increases the number of axo-shaft synapses, directly located on dendritic shafts. Interestingly, these changes were partially blocked by chronic antidepressant pretreatment, as previously show for glutamate release and transmission (81), thus providing a parallel between the modulation of excitatory transmission induced by antidepressants and changes in structural remodeling. Moreover, our findings provide a first evidence that activity-dependent synaptogenesis of small synapses can occur *ex novo* in PFC, as early as 40 min after a severe stressful event, confirming the remarkable dynamics of synapse structure in response to stressful events in this brain area.

In line with these data, the plasticity enhancing effect induced by acute forced swim stress and elevated-platform stress in PFC glutamate transmission was associated with enhanced working memory in the T-maze delayed alternation task when examined 4 h or 1 day after stress, but not 2 days after stress (87). The enhancement of working memory, as for the increase in glutamate transmission, was shown to be dependent on glucocorticoid receptors and on the activation of glucocorticoid receptor and glucocorticoid-inducible kinase dependent mechanisms (88).

Finally, in a separate study, acute forced swim stress was found to induce a significant retraction of apical (not basal) branches, measured 3 days after stress (44). However, the remodeling of dendritic arbor was found to be selectively located in infralimbic, but not prelimbic PFC, suggesting that pyramidal neurons within infralimbic PFC are highly sensitive to forced swim stress.

Conclusion

Compelling evidence strongly suggests that long-term changes in brain areas and circuits, mediating complex cognitive and emotional behaviors, represent the biological underpinnings of mood and anxiety disorders. Stressful life events deeply affect brain function and, especially when stress exposure is intense, chronic, uncontrollable, or overwhelming, it represents a major risk factor for many diseases, including neuropsychiatric disorders.

The stress response is a complex physiological process involving hormonal, neurochemical, and metabolic mechanisms. Indeed, although PFC was consistently reported to be a brain area particularly sensitive to stress, it is important to notice that stress induces changes in connectivity and plasticity, not only within PFC but also in other areas, such as hippocampus and amygdala, and between different areas [as a review, see Ref. (92)]. Intriguingly, the effects of stress seem to be region-specific. Indeed, as for PFC, in hippocampus acute stress was shown to deeply affect glutamatergic transmission in preclinical models. However, differently from PFC, in hippocampus the activation of synaptic corticosterone receptors, and particularly mineralocorticoid receptor, was found to be sufficient to induce rapid enhancement of glutamate release and synaptic transmission, through completely non-genomic mechanisms (93, 94). On the other hand, opposite effects were noticed in amygdala, where the enhancement of glutamate transmission induced by stress in rodents is accompanied by increased dendritic complexity (95, 96). Moreover, PFC, hippocampus, and amygdala also critically participate in orchestrating the hypothalamic–pituitary–adrenal (HPA) axis response to stress, thus modulating the physiological stress response (97). Understanding how these stress-related networks operate could be helpful in uncovering pathways mediating pathological stress-related conditions.

As shown above, although the evidence is far from conclusive, the acute and delayed (e.g., after repeated or chronic stress) outcome on structural and functional features of the glutamate system could be different and often opposite, at least in the PFC. On one side, stressful events rapidly enhance glutamate release and excitatory transmission and may facilitate plasticity and PFC-dependent behavior, while chronic stress and long-term effects of some acute stressors induce a reduction of excitatory transmission, atrophy/remodeling of dendrites and loss of synapses, accompanied by behavioral impairment. Therefore, the structural and functional changes in excitatory circuitry may follow a biphasic process, during which, at some unknown points, the stress response turns from increased excitatory activation into its opposite [**Figure 2**; see Ref. (78, 98, 99) for a discussion]. Thus, upon severe acute stressful stimulation, HPA axis response is triggered, as shown by elevated glucocorticoids levels, in concert with strong induction of PFC function. This overall marked potentiation, most likely promoted to face the initial threat and facilitate induction of the memory of the stressor, may subsequently produce progressive exhaustion of the system, which in turn results into deep impairment of mPFC-mediated function and neuroarchitecture. Therefore, the biphasic effect of stress on synaptic transmission, morphology, and behavioral performance may be considered a compensatory physiologically adaptive response to environmental stressors. However, if the stress response is inadequate or dysregulated, because the stressor is prolonged or overcomes the coping capability of the system, the structural/functional changes may disrupt homeostasis, thus increasing the risk to develop a stress-related pathology (35, 99).

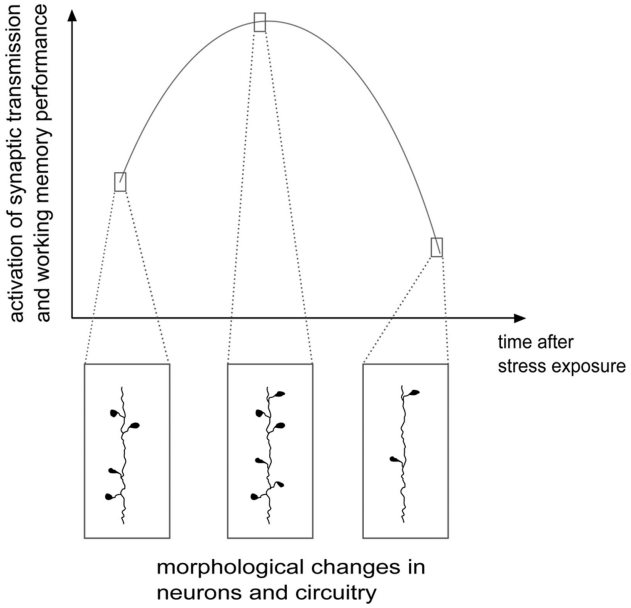

FIGURE 2 | Hypothetical scheme of structural/functional changes induced by stress in the glutamate system: a biphasic process. Stress and corticosterone were shown to induce enhancement of excitatory synaptic transmission and increase in the number of spines and synapses, often accompanied by cognitive enhancement, in the first several minutes and hours. Later on, at least 24 h after application of the stressor, a phase of inhibition follows, with reduction of synaptic transmission, dendritic atrophy and remodeling, loss of spines and synapses and negative effects on cognitive functions. See text for details. Adapted from Musazzi et al. (98).

Understanding what regulates the turning point between a physiological adaptive stress response and the beginning of maladaptive remodeling will be crucial in the study of pathophysiology of neuropsychiatric stress-related disorders. In this context, considering that a high majority of individuals, although exposed to traumatic experiences during lifespan, do not develop stress-related neuropsychiatric disorders, a better knowledge of the molecular/cellular effectors regulating the individual susceptibility to stress could be of great help to mitigate the detrimental effects of external threats and/or to increase the resilience of individuals to stressful events (100).

Author Contributions

All authors contributed to the design and content of the manuscript as well as the first draft of the manuscript and all subsequent revisions. All authors have approved the final version of the manuscript.

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References

1. Bunney WE, Davis JM. Norepinephrine in depressive reactions. A review. *Arch Gen Psychiatry* (1965) **13**:483–94. doi:10.1001/archpsyc.1965.01730060001001
2. Schildraut JJ. The catecholamine hypothesis of affective disorders: a review of supporting evidence. *Am J Psychiatry* (1965) **122**:509–22. doi:10.1176/ajp.122.5.509
3. Duman RS, Aghajanian GK. Synaptic dysfunction in depression: potential therapeutic targets. *Science* (2012) **338**:68–72. doi:10.1126/science.1222939
4. Sanacora G, Treccani G, Popoli M. Towards a glutamate hypothesis of depression: an emerging frontier of neuropsychopharmacology for mood disorders. *Neuropharmacology* (2012) **62**:63–77. doi:10.1016/j.neuropharm.2011.07.036
5. Musazzi L, Treccani G, Mallei A, Popoli M. The action of antidepressants on the glutamate system: regulation of glutamate release and glutamate receptors. *Biol Psychiatry* (2013) **73**:1180–8. doi:10.1016/j.biopsych.2012.11.009
6. Gorman JM, Docherty JP. A hypothesized role for dendritic remodeling in the etiology of mood and anxiety disorders. *J Neuropsychiatry Clin Neurosci* (2010) **22**:256–64. doi:10.1176/appi.neuropsych.22.3.256
7. Korgaonkar MS, Fornito A, Williams LM, Grieve SM. Abnormal structural networks characterize major depressive disorder: a connectome analysis. *Biol Psychiatry* (2014) **76**:567–74. doi:10.1016/j.biopsych.2014.02.018
8. Price JL, Drevets WC. Neurocircuitry of mood disorders. *Neuropsychopharmacology* (2010) **35**:192–216. doi:10.1038/npp.2009.104
9. Stuss DT, Knight RT. *Principles of Frontal Lobe Function*. New York, NY: Oxford University Press (2002).
10. Küçükibrahimoglu E, Saygin MZ, Caliskan M, Kaplan OK, Unsal C. Gören MZ The change in plasma GABA, glutamine and glutamate levels in fluoxetine- or S-citalopram-treated female patients with major depression. *Eur J Clin Pharmacol* (2009) **65**:571–7. doi:10.1007/s00228-009-0650-7
11. Hashimoto K, Sawa A, Iyo M. Increased levels of glutamate in brains from patients with mood disorders. *Biol Psychiatry* (2007) **62**:1310–6. doi:10.1016/j.biopsych.2007.03.017
12. Beneyto M, Kristiansen LV, Oni-Orisan A, McCullumsmith RE, Meador-Woodruff JH. Abnormal glutamate receptor expression in the medial temporal lobe in schizophrenia and mood disorders. *Neuropsychopharmacology* (2007) **32**:1888–902. doi:10.1038/sj.npp.1301312
13. Beneyto M, Meador-Woodruff JH. Lamina-specific abnormalities of NMDA receptor-associated postsynaptic protein transcripts in the prefrontal cortex in schizophrenia and bipolar disorder. *Neuropsychopharmacology* (2008) **33**:2175–86. doi:10.1038/sj.npp.1301604
14. Yüksel C, Öngür D. Magnetic resonance spectroscopy studies of glutamate-related abnormalities in mood disorders. *Biol Psychiatry* (2010) **68**:785–94. doi:10.1016/j.biopsych.2010.06.016
15. Luykx JJ, Laban KG, van den Heuvel MP, Boks MP, Mandl RC, Kahn RS, et al. Region and state specific glutamate downregulation in major depressive disorder: a meta-analysis of (1)H-MRS findings. *Neurosci Biobehav Rev* (2012) **36**:198–205. doi:10.1016/j.neubiorev.2011.05.014
16. Maddock RJ, Buonocore MH. MR spectroscopic studies of the brain in psychiatric disorders. *Curr Top Behav Neurosci* (2012) **11**:199–251. doi:10.1007/7854_2011_197
17. Auer DP, Pütz B, Kraft E, Lipinski B, Schill J, Holsboer F. Reduced glutamate in the anterior cingulate cortex in depression: an in vivo proton magnetic resonance spectroscopy study. *Biol Psychiatry* (2000) **47**:305–13. doi:10.1016/S0006-3223(99)00159-6
18. Michael N, Gösling M, Reutemann M, Kersting A, Heindel W, Arolt V, et al. Metabolic changes after repetitive transcranial magnetic stimulation (rTMS) of the left prefrontal cortex: a sham-controlled proton magnetic resonance spectroscopy (1H MRS) study of healthy brain. *Eur J Neurosci* (2003) **17**:2462–8. doi:10.1046/j.1460-9568.2003.02683.x
19. Michael N, Erfurth A, Ohrmann P, Arolt V, Heindel W, Pfleiderer B. Neurotrophic effects of electroconvulsive therapy: a proton magnetic resonance study of the left amygdalar region in patients with treatment-resistant depression. *Neuropsychopharmacology* (2003) **28**:720–5. doi:10.1038/sj.npp.1300085
20. Hasler G, van der Veen JW, Tumonis T, Meyers N, Shen J, Drevets WC. Reduced prefrontal glutamate/glutamine and gamma-aminobutyric acid levels in major depression determined using proton magnetic resonance spectroscopy. *Arch Gen Psychiatry* (2007) **64**:193–200. doi:10.1001/archpsyc.64.2.193
21. Grimm S, Luborzewski A, Schubert F, Merkl A, Kronenberg G, Colla M, et al. Region-specific glutamate changes in patients with unipolar depression. *J Psychiatr Res* (2012) **46**:1059–65. doi:10.1016/j.jpsychires.2012.04.018
22. Zhang J, Narr KL, Woods RP, Phillips OR, Alger JR, Espinoza RT. Glutamate normalization with ECT treatment response in major depression. *Mol Psychiatry* (2013) **18**:268–70. doi:10.1038/mp.2012.46
23. Li XB, Tang YL, Wang CY, de Leon J. Clozapine for treatment-resistant bipolar disorder: a systematic review. *Bipolar Disord* (2014). doi:10.1111/bdi.12272
24. Bhagwagar Z, Wylezinska M, Jezzard P, Evans J, Ashworth F, Sule A, et al. Reduction in occipital cortex gamma-aminobutyric acid concentrations in medication-free recovered unipolar depressed and bipolar subjects. *Biol Psychiatry* (2007) **61**:806–12. doi:10.1016/j.biopsych.2006.08.048
25. Rajkowska G, Miguel-Hidalgo JJ, Wei J, Dilley G, Pittman SD, Meltzer HY, et al. Morphometric evidence for neuronal and glial prefrontal cell pathology in major depression. *Biol Psychiatry* (1999) **45**:1085–98. doi:10.1016/S0006-3223(99)00041-4
26. Lorenzetti V, Allen NB, Fornito A, Yücel M. Structural brain abnormalities in major depressive disorder: a selective review of recent MRI studies. *J Affect Disord* (2009) **117**:1–17. doi:10.1016/j.jad.2008.11.021
27. Grieve SM, Korgaonkar MS, Koslow SH, Gordon E, Williams LM. Widespread reductions in gray matter volume in depression. *Neuroimage Clin* (2013) **3**:332–9. doi:10.1016/j.nicl.2013.08.016
28. Li M, Metzger CD, Li W, Safron A, van Tol MJ, Lord A, et al. Dissociation of glutamate and cortical thickness is restricted to regions subserving trait but not state markers in major depressive disorder. *J Affect Disord* (2014) **169**:91–100. doi:10.1016/j.jad.2014.08.001
29. Han KM, Choi S, Jung J, Na KS, Yoon HK, Lee MS, et al. Cortical thickness, cortical and subcortical volume, and white matter integrity in patients with their first episode of major depression. *J Affect Disord* (2014) **155**:42–8. doi:10.1016/j.jad.2013.10.021
30. Salvadore G, Nugent AC, Lemaitre H, Luckenbaugh DA, Tinsley R, Cannon DM, et al. Prefrontal cortical abnormalities in currently depressed versus currently remitted patients with major depressive disorder. *Neuroimage* (2011) **54**:2643–51. doi:10.1016/j.neuroimage.2010.11.011
31. Cotter D, Mackay D, Chana G, Beasley C, Landau S, Everall IP. Reduced neuronal size and glial cell density in area 9 of the dorsolateral prefrontal cortex in subjects with major depressive disorder. *Cereb Cortex* (2002) **12**:386–94. doi:10.1093/cercor/12.4.386
32. Tang Y, Wang F, Xie G, Liu J, Li L, Su L, et al. Reduced ventral anterior cingulate and amygdala volumes in medication-naïve females with major depressive disorder: a voxel-based morphometric magnetic resonance imaging study. *Psychiatry Res* (2007) **156**:83–6. doi:10.1016/j.pscychresns.2007.03.005
33. Wagner G, Koch K, Schachtzabel C, Reichenbach JR, Sauer H, Schlosser RG. Enhanced rostral anterior cingulate cortex activation during cognitive control is related to orbitofrontal volume reduction in unipolar depression. *J Psychiatry Neurosci* (2008) **33**:199–208.
34. Kang HJ, Voleti B, Hajszan T, Rajkowska G, Stockmeier CA, Licznerski P, et al. Decreased expression of synapse-related genes and loss of synapses in major depressive disorder. *Nat Med* (2012) **18**:1413–7. doi:10.1038/nm.2886
35. McEwen BS. Stress, sex, and neural adaptation to a changing environment: mechanisms of neuronal remodeling. *Ann N Y Acad Sci* (2010) **1204**:E38–59. doi:10.1111/j.1749-6632.2010.05568.x
36. Overstreet DH. Modeling depression in animal models. *Methods Mol Biol* (2012) **829**:125–44. doi:10.1007/978-1-61779-458-2_7
37. Willner P, Scheel-Krüger J, Belzung C. The neurobiology of depression and antidepressant action. *Neurosci Biobehav Rev* (2013) **37**:2331–71. doi:10.1016/j.neubiorev.2012.12.007
38. Schmitt A, Malchow B, Hasan A, Falkai P. The impact of environmental factors in severe psychiatric disorders. *Front Neurosci* (2014) **8**:19. doi:10.3389/fnins.2014.00019
39. Cook SC, Wellman CL. Chronic stress alters dendritic morphology in rat medial prefrontal cortex. *J Neurobiol* (2004) **60**:236–48. doi:10.1002/neu.20025
40. Silva-Gómez AB, Rojas D, Juárez I, Flores G. Decreased dendritic spine density on prefrontal cortical and hippocampal pyramidal neurons in postweaning socialisolation rats. *Brain Res* (2003) **983**:128–36. doi:10.1016/S0006-8993(03)03042-7
41. Radley JJ, Sisti HM, Hao J, Rocher AB, McCall T, Hof PR, et al. Chronic behavioral stress induces apical dendritic reorganization in pyramidal neurons

of the medial prefrontal cortex. *Neuroscience* (2004) **125**:1–6. doi:10.1016/j.neuroscience.2004.01.006
42. Brown SM, Henning S, Wellman CL. Mild, short-term stress alters dendritic morphology in rat medial prefrontal cortex. *Cereb Cortex* (2005) **15**:1714–22. doi:10.1093/cercor/bhi048
43. Radley JJ, Rocher AB, Janssen WG, Hof PR, McEwen BS, Morrison JH. Reversibility of apical dendritic retraction in the rat medial prefrontal cortex following repeated stress. *Exp Neurol* (2005) **196**:199–203. doi:10.1016/j.expneurol.2005.07.008
44. Izquierdo A, Wellman CL, Holmes A. Brief uncontrollable stress causes dendritic retraction in infralimbic cortex and resistance to fear extinction in mice. *J Neurosci* (2006) **26**:5733–8. doi:10.1523/JNEUROSCI.0474-06.2006
45. Liston C, Miller MM, Goldwater DS, Radley JJ, Rocher AB, Hof PR, et al. Stress-induced alterations in prefrontal cortical dendritic morphology predict selective impairments in perceptual attentional set-shifting. *J Neurosci* (2006) **26**:7870–4. doi:10.1523/JNEUROSCI.1184-06.2006
46. Manikandan S, Padma MK, Srikumar R, Jeya Parthasarathy N, Muthuvel A, Sheela Devi R. Effects of chronic noise stress on spatial memory of rats in relation to neuronal dendritic alteration and free radical-imbalance in hippocampus and medial prefrontal cortex. *Neurosci Lett* (2006) **399**:17–22. doi:10.1016/j.neulet.2006.01.037
47. Radley JJ, Arias CM, Sawchenko PE. Regional differentiation of the medial prefrontal cortex in regulating adaptive responses to acute emotional stress. *J Neurosci* (2006) **26**:12967–76. doi:10.1523/JNEUROSCI.4297-06.2006
48. Michelsen KA, van den Hove DL, Schmitz C, Segers O, Prickaerts J, Steinbusch HW. Prenatal stress and subsequent exposure to chronic mild stress influence dendritic spine density and morphology in the rat medial prefrontal cortex. *BMC Neurosci* (2007) **8**:107. doi:10.1186/1471-2202-8-107
49. Perez-Cruz C, Müller-Keuker JI, Heilbronner U, Fuchs E, Flügge G. Morphology of pyramidal neurons in the rat prefrontal cortex: lateralized dendritic remodeling by chronic stress. *Neural Plast* (2007) **2007**:46276. doi:10.1155/2007/46276
50. Perez-Cruz C, Simon M, Czéh B, Flügge G, Fuchs E. Hemispheric differences in basilar dendrites and spines of pyramidal neurons in the rat prelimbic cortex: activity- and stress-induced changes. *Eur J Neurosci* (2009) **29**:738–47. doi:10.1111/j.1460-9568.2009.06622.x
51. Radley JJ, Rocher AB, Rodriguez A, Ehlenberger DB, Dammann M, McEwen BS, et al. Repeated stress alters dendritic spine morphology in the rat medial prefrontal cortex. *J Comp Neurol* (2008) **507**:1141–50. doi:10.1002/cne.21588
52. Dias-Ferreira E, Sousa JC, Melo I, Morgado P, Mesquita AR, Cerqueira JJ, et al. Chronic stress causes frontostriatal reorganization and affects decision-making. *Science* (2009) **325**:621–5. doi:10.1126/science.1171203
53. Garrett JE, Wellman CL. Chronic stress effects on dendritic morphology in medial prefrontal cortex: sex differences and estrogen dependence. *Neuroscience* (2009) **162**:195–207. doi:10.1016/j.neuroscience.2009.04.057
54. Hains AB, Vu MA, Maciejewski PK, van Dyck CH, Gottron M, Arnsten AF. Inhibition of protein kinase C signaling protects prefrontal cortex dendritic spines and cognition from the effects of chronic stress. *Proc Natl Acad Sci U S A* (2009) **106**:17957–62. doi:10.1073/pnas.0908563106
55. Shansky RM, Hamo C, Hof PR, McEwen BS, Morrison JH. Stress-induced dendritic remodeling in the prefrontal cortex is circuit specific. *Cereb Cortex* (2009) **19**:2479–84. doi:10.1093/cercor/bhp003
56. Hill MN, McLaughlin RJ, Pan B, Fitzgerald ML, Roberts CJ, Lee TT, et al. Recruitment of prefrontal cortical endocannabinoid signaling by glucocorticoids contributes to termination of the stress response. *J Neurosci* (2011) **31**:10506–15. doi:10.1523/JNEUROSCI.0496-11.2011
57. Martin KP, Wellman CL. NMDA receptor blockade alters stress-induced dendritic remodeling in medial prefrontal cortex. *Cereb Cortex* (2011) **21**:2366–73. doi:10.1093/cercor/bhr021
58. Eiland L, Ramroop J, Hill MN, Manley J, McEwen BS. Chronic juvenile stress produces corticolimbic dendritic architectural remodeling and modulates emotional behavior in male and female rats. *Psychoneuroendocrinology* (2012) **37**:39–47. doi:10.1016/j.psyneuen.2011.04.015
59. Suenaga T, Yukie M, Gao S, Nakahara D. Sex-specific effects of prenatal stress on neuronal development in the medial prefrontal cortex and the hippocampus. *Neuroreport* (2012) **23**:430–5. doi:10.1097/WNR.0b013e3283529805
60. Wang YC, Ho UC, Ko MC, Liao CC, Lee LJ. Differential neuronal changes in medial prefrontal cortex, basolateral amygdala and nucleus accumbens after postweaning social isolation. *Brain Struct Funct* (2012) **217**:337–51. doi:10.1007/s00429-011-0355-4
61. Chocyk A, Bobula B, Dudys D, Przyborowska A, Majcher-Maslanka I, Hess G, et al. Early-life stress affects the structural and functional plasticity of the medial prefrontal cortex in adolescent rats. *Eur J Neurosci* (2013) **38**:2089–107. doi:10.1111/ejn.12208
62. Gutiérrez-Rojas C, Pascual R, Bustamante C. Prenatal stress alters the behavior and dendritic morphology of the medial orbitofrontal cortex in mouse offspring during lactation. *Int J Dev Neurosci* (2013) **31**:505–11. doi:10.1016/j.ijdevneu.2013.05.008
63. Markham JA, Mullins SE, Koenig JI. Periadolescent maturation of the prefrontal cortex is sex-specific and is disrupted by prenatal stress. *J Comp Neurol* (2013) **521**:1828–43. doi:10.1002/cne.23262
64. Liu RJ, Aghajanian GK. Stress blunts serotonin- and hypocretin-evoked EPSCs in prefrontal cortex: role of corticosterone-mediated apical dendritic atrophy. *Proc Natl Acad Sci U S A* (2008) **105**:359–64. doi:10.1073/pnas.0706679105
65. Goldwater DS, Pavlides C, Hunter RG, Bloss EB, Hof PR, McEwen BS, et al. Structural and functional alterations to rat medial prefrontal cortex following chronic restraint stress and recovery. *Neuroscience* (2009) **164**:798–808. doi:10.1016/j.neuroscience.2009.08.053
66. Li N, Liu RJ, Dwyer JM, Banasr M, Lee B, Son H, et al. Glutamate N-methyl-D-aspartate receptor antagonists rapidly reverse behavioral and synaptic deficits caused by chronic stress exposure. *Biol Psychiatry* (2011) **69**:754–61. doi:10.1016/j.biopsych.2010.12.015
67. Liston C, Cichon JM, Jeanneteau F, Jia Z, Chao MV, Gan WB. Circadian glucocorticoid oscillations promote learning-dependent synapse formation and maintenance. *Nat Neurosci* (2013) **16**:698–705. doi:10.1038/nn.3387
68. Wellman CL. Dendritic reorganization in pyramidal neurons in medial prefrontal cortex after chronic corticosterone administration. *J Neurobiol* (2001) **49**:245–53. doi:10.1002/neu.1079
69. Koot S, Koukou M, Baars A, Hesseling P, van't Klooster J, Joëls M, et al. Corticosterone and decision-making in male Wistar rats: the effect of 9 corticosterone application in the infralimbic and orbitofrontal cortex. *Front Behav Neurosci* (2014) **8**:127. doi:10.3389/fnbeh.2014.00127
70. Bijlsma EY, van Leeuwen ML, Westphal KG, Olivier B, Groenink L. Local repeated corticotropin-releasing factor infusion exacerbates anxiety- and fear-related behavior: differential involvement of the basolateral amygdala and medial prefrontal cortex. *Neuroscience* (2011) **173**:82–92. doi:10.1016/j.neuroscience.2010.11.026
71. Chiba S, Numakawa T, Ninomiya M, Richards MC, Wakabayashi C, Kunugi H. Chronic restraint stress causes anxiety- and depression-like behaviors, downregulates glucocorticoid receptor expression, and attenuates glutamate release induced by brain-derived neurotrophic factor in the prefrontal cortex. *Prog Neuropsychopharmacol Biol Psychiatry* (2012) **39**:112–9. doi:10.1016/j.pnpbp.2012.05.018
72. Wei J, Yuen EY, Liu W, Li X, Zhong P, Karatsoreos IN, et al. Estrogen protects against the detrimental effects of repeated stress on glutamatergic transmission and cognition. *Mol Psychiatry* (2014) **19**:588–98. doi:10.1038/mp.2013.83
73. Gilabert-Juan J, Castillo-Gomez E, Guirado R, Moltó MD, Nacher J. Chronic stress alters inhibitory networks in the medial prefrontal cortex of adult mice. *Brain Struct Funct* (2013) **218**:1591–605. doi:10.1007/s00429-012-0479-1
74. Yuen EY, Wei J, Liu W, Zhong P, Li X, Yan Z. Repeated stress causes cognitive impairment by suppressing glutamate receptor expression and function in prefrontal cortex. *Neuron* (2012) **73**:962–77. doi:10.1016/j.neuron.2011.12.033
75. Yuen EY, Zhong P, Li X, Wei J, Yan Z. Restoration of glutamatergic transmission by dopamine D4 receptors in stressed animals. *J Biol Chem* (2013) **288**:26112–20. doi:10.1074/jbc.M112.396648
76. Moghaddam B. Stress activation of glutamate neurotransmission in the prefrontal cortex: implications for dopamine-associated psychiatric disorders. *Biol Psychiatry* (2002) **51**:775–87. doi:10.1016/S0006-3223(01)01362-2
77. Veeraiah P, Noronha JM, Maitra S, Bagga P, Khandelwal N, Chakravarty S, et al. Dysfunctional glutamatergic and γ-aminobutyric acidergic activities in prefrontal cortex of mice in social defeat model of depression. *Biol Psychiatry* (2014) **76**:231–8. doi:10.1016/j.biopsych.2013.09.024
78. Popoli M, Yan Z, McEwen BS, Sanacora G. The stressed synapse: the impact of stress and glucocorticoids on glutamate transmission. *Nat Rev Neurosci* (2012) **13**:22–37. doi:10.1038/nrn3138

79. Licznerski P, Duman RS. Remodeling of axo-spinous synapses in the pathophysiology and treatment of depression. *Neuroscience* (2013) **251**:33–50. doi:10.1016/j.neuroscience.2012.09.057
80. Hascup ER, Hascup KN, Stephens M, Pomerleau F, Huettl P, Gratton A, et al. Rapid microelectrode measurements and the origin and regulation of extracellular glutamate in rat prefrontal cortex. *J Neurochem* (2010) **115**:1608–20. doi:10.1111/j.1471-4159.2010.07066.x
81. Musazzi L, Milanese M, Farisello P, Zappettini S, Tardito D, Barbiero VS, et al. Acute stress increases depolarization-evoked glutamate release in the rat prefrontal/frontal cortex: the dampening action of antidepressants. *PLoS One* (2010) **5**:e8566. doi:10.1371/journal.pone.0008566
82. Bonanno G, Giambelli R, Raiteri L, Tiraboschi E, Zappettini S, Musazzi L, et al. Chronic antidepressants reduce depolarization-evoked glutamate release and protein interactions favoring formation of SNARE complex in hippocampus. *J Neurosci* (2005) **25**:3270–9. doi:10.1523/JNEUROSCI.5033-04.2005
83. Treccani G, Musazzi L, Perego C, Milanese M, Nava N, Bonifacino T, et al. Stress and corticosterone increase the readily releasable pool of glutamate vesicles in synaptic terminals of prefrontal and frontal cortex. *Mol Psychiatry* (2014) **19**:433–43. doi:10.1038/mp.2014.5
84. Nava N, Treccani G, Liebenberg N, Chen F, Popoli M, Wegener G, et al. Chronic desipramine prevents acute stress-induced reorganization of medial prefrontal cortex architecture by blocking glutamate vesicle accumulation and excitatory synapse increase. *Int J Neuropsychopharmacol* (2014). doi:10.1093/ijnp/pyu085
85. Pillai AG, Henckens MJ, Fernández G, Joëls M. Delayed effects of corticosterone on slow after-hyperpolarization potentials in mouse hippocampal versus prefrontal cortical pyramidal neurons. *PLoS One* (2014) **9**:e99208. doi:10.1371/journal.pone.0099208
86. Treccani G, Musazzi L, Perego C, Milanese M, Nava N, Bonifacino T, et al. Acute stress rapidly increases the readily releasable pool of glutamate vesicles in prefrontal and frontal cortex through non-genomic action of corticosterone. *Mol Psychiatry* (2014) **19**:401. doi:10.1038/mp.2014.5
87. Yuen EY, Liu W, Karatsoreos IN, Feng J, McEwen BS, Yan Z. Acute stress enhances glutamatergic transmission in prefrontal cortex and facilitates working memory. *Proc Natl Acad Sci U S A* (2009) **106**:14075–9. doi:10.1073/pnas.0906791106
88. Yuen EY, Liu W, Karatsoreos IN, Ren Y, Feng J, McEwen BS, et al. Mechanisms for acute stress-induced enhancement of glutamatergic transmission and working memory. *Mol Psychiatry* (2011) **16**:156–70. doi:10.1038/mp.2010.50
89. Lee JB, Wei J, Liu W, Cheng J, Feng J, Yan Z. Histone deacetylase 6 gates the synaptic action of acute stress in prefrontal cortex. *J Physiol* (2012) **590**:1535–46. doi:10.1113/jphysiol.2011.224907
90. Holtmaat A, Svoboda K. Experience-dependent structural synaptic plasticity in the mammalian brain. *Nat Rev Neurosci* (2009) **10**:647–58. doi:10.1038/nrn2699
91. Leuner B, Gould E. Dendritic growth in medial prefrontal cortex and cognitive flexibility are enhanced during the postpartum period. *J Neurosci* (2010) **30**:13499–503. doi:10.1523/JNEUROSCI.3388-10.2010
92. Leuner B, Shors TJ. Stress, anxiety, and dendritic spines: what are the connections? *Neuroscience* (2013) **251**:108–19. doi:10.1016/j.neuroscience.2012.04.021
93. Karst H, Berger S, Turiault M, Tronche F, Schütz G, Joëls M. Mineralocorticoid receptors are indispensable for nongenomic modulation of hippocampal glutamate transmission by corticosterone. *Proc Natl Acad Sci U S A* (2005) **102**:19204–7. doi:10.1073/pnas.0507572102
94. Joëls M, Sarabdjitsingh A, Karst H. Unraveling the time domains of corticosteroid hormone influences on brain activity: rapid, slow, and chronic modes. *Pharmacol Rev* (2012) **64**:901–38. doi:10.1124/pr.112.005892
95. Duman CH, Duman RS. Spine synapse remodeling in the pathophysiology and treatment of depression. *Neurosci Lett* (2015). doi:10.1016/j.neulet.2015.01.022
96. Kim H, Yi JH, Choi K, Hong S, Shin KS, Kang SJ. Regional differences in acute corticosterone-induced dendritic remodeling in the rat brain and their behavioral consequences. *BMC Neurosci* (2014) **15**:65. doi:10.1186/1471-2202-15-65
97. Lucassen PJ, Pruessner J, Sousa N, Almeida OF, Van Dam AM, Rajkowska G, et al. Neuropathology of stress. *Acta Neuropathol* (2014) **127**:109–35. doi:10.1007/s00401-013-1223-5
98. Musazzi L, Treccani G, Perego C, Nava N, Nyengaard JR, Popoli M. Synaptic stress, changes in glutamate transmission and circuitry, and psychopatology. In: Popoli M, Dimond D, Sanacora G, editors. *Synaptic Stress and Pathogenesis of Neuropsychiatric Disorders*. Springer Press (2014). p. 33–52.
99. McEwen BS, Morrison JH. The brain on stress: vulnerability and plasticity of the prefrontal cortex over the life course. *Neuron* (2013) **79**:16–29. doi:10.1016/j.neuron.2013.06.028
100. Karatsoreos IN, McEwen BS. Psychobiological allostasis: resistance, resilience and vulnerability. *Trends Cogn Sci* (2011) **15**:576–84. doi:10.1016/j.tics.2011.10.005

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Chronic subordinate colony housing paradigm: a mouse model to characterize the consequences of insufficient glucocorticoid signaling

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Chronic, in particular chronic psychosocial, stress is a burden of modern societies and known to be a risk factor for numerous somatic and affective disorders (in detail referenced below). However, based on the limited existence of appropriate, and clinically relevant, animal models for studying the effects of chronic stress, the detailed behavioral, physiological, neuronal, and immunological mechanisms linking stress and such disorders are insufficiently understood. To date, most chronic stress studies in animals employ intermittent exposure to the same (homotypic) or to different (heterotypic) stressors of varying duration and intensity. Such models are only of limited value, since they do not adequately reflect the chronic and continuous situation that humans typically experience. Furthermore, application of different physical or psychological stimuli renders comparisons to the mainly psychosocial stressors faced by humans, as well as between the different stress studies almost impossible. In contrast, rodent models of chronic psychosocial stress represent situations more akin to those faced by humans and consequently seem to hold more clinical relevance. Our laboratory has developed a model in which mice are exposed to social stress for 19 continuous days, namely the chronic subordinate colony housing (CSC) paradigm, to help bridge this gap. The main aim of the current review article is to provide a detailed summary of the behavioral, physiological, neuronal, and immunological consequences of the CSC paradigm, and wherever possible relate the findings to other stress models and to the human situation.

Keywords: chronic psychosocial stress, chronic subordinate colony housing, somatic and affective disorders, decreased glucocorticoid signaling, hypocorticism

INTRODUCTION

THE STRESS CONCEPT

In the nineteenth century, the French physiologist Claude Bernard (1813–1878) noticed that relative constancy of the internal environment is critical for the functional integrity of an organism. Later, in his "emergency concept", Walter Cannon (1871–1945) described the disruption of this internal equilibrium, thereafter referred to as homeostasis (1), by fear- or rage-induced "fight or flight" reactions. In 1936, it was Hans Selye (1907–1982), who first defined stress, and the stress response, as "the non-specific response of the body to any physical demand" (2), and made the distinction between "stress" and the "stressor" (3). According to him, "stressors" are defined as specific challenges that cause a physiological "stress" response (3). Until now, an overwhelming number of studies have focused on the physiological, in particular neuroendocrine, and behavioral consequences of an acute stress response, which are, in general, well understood.

Thus, it is commonly accepted that the physiological and behavioral responses to acute stressors are adaptive, and important to reinstate body homeostasis [(4–6); for review see (7, 8)]. While physical stressors are, thereby, defined as external challenges to homeostasis, psychological stressors are stated as the anticipation, justified or not, of a challenge to homeostasis (9). In contrast, repeated or chronic stressor exposure over several weeks or months, and the prolonged attempt of the body to reinstate homeostasis during this time – a process referred to as allostasis [for review see (5, 10)] – is thought to result in alterations of numerous body and brain systems, finally resulting in a disease state [(11, 12); for review see (5)]. However, although chronic stress-induced alterations in neuroendocrine, emotional, and immune parameters are likely to play a major role in the etiology of numerous diseases including anxiety and depressive disorders, chronic inflammatory disorders, or cancer [(13–21); for review see (22–27)], the detailed underlying mechanisms are less well understood due, at least in part, to the shortage of appropriate animal models.

Physiological responses to an acute stressor

In response to any acute stressor, two major stress systems become activated, namely the autonomic nervous system, especially its sympathetic (SNS) branch, and the hypothalamo–pituitary–adrenocortical (HPA) axis. Stimulation of these emergency systems, which differ in both their time course and processing,

reflects the body's attempt to deal with the immediate situation and to restore homeostasis [for review see (5, 10)]. Activation of the SNS occurs rapidly, within seconds, via exclusively neuronal pathways originating in the thoracolumbal regions of the spinal cord (splanchnic nerve), and results in the release of adrenaline from chromaffin cells of the adrenal medulla into the blood. Elevated adrenaline levels in the circulation act in synergy with an increased sympathetic noradrenergic innervation of essentially all organs in the body [referenced in (28, 29)]. As a result, cardiovascular and catabolic functions are promoted, and processes not vital in the immediate situation, such as anabolic processes and digestion, are inhibited.

In addition to the SNS, there is a slightly delayed activation of the HPA axis in response to acute stressors. The stimulation of the HPA axis is triggered by the secretion of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) from parvocellular neurons of the paraventricular nucleus (PVN) of the hypothalamus into the portal blood stream of the pituitary stalk. CRH and AVP promote the synthesis and the secretion of adrenocorticotropic hormone (ACTH) from anterior pituitary corticotroph cells into the peripheral blood, which, in turn, stimulates cortical cells of the adrenal gland to produce and secrete glucocorticoids [GCs; cortisol in humans, corticosterone (CORT) in rats and mice] into the circulation. Within minutes of activation, termination of an acute HPA axis response is achieved by efficient negative feedback inhibition via GC acting at GC receptors and mineralocorticoid receptors at several brain levels (30). The degree and temporal dynamics of HPA axis activation are strongly dependent on the quality, intensity, and duration of the acute stressor (11, 31, 32). In addition, the acute neuroendocrine stress response was shown to be dependent on the time of day (33–35), age of an individual [(36); for review see (37)], reproductive status of an individual [e.g., in the peripartum period (38–40)], genetic background (41–45), and stressor exposure during life history (46, 47).

Taken together, stressor-induced activation of the SNS and HPA axis contribute to the restoration of the "internal equilibrium" by rapid mobilization of metabolic resources (glucose, oxygen); processes that are adaptive and essential for survival.

Behavioral responses to an acute stressor

In addition to, and facilitated by, the rapid activation of physiological systems (SNS, HPA axis) in response to an acute stressor, there is an instant behavioral response, such as arousal, anxiety/fear, or aggression. This behavioral flexibility is regulated via activation of a number of brain regions, including cortical areas, limbic regions, and the brainstem (48–50). A region of particular importance is the lateral septal area, which is thought to segregate the autonomic, neuroendocrine, and behavioral responses (51). In humans, the behavioral (emotional) response to acute stressor exposure is an important measure of mental health. It is mainly quantified retrospectively via questionnaires (52–54) or by analyzing behavioral patterns known to be linked with distress during, for instance, public speaking (55–57). In laboratory animal models, a variety of behavioral tests have been established in order to quantify signs of arousal (e.g., measurement of homecage activity/locomotion), fear and anxiety-/social anxiety-related behavior [e.g., novelty-supressed feeding, shock pole burying, elevated plus-maze (EPM) test, light–dark box (LDB) test, open arm exposure test, open field test, social preference/avoidance test (SPAT), elevated platform (EPF) exposure, resident-intruder test, Vogel test, 4-plate test, marble burying, stress-induced hyperthermia, contextual/cued fear conditioning, acoustic startle], learning deficits (e.g., Morris water maze, Y/T-maze, holeboard, Barnes maze), anhedonia (e.g., sucrose preference test, progressive ratio responding, psychostimulant-induced hyperactivity, female urine sniffing test), memory skills (contextual-/cued fear conditioning), aggression (resident-intruder test), and active versus passive stress coping strategies [e.g., forced swim test (FST), tail suspension test (TST), learned helplessness] [for reviews dealing with these, and additional tests to assess such behaviors see (58–65)]. Similar to the physiological stress response, the behavioral stress response is strongly dependent on the time of day of stressor exposure (33), quality, intensity, and duration of the stressor (66–70), as well as the genetic and environmental background of the organism (41, 46, 47, 71–74).

CHRONIC STRESS IN HUMANS

Mal-adaptive consequences of chronic stressor exposure

While the acute stressor-induced changes described in the sections above are adaptive, chronic activation of the two stress systems poses an acknowledged risk factor for numerous disorders, including somatic disorders, like cardiovascular diseases [(75–79); for review see (80)], chronic fatigue syndrome (81), fibromyalgia (82), bronchial asthma (83, 84), atopic dermatitis [for review see (85)], arthritis [(86); for review see (87)], inflammatory bowel disease (IBD) [(13, 14, 16, 18, 20, 21); for review see (22, 25, 26)], stomach ulcers (86), diarrhea and digestive problems (86, 88), chronic pelvic and abdominal pain (86, 88), infections (86, 88–90), headaches (86, 88), impaired wound healing (91–93), cancerogenesis [(17); for review see (27, 94)], as well as affective disorders, like anxiety disorders and depression [(95–98); for review see (24, 58, 60, 99, 100)]. While the underlying etiology of these diseases are not fully understood, due at least in part to a lack of animal models, chronic stress-induced dysregulation of almost all psycho-neuro-immunological systems including the HPA axis, the autonomic nervous system, the immune and cardiovascular systems, and emotional and cognitive brain circuits is highly likely to contribute to the complex, and multifactorial, etiology of such disorders. On closer inspection, one mechanism that appears to be common throughout all of these diseases and chronic stress models is altered GC signaling.

The link between chronic stress and impaired GC signaling

Raison and Miller defined decreased GC signaling as "any state in which the potential of GC is inadequate to restrain relevant stress-responsive systems". Such inappropriate GC signaling can be the result of decreased hormone bioavailability (hypocorticism), attenuated GC sensitivity/enhanced GC resistance of target cells, or the combination of both [for review see (101)]. Although HPA axis hyperactivity (hypercorticism) has been generally linked to prolonged, or chronic, stressor exposure, there is accumulating evidence for additional, even opposite alterations [for review see (23)]. In this respect, chronic stress-induced hypocorticism

regained consideration after being more or less ignored up until the beginning of the 2000s. For example, Friedman and colleagues in the early 1970s described decreased plasma and urinary cortisol levels in parents of children suffering from neoplastic disease, with a paradoxical decrease during periods of heightened stress (102). Lower basal GC levels were further reported in high work load employees (103) and patients suffering from post-traumatic stress disorder [for review see (104)]. While elevated basal GC levels have been repeatedly linked to stress-related disorders like major depression [(105); for review see (100, 106)], the overall GC signaling in these patients has been shown to be decreased both *in vivo* and *in vitro* as a consequence of GC insensitivity [for review see (106, 107)]. Taken together, this growing body of evidence has led to greater acceptance of the idea that chronic stress experiences in adulthood result in an insufficient GC signaling.

In addition, chronic stress experienced early in life, like loss of parents, emotional neglect, maltreatment, or abuse have also been linked to a reduced GC signaling capacity in humans. In this context, it has been shown that women maltreated during early life exhibited lower basal and ACTH-induced plasma cortisol levels, an effect that was probably mediated by adrenal dysregulation (108, 109). However, whether the reduction in the overall GC signaling poses a central and causal mechanism by which chronic stress causes the variety of somatic and affective disorders described above is still unknown, but likely.

Many stress-related disorders are linked to a decrease in GC signaling

Although a causal involvement still has to be proven, as stated above, several chronic stress-related pathologies have been shown to be concurrent with reduced GC signaling in a growing number of studies. For example, hypocorticism has been described in patients suffering from burnout and chronic fatigue syndrome, fibromyalgia, chronic pelvic pain, and geriatric depression [(105, 110–112; for review see (23)]. Low levels of plasma GC have been further reported when suffering from inflammatory disorders, including rheumatoid arthritis [for review see (113)] or asthma (114). In line with this, elevated levels of pro-inflammatory cytokines have been reported in patients suffering from acute GC deficiency after surgical removal of adrenal cortical tissue (115). Moreover, it has recently been shown that obese women have lower cortisol levels during pregnancy (116). Interestingly, based on human and animal studies, it has been hypothesized that the onset of IBD might be associated with hypo- rather than hypercorticism [for review see (117, 118)]. This is further supported by a recent finding showing an impaired HPA axis reactivity in 25% of Crohn's patients during exposure to the ultra-low dose ACTH test (119). In addition, a positive correlation between plasma cortisol levels and the time patients were off steroid treatment has recently been described (120). Finally, Rodriguez and coworkers speculated that a down-regulated cortisol response to intero- and exteroceptive stressors might predispose patients suffering from irritable bowel syndrome to chronic inflammatory conditions, such as asthma, rheumatoid arthritis, or IBD (121).

Besides hypocorticism, GC resistance has been speculated to contribute to the reduced GC signaling and the pro-inflammatory immune shift in patients suffering from chronic stress-related pathologies (122). As mentioned above, the disorder that best fits this context is major depression [for review see (101)], as patients show a reduced response to GC both *in vivo* and *in vitro* [for review see (106, 107)], which is believed to be mediated, at least in part, by decreased GC receptor expression and/or functionality [(123–125); for review see (107)] . GC resistance has further been diagnosed in a subset of patients suffering from typically chronic inflammatory disorders like ulcerative colitis and Morbus Crohn [(126); for review see (127)], as well as rheumatoid arthritis (128).

To causally demonstrate that chronic psychosocial stress promotes the development of, at least some, somatic and affective disorders via a reduction in overall GC signaling, it is necessary to have appropriate animal stress models, which mimic the human situation in an adequate way. Thus, animal models are warranted that are of chronic psychosocial nature, to show face validity, and cause both somatic and affective disorders, as well as result in a reduced GC signaling (ideally both hypocorticism and decreased GC sensitivity), to provide predictive validity. Given that the vast majority of somatic and affective disorders are multifactorial diseases, for which the underlying etiological factors are only poorly understood, most of the animal models fail to satisfy construct validity. However, if insufficient GC signaling is indeed causally involved in the development of many such diseases, animal models resulting in either hypocorticism or a decreased GC sensitivity, or both, could be considered primarily as models displaying construct validity.

In the following paragraphs, we will in detail describe the chronic subordinate colony housing (CSC) paradigm, which fulfills all the criteria outlined above and, thus, represents an adequate and preclinically validated (face and predictive validity) animal model to investigate the underlying mechanisms related to chronic psychosocial stress-induced impaired GC signaling and its involvement in somatic and affective disorders.

CHRONIC SUBORDINATE COLONY HOUSING

GENERAL DESCRIPTION AND EXPERIMENTAL DETAILS

The CSC paradigm combines chronic, psychological, and social aspects of stress and, thus, represents a highly potent animal model to mimic the type of health compromising stressors faced by humans (high face validity). CSC further promotes development of both somatic and affective disorders, results in a reduced GC signaling (high predictive/construct validity) and, thus, provides a powerful experimental tool to study the mechanisms underlying several relevant stress-induced pathologies. Notably, given that the CSC paradigm is simply based on the fact that male mice instinctively establish a certain hierarchical structure within their colony, it additionally resembles the natural way of life of a male mouse in the wild [(129); for review see (9)]. However, before we detail the physiological, immunological, and behavioral consequences of the CSC paradigm unraveled to date, we will briefly introduce the experimental details of this chronic psychosocial stress model.

During CSC (for details see **Figure 1**), experimental mice (CSC mice) live in chronic subordination to a dominant resident mouse for 19 consecutive days (130). In detail, four CSC mice are put into the homecage of a larger male resident mouse on day 1 of the CSC paradigm, resulting in immediate subordination of the four intruder CSC mice. To avoid habituation, all four CSC mice

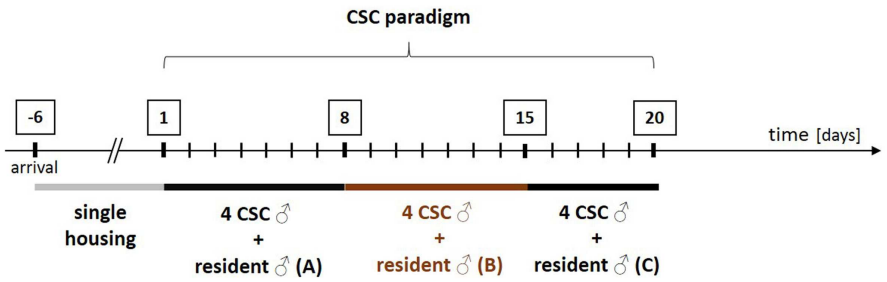

FIGURE 1 | Schematic illustration of the experimental design of the chronic subordinate colony housing (CSC) paradigm. After arrival, all experimental male mice are housed singly for 1 week before they are assigned to the single-housed control (SHC) or the CSC group in a weight matched manner. In order to induce chronic psychosocial stress, CSC mice are housed together with a larger dominant male for 19 consecutive days. In detail, four experimental CSC mice are introduced into the homecage (polycarbonate observation cage; 38 cm × 22 cm × 35 cm) of resident A on day 1 of CSC, resulting in immediate subordination of the four intruder CSC mice. The latter are then housed together with this dominant resident (A) for eight consecutive days. On day 8, and again on day 15, of CSC, the four experimental CSC mice are transferred into the homecages of resident B (day 8) and resident C (day 15) to avoid habituation. Before the CSC procedure, the future dominant males are tested for their aggressive behavior. Males that start to injure their opponents by excessive and harmful bites during testing are not used.

are transferred into the homecage of a novel larger male resident mouse on days 8 and 15.

All resident males are tested before CSC housing for their aggressive behavior and males that injure their opponents by excessive aggression (e.g., harmful bites) are not used. Notably, although this procedure strongly reduces the number of bite wounds delivered by the residents during CSC exposure, it does not 100% prevent them. As a matter of routine, the subordinate position of each CSC mouse is confirmed by behavioral analysis of the first 30 min after setting up the CSC colonies on days 1, 8, and 15 (131). Resident males reliably (>99% of CSC colonies) obtain the dominant position by displaying offensive behaviors toward the CSC mice, such as chasing, mounting, or attacking their four cage mates (131). In contrast, CSC mice can be considered as "subordinates" based on their defensive behaviors, including flight and submissive upright (131). So far, the CSC model has reproducibly been shown to work in different mouse strains, namely, C57BL/6 mice (130), BALB/c mice (132), and CD1 mice (41). Moreover, CSC effects are independent from the background of the residents, as the physiological, immunological, and behavioral consequences of the CSC paradigm are comparable using either C57BL/6 (130) or the male offspring of CD1 female mice [bred at the Max Planck Institute of Psychiatry in Munich (Germany) for high anxiety-related behavior (HAB mice)] and male C57BL/6 mice as dominant animals (133, 134). Recent own unpublished data reveal that using male CD1 mice as residents allows reliable reproduction of known CSC effects (see **Figure 2**).

An important issue for the design of chronic psychosocial stress paradigms is the choice of adequate same-aged controls, with single housing [single-housed control (SHC) mice] or group housing [group-housed control (GHC) mice] being widely used options. For CSC experiments, we employ SHC mice based on own data indicating that group housing *per se* poses a stressful condition for male mice. Surprisingly, similar physiological and behavioral alterations after 3 weeks of GHC or CSC were observed, leading us to believe that the novel hierarchy formed by GHC mice is as almost as stressful as being subordinated by a dominant resident. For example, lower body weight gain and increased state anxiety were found in both CSC and GHC compared with SHC mice (135). In detail, the number and time of head dips and distance traveled on the open arm of the EPM were reduced in both CSC and GHC compared with SHC (135) mice; parameters related to risk assessment, anxiety, exploratory (136), and locomotor behavior (137), respectively. Given that isolation has been shown to lack effects on stress-related immune and/or endocrine functions in male mice by other stress laboratories (138, 139), single housing seems to be the most appropriate non-stressful control condition in non-sibling male mice. In line with this, Blanchard et al. [for review see (140)] and Palanza [for review see (141)] proposed that isolation is more stressful for female mice, while social grouping is more stressful for male mice. In males, any kind of group housing is likely to be accompanied by the establishment of subtle hierarchies with the result that in each cage dominant and more or less subordinate cage mates can be found (135).

CSC-INDUCED CONSEQUENCES

Endocrine changes

Adrenal gland, pituitary, and acute stress reactivity. The CSC paradigm has been shown to result in profound and reproducible physiological changes, including a significant (41, 46, 130, 133, 142) and long-lasting (at least until day 8 after termination of CSC) (70) enlargement of the adrenal glands. This increase in absolute adrenal mass is mediated by cell hyperplasia (133), without alterations in adrenal cholesterol delivery pathways [cortical lipid droplets; protein expression of hormone-sensitive lipase, 3-hydroxy-3-methylglutaryl coenzyme A reductase, and low-density lipoprotein receptor, with the exception of the scavenger receptor class B type 1 protein, which was increased following CSC exposure (133)]. Notably, in an adrenocortical cell the CORT precursor molecule cholesterol can among others be derived from (i) hormone-sensitive lipase-mediated hydrolyzation of cholesteryl esters, stored in lipid droplets within the cytoplasm of mostly zona fasciculata cells [for review see (143)], (ii) hormone-sensitive lipase-mediated hydrolyzation of cholesteryl esters "selectively" taken up from high-density lipoproteins and low-density

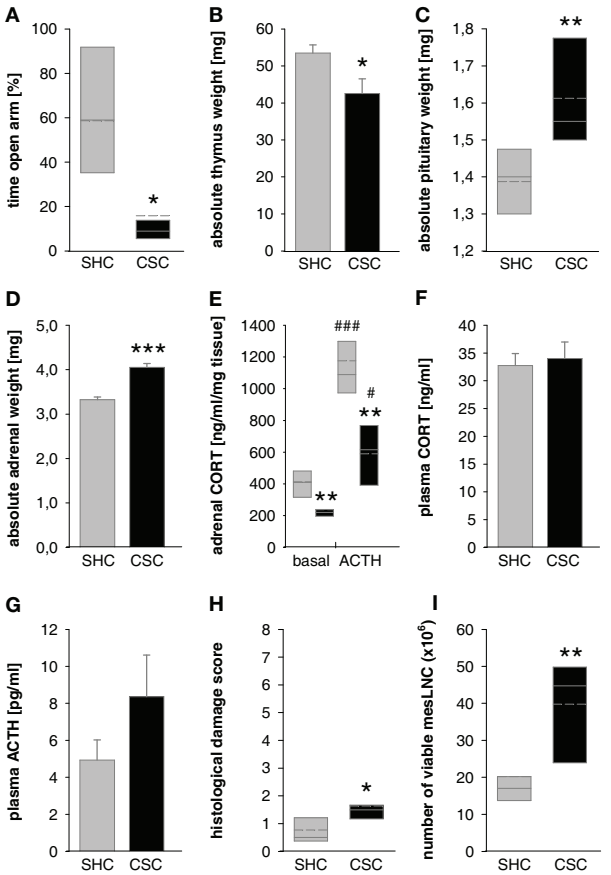

FIGURE 2 | Effects of 19 days of CSC exposure on behavioral, physiological, and immunological parameters. Dominant resident male mice originated from the CD1 strain, CSC mice from the C57BL/6 strain. CSC exposure resulted in increased anxiety-related behavior measured on the elevated plus-maze [EPM; **(A)**] without affecting locomotor activity (data not shown), decreased thymus weight **(B)**, increased pituitary weight **(C)**, increased adrenal weight **(D)**, reduced adrenal *in vitro* ACTH sensitivity **(E)**, unaffected basal morning plasma corticosterone [CORT; **(F)**], a tendency toward increased basal morning plasma adrenocorticotropic hormone [ACTH; **(G)**], increased histological damage in colonic tissue **(H)**, and increased number of viable mesenteric lymph node cells [mesLNC; **(I)**]. ▒ SHC ($n = 7$–8); ■ CSC ($n = 7$–8). Parametric data are represented as mean + SEM. Non-parametric data are represented in box-plot diagrams. Boxes signify the upper and lower quartiles, the median is represented as solid line, and the mean as dashed line within each box. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus respective SHC; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ versus respective basal values.

lipoprotein via the scavenger receptor class B type 1 [(144, 145); for review see (146–148)], (iii) lysosomal acid lipase-mediated hydrolyzation of cholesteryl esters from low-density lipoprotein taken up endocytotically via the low-density lipoprotein receptor (149), and (iv) endogenous *de novo* synthesis from acetyl coenzyme A via the 3-hydroxy-3-methylglutaryl coenzyme A reductase [for review see (150)]. Thus, given the elevated adrenal weight following 19 days of CSC, the molecular and cellular changes reported above in CSC versus SHC mice at the level of the adrenal glands strongly suggest an enhanced adrenal availability and/or mobilization capacity of the CORT precursor molecule cholesterol and, consequently, an increased adrenal functionality in CSC versus SHC mice. In line, analysis of plasma high-density lipoprotein cholesterol and low-density lipoprotein cholesterol revealed increased levels of the latter in CSC versus SHC mice. Similarly, a comparable or even increased relative expression of melanocortin 2 receptor protein and melanocortin 2 receptor accessory protein mRNA, as well as of steroidogenic acute regulatory protein mRNA, side-chain cleavage enzyme mRNA, 11β-hydroxylase and aldosterone-synthase mRNA – the latter enzymes are known to be essential in the progress of CORT synthesis from its precursor cholesterol [for review see (151, 152)] and to be controlled by ACTH signaling [for review see (153, 154)] – support the idea of an overall increased adrenal functionality following CSC exposure.

In confirmation of this hypothesis, mice exposed to 19 days of CSC indeed show exaggerated plasma CORT concentrations, as well as an increased adrenal CORT content, when killed 5 min following termination of a mild acute heterotypic stressor (EPF) at the beginning of the light phase, despite the increase in plasma ACTH concentrations not differing from EPF-exposed SHC mice (142). Notably, when exposed to a more severe acute heterotypic stressor, i.e., 6-min of forced swimming, CSC mice even show an exaggerated ACTH response compared with SHC mice (155), likely to further enhance HPA axis reactivity toward a novel and severe enough heterotypic challenge in CSC mice. A facilitated ACTH response to a novel heterotypic challenge is thereby in line with other chronic stress studies [(156); for review see (157)].

In line with what we found at the adrenal level, the increased capability of the CSC pituitary gland to produce and secret ACTH is mediated at least partly by corticotroph cell hyperplasia (155). Non-compromised functionality of those newly formed cells was suggested by comparable relative pituitary pro-opiomelanocortin protein expression between CSC and SHC mice (155). Interestingly, the idea that during conditions of prolonged/chronic stress AVP becomes the main pituitary ACTH secretagogue [for review see (157)] is supported by unaltered relative pituitary AVP1b receptor and decreased CRH receptor 1 protein expression in CSC versus SHC mice (155). Taken together, these data suggest that these newly formed corticotrophs shift their sensitivity from CRH to AVP. Increased AVP output at the level of the PVN – as suggested by other studies dealing with repeated/chronic stressor exposure [for review see (157)] – does not appear to enhance pituitary AVP stimulation and, thus, to contribute to the increased ACTH drive in CSC mice, as the number of AVP positive parvocellular PVN neurons is comparable between CSC and SHC mice (155). In line, mRNA expression of AVP is even lower in the PVN of CSC versus SHC mice (131), while CRH mRNA is not affected (130). Furthermore, neuronal activation in the parvocellular PVN (predominantly AVP and CRH neurons) is lower in CSC versus SHC mice following acute heterotypic stressor exposure (open arm; 5 min) (135). Similarly, the contributing role of changes in pituitary negative feedback inhibition to the increased ACTH secretion in CSC mice seems to be negligible, as the dexamethasone suppression test indicated a fully functional feedback system (155). Notably, the latter finding clearly indicates that a decrease in pituitary cytoplasmic GC receptor protein expression, as seen in CSC versus SHC mice (155), cannot generally be interpreted as an

impairment of negative feedback function. This is of considerable importance for the stress field in general, as in many published studies this was common practice.

In addition to the above described data assessed on day 20 of CSC (after 19 days of CSC), a time course analysis revealed that relative adrenal mass was significantly increased in stressed mice at all time-points assessed (24, 48 h, days 7, 14, 20) during the 19 days of CSC exposure (130). A more recent study, confirming the increase in relative adrenal weight following 48 h of CSC, extended these findings to demonstrate that even 10 h of CSC are sufficient to cause such changes (134). However, in contrast to relative weight, absolute adrenal weight during this initial phase of CSC was increased after 10 h, but not 48 h, of CSC exposure. Considering the reduction in body weight at both these time-points (70, 132, 134), this clearly indicates that the increase in relative adrenal weight observed following 48 h of CSC is exclusively due to changes in body weight and not to changes at the adrenal level *per se*. Given the reliable increase of absolute adrenal mass following 19 days of CSC described earlier (41, 46, 130, 133, 142), these data for the first time indicate that the adrenal glands of an organism exposed to chronic psychosocial stress enlarge during the very initial phase of stressor exposure, normalize after about 48 h of continuous challenge and, given the stressor still persists, start to enlarge again.

Basal plasma CORT, ACTH and noradrenaline, and GC signaling. Interestingly, these changes in absolute adrenal weight during the initial phase of CSC seem to run in parallel with the fluctuations of basal morning plasma CORT levels. Following 10 h of CSC exposure, plasma morning CORT (132, 134, 158), as well as absolute adrenal mass (134), are significantly increased, whereas following 48 h both parameters return to baseline values. Thus, it seems that reversing the early increase in adrenal mass in CSC mice (10 h) poses some kind of adaptive mechanism, contributing, together with a reduction of stimulatory adrenal input (ACTH) from the pituitary and a possibly increased CORT metabolism (134), to prevent the organism from prolonged exposure to elevated plasma CORT concentrations. This is supported by studies showing a positive correlation between plasma CORT and adrenal weight under stress conditions in rats (159, 160). Chronically elevated CORT concentrations are known to have deleterious health consequences [(161); for review see (162–164)] and to cause increased anxiety- and depressive-like behavior in rats. Moreover, as greater insights can be obtained from rodent studies, chronically high CORT levels have also been shown to affect the brain serotonergic system (165), as well as to rapidly and dramatically increase body weight gain, adiposity, plasma leptin, insulin, and triglyceride levels, and also to decrease homecage locomotion (166) when delivered via the drinking water. However, future studies are needed to clarify whether these early adaptive changes in absolute adrenal weight during CSC exposure are mediated by hyper-/hypotrophy or by hyperplasia/apoptosis of adrenal cells.

Interestingly, although absolute adrenal mass increases again subsequent to the 48 h time point, plasma morning CORT concentrations on days 7, 14, 20 stay still comparable to those of SHC mice (41, 46, 130, 142). This suggests a mechanism different from the one involved during the initial phase of chronic stressor exposure to prevent the deleterious consequences of hypercorticism in the later phases of chronic stressor exposure. Given that isolated adrenal cells (130), as well as adrenal explants (142), from mice exposed to 19 days of CSC show a reduced *in vitro* CORT release when treated with different ACTH doses, it is likely that this is implemented via a reduced ACTH sensitivity of cortical adrenal cells. Notably, adrenal ACTH sensitivity seems to be not only diminished under *in vitro* conditions, as unaffected basal morning plasma CORT in 19-day CSC mice is paralleled by elevated plasma ACTH in comparison with SHC mice (41, 155). Support for this second mechanism to play a role only after prolonged stressor exposure and not to contribute to the initial normalization of plasma CORT is provided by our finding that adrenal *in vitro* ACTH sensitivity of CSC mice was not different from SHC at the 10 and 48 h time point (134).

While the reduction in adrenal ACTH sensitivity in the presence of increased absolute adrenal mass and plasma ACTH seems to ensure normal basal morning CORT concentrations, it is likely to promote the basal evening hypocorticism detected in 19-day CSC mice (130). SHC mice were able to show the expected (167, 168) diurnal rise in plasma GC concentrations at the beginning of their active period, whereas CSC mice were not and, thus, had lower plasma CORT concentrations than respective SHC mice in the evening of day 20 of CSC (130). The resulting decline in GC signaling was further amplified by a CSC-induced reduction in GC sensitivity. The latter was described in both lipopolysaccharide-stimulated splenocytes (130) and plate-bound anti-CD3-stimulated T helper (Th) 2 cells from peripheral lymph nodes (169) of 19-day CSC compared with SHC mice. Thus, given the accumulating evidence that a reduction in GC signaling might be involved in the development of somatic and affective disorders linked with an inflammatory component [for review see (101, 170–172)], it is likely that adrenal changes seen during CSC exposure, although preventing the negative consequences of prolonged hypercorticism, contribute to the development of spontaneous colitis (130, 158, 173), hepatic inflammation (174), increased anxiety-related behavior (41, 46, 70, 130, 131, 135, 142, 173, 175), hyperactivity (70), and the increased risk of inflammation-related colorectal cancer (CRC) (176). In support, we previously showed additive effects of early life stress (repeated maternal separation) and CSC exposure on both the development of hypocorticism and on the severity of a chemically induced colitis (46). To assess, adrenalectomy with CORT replacement needs to be performed to see what CSC-induced behavioral and physiological effects remain.

Notably, as already discussed above in detail, this decreased adrenal ACTH sensitivity is not preventing CSC mice from showing an exaggerated CORT response to subsequent EPF exposure, suggesting an additional, yet unknown, factor that is enhanced in CSC mice during acute heterotypic stressor exposure, unaffected by the diurnal rhythm, which rescues the attenuated adrenal ACTH responsiveness. For example, sympathetic innervation of the adrenal medulla via the splanchnic nerve is known to play a critical role in modulating adrenocortical sensitivity to ACTH (177–179). Following activation, adrenal medullary cells secrete neurotransmitters and neuropeptides such as adrenaline/noradrenaline, neuropeptide Y, vasoactive intestinal

peptide, or substance P, which may, in a paracrine manner [for review see (180, 181)], influence adrenocortical CORT secretion. Moreover, neuropeptides such as prolactin and oxytocin (OXT), which are released during various types of acute stressor exposures [(39,182); for review see (183)], act as direct CORT secretagogues (184–186). Therefore, instead of rescuing ACTH signaling, it is also possible that this unknown factor is a CORT secretagogue itself, thereby simply replacing ACTH in the process of adrenal activation during heterotypic stressor exposure. However, future studies are required to elucidate the identity of this currently unknown determinant.

In contrast to the reduction of basal adrenal cortex function, increased basal plasma noradrenaline concentrations following CSC (130) indicate an over-activated adrenal medulla and, thus, uncoupling the activity of the HPA axis and the SNS during chronic psychosocial stressor exposure. As the concerted action of steroid hormones and neurotransmitters of the SNS is crucial for optimal immunosuppression, uncoupling of the HPA axis and the SNS is likely to further promote pro-inflammatory processes (187). Thus, future studies focusing on the role of the SNS in CSC-induced pathology are warranted.

Summary. In summary, exposure to CSC initially (10–24 h) triggers a pronounced HPA axis response, resulting in increased absolute adrenal mass and elevated basal morning plasma GC concentrations. Following 48 h of continuous CSC exposure, basal morning GC concentrations return to basal levels again, mediated most likely by a combination of decreased stimulatory input from the pituitary, enhanced CORT metabolism, and restoration of normal adrenal mass. Interestingly, during further stressor continuation, the recurrence of rising absolute adrenal mass is not paralleled by increased (morning), but rather decreased (evening) basal plasma GC concentrations, mediated at least partly via a pronounced reduction in adrenal ACTH responsiveness.

Body weight changes

While decreased body weight gain has been reported in many studies investigating the effects of repeated/chronic stressor exposure (67, 188–191), other studies have reported no alteration in body weight development (192–195). In line, the effects of CSC exposure on body weight development are not fully consistent, resulting in either decreased (130, 131, 135, 169, 173, 176) or unaffected body weight gain (41, 46, 70). Therefore, while CSC seems to reliably diminish body weight gain during the initial phase of CSC exposure (46, 70, 131, 132, 134), this in some sets of mice normalizes over the final days of stressor exposure. Notably, CSC mice further gained significantly more weight in the week after stressor termination than unstressed SHC controls leading to a normalization or even increased bodyweight of CSC versus SHC mice (70). Similar findings have previously been reported following subjection to the visible burrow system (196) and repeated social defeat (194) and may represent a general phenomenon following prolonged stressor exposure. This increase in body weight after termination of chronic stress may be an adaptive mechanism for ensuring sufficient resources in preparation for subsequent stressful events. Together, these data emphasize the necessity to further investigate the link between repeated/chronic stressor exposure and changes in body weight and, suggest that caution should be exhibited when interpreting a lack of reduced body weight gain as a sign of a non-effective chronic stress paradigm.

Somatic disorders

In addition to the consequences on endocrine parameters and body weight development, CSC represents an established model to study the immunological consequences of chronic psychosocial stress exposure. In agreement with other chronic social stress paradigms (188, 189, 197–201), CSC causes thymic involution, first detected after 24 h (130), which is in line with the thymus atrophy reported in rats following 24 h of resident-intruder confrontations (189, 201).

Inflammation. Interestingly, and again in line with others (198, 202, 203), CSC causes splenomegaly (41) and reduced *in vitro* GC sensitivity in isolated and lipopolysaccharide-stimulated splenocytes (130). Given that this is paralleled by pronounced immune activation in the social disruption (SDR) paradigm (203–205), it is very likely that the systemic immune status of CSC mice is enhanced as well. GC resistance of IL-4 producing Th2 cells, a reduced number of regulatory T cells, and an increased T cell effector function, all detected in peripheral lymph nodes following 19 days of CSC exposure (169) support this idea. Moreover, CSC mice show higher hepatic tumor necrosis factor alpha, monocyte chemotactic protein 1, and heme oxygenase mRNA expression, indicating noticeable oxidative stress and hepatic inflammation (174), and develop a more severe colitis when subsequently treated with dextran-sulfate sodium (DSS, 1%, 7 days) (46, 173). The latter was indicated by increased body weight loss, inflammatory reduction of colon length, and histological damage score in CSC versus SHC mice after 8 days of DSS treatment.

Interestingly, unlike SHC, CSC mice already on the second day of DSS treatment demonstrate an increased cytokine secretion from isolated and plate-bound anti-CD3-stimulated mesenteric lymph node cells (173), suggesting chronic subordination itself to trigger the development of a colonic inflammation. In support, stimulated cytokine secretion from isolated mesenteric lymph node cells is increased in non-DSS treated CSC mice 8 days following stressor termination (173). Finally, confirming chronic subordination-induced spontaneous colitis, CSC mice display an increased histological damage score in the colon – first detectable after 14 days of CSC (130), number of colonic macrophages, dendritic, and Th cells (158), and cytokine secretion from *in vitro* stimulated mesenteric lymph node (130) and lamina propria mononuclear cells (158). Notably, comparable to the CSC paradigm, a modified version of the SDR paradigm (202–204, 206) has recently been shown to also cause mild histological colonic damage in male mice (195).

Based on the absent CORT increase in the plasma of CSC mice on the second day of DSS treatment – despite increased *in vitro* stimulated cytokine secretion from mesenteric lymph node cells at this time – we recently hypothesized that CSC-induced adrenal insufficiency contributes to the increased severity of DSS-induced colitis [for review see (118)]. In contrast, increased cytokine secretion from *in vitro* stimulated mesenteric lymph node cells of SHC mice was first detected on the eighth day of DSS treatment,

and immediately paralleled by high plasma CORT concentrations (173). However, the adrenal hyper-reactivity toward heterotypic stressors in CSC versus SHC mice clearly argues against a general break down of adrenal functioning in CSC mice. Thus, it is rather likely that cytokine levels secreted from mesenteric lymph node cells *in vivo* during DSS treatment were not high enough to spill over into the systemic circulation and, thereby, activate the HPA axis until day 4 of DSS treatment in both CSC and SHC mice. In turn, a more pronounced plasma CORT increase in CSC versus SHC mice on day 8 of DSS treatment suggests elevated systemic cytokine levels in both groups, and indicates HPA axis hyper-reactivity in CSC mice also to occur in response to heterotypic stressors of an inflammatory nature, given the assumption that inflammation is severe enough to activate the HPA axis.

Support for decreased basal GC signaling – caused by basal hypocorticism and/or GC resistance (130, 169) – to promote CSC-induced aggravation of DSS-induced colitis is provided by the finding that the combination of early-life stress (maternal separation, MS; 3 h/day, from postnatal day 1–14) and 19 days of CSC during adulthood has additive effects on DSS-induced colitis. In contrast to CSC mice, which are only unable to adequately increase plasma CORT at the beginning of the dark/active phase, mice exposed to both MS and CSC suffer from hypocorticism even during the morning hours (46).

With respect to the mechanisms underlying the development of CSC-induced spontaneous colitis, assessment of several functional levels of the colon following the initial stress phase (10 h of CSC) revealed a pronounced, adrenal hormone-mediated, local immune suppression in colonic tissue; probably allowing luminal- and translocated-bacteria to proliferate without constraint (158). Immune suppression was indicated by a reduced cytokine and immunoglobulin A secretion from isolated and anti-CD3/IL-2-stimulated lamina propria mononuclear cells, a decreased percentage of $CD3^+$ cells within all isolated lamina propria mononuclear cells, a decreased pro-inflammatory colonic cytokine mRNA expression, and a lower number of $F4/80^+$ macrophages, $CD11c^+$ dendritic cells, $CD3^+$ T cells, and $CD4^+$ Th cells in colonic tissue of CSC compared with SHC mice. Whether or not this effect is mediated by cortical GC or medullary catecholamines still needs further investigation. The early decrease in colonic IgA secretion in combination with an obsolescent mucosa, indicated by reduced epithelial cell proliferation and apoptosis, additionally suggested the initiation of impaired epithelial barrier functions (158). In line, 10 h of CSC resulted in a reduced/deficient mucus production of colonic epithelial cells. Surprisingly, and in contrast to early CSC-induced immune suppression, our data clearly indicated that the reduction in epithelial barrier functions was not mediated by adrenal hormones. Given that intact local immune and epithelial barrier functions are essential for the control of commensal flora, it was not surprising to detect an increased bacterial load in colonic tissue and in stool samples from CSC mice following 10 h of stressor exposure. Furthermore, experiments employing prolonged antibiotic treatment have revealed a causal role of such bacterial translocation/proliferation during the initial phase of CSC in the initiation/induction of colonic inflammation (158). However, using adrenalectomized mice, we showed that the immunosuppressive effects of high levels of adrenal hormones during the initial CSC phase were required to develop a moderate colitis into a full-blown form (158). Direct evidence showing that the over-active immune system in the later stages of CSC, i.e., when hypocorticism (46, 130) and GC resistance (130, 169) have developed, targets this elevated presence of bacterial antigens in the colonic tissue of CSC mice, leading to the observed colitis (130, 158), still needs to be provided.

Inflammation-related colon carcinogenesis. Given that chronic stress is an acknowledged risk factor for numerous disorders, including IBD [(13, 14, 16, 18, 20, 21); for review see (22, 25, 26)] and cancer [(17); for review see (27)], and that CRC poses one of the most serious complications in IBD patients [(207); for review see (208, 209)], it is not surprising that CSC, besides causing spontaneous colitis (130, 158), also increases the risk for inflammation-related CRC. Combining a novel colitis-related CRC mouse model – in which CRC is initiated with azoxymethane and promoted by repeated cycles of DSS administration (210) – with CSC exposure, revealed that CSC mice show accelerated development of macroscopic suspect lesions, as well as a trend toward an increased incidence of low- and/or high-grade colonic dysplasia (176). Although only a small fraction of these polyps may finally become malignant, there is evidence indicating that a large majority of colorectal carcinomas develop from these adenomatous polyps (211). Similarly, humans who develop severe dysplasia in adenomas are considered to be at increased risk of developing cancer (211). CSC mice further showed an increased number of Ki-67^+ and a decreased number of $TUNEL^+$ colonic epithelial cells, indicating abnormal patterns of cell replication, as detected in several clinical conditions associated with an increased risk for colorectal malignancies [for review see (212)]. A reduction in epithelial cell apoptosis already following 10 h of CSC (158), thereby, indicates that this effect is fast in onset and, hence, likely to be causally involved in CSC-induced promotion of azoxymethane/DSS-induced CRC. The latter was further indicated by increased colonic mRNA and/or protein expression of liver receptor homolog-1, β-catenin, cyclooxygenase II, and tumor necrosis factor alpha in CSC compared with SHC mice. Both liver receptor homolog-1 (213) and β-catenin (214) are involved in the control of intestinal cell renewal, and known to be involved in gastrointestinal tumor development (215–217). The same is true for cyclooxygenase II, which modulates apoptosis, angiogenesis, and tumor invasiveness [for review see (218)] and is over-expressed in approximately 80% of CRC and 40% of colorectal adenomas relative to normal mucosa (219). Tumor necrosis factor has been shown to promote signaling via the β-catenin pathway, thereby contributing to tumor development in the gastric mucosa (220).

Interestingly, a shift from protective Th cells to regulatory T cells was recently hypothesized to mediate the increased susceptibility of mice to UV-induced skin cancer following repeated immobilization (6 h/day over 3 weeks) (12). Similarly, increased regulatory T cell infiltration into the tumor bed, predicted reduced survival in cancer-bearing patients [for review see (221)]. Therefore, development of GC resistance in Th2-, but not Th1-, cell subpopulations during 19 days of CSC (169), causing a potential down-regulation of tumor protective Th1 immunity during repeated post-CSC DSS cycles (heterotypic immune stressors; for

details see before), might be involved in CSC-induced CRC progression. An increased number of colonic $CD4^+$ Th cells and percentage of $CD3^+$ mesenteric lymph node cells in CSC versus SHC mice, but a decreased colonic interferon-γ mRNA expression coupled with an unaltered interferon-γ secretion from stimulated mesenteric lymph node cells support this hypothesis. An increased colonic FoxP3 mRNA expression, as well as number of $CD3^+$/$Foxp3^+$ double-positive mesenteric lymph node cells, following CSC further suggests enhanced immune regulation. Together with the above described reduction in regulatory T cell counts in peripheral lymph node tissue immediately following termination of CSC (169), these data either suggest tissue specificity of CSC effects or that regulatory T cell numbers normalize and even increase gradually following CSC, ameliorating CSC effects on subsequent inflammatory episodes (repeated DSS treatment) but promoting those on CRC development. Notably, body weight development of CSC and SHC mice during second and third DSS cycles is comparable, indicative of an equally severe colitis, whereas three CSC but no SHC mice were dying off too severe colitis during the first DSS cycle.

Affective disorders

Hyperactivity. Humans exposed to severe stressors are at increased risk for developing affective disorders, including posttraumatic stress disorder, which is characterized by pronounced and long-lasting hyperarousal, among other symptoms [for review see (222)]. A link between stress and hyperactivity is also suggested by studies revealing that prenatal stress in humans is associated with attention deficits, hyperarousal, and hyperactivity during childhood [(223); for review see (224)]. Poor school and social functioning, behavioral problems, and parental conflicts, all representing chronic psychosocial stressors, are further well-known factors predicting persistence of childhood attention deficit hyperactivity disorder into adolescence and adulthood (for review see 224). Moreover, bipolar disorder, which affects between 1–3% of the population, is characterized by a cycling between depressive episodes and periods of overactivity, termed mania. Given this strong overactivity component, it is not surprising that the majority of animal models used to study mania have focused on manipulations leading to hyperactivity, e.g., psychostimulant-induced hyperlocomotion [for review see (60, 225, 226)]. In contrast, studies employing animal models of repeated/chronic stress more or less consistently report a stress-induced reduction in locomotor activity, both in the homecage (33, 227) and in a novel environment during behavioral testing (67, 188, 200, 228, 229). Notably, while locomotor activity during behavioral testing (e.g., EPM) should ideally be dissociated from the anxiety state of the animal, altered locomotion is a general confound in such tests. For instance, reduced locomotion during EPM testing of rats following a single cat exposure might also be interpreted as reduced exploration due to increased levels of predator-induced anxiety-related behavior (230). Moreover, highly-anxious rodents (rats bred for high-anxiety-related behavior, HAB rats) show a gender-independent decrease in the number of line crossings in the dark compartment during LDB testing compared with their respective low anxious counterparts (rats bred for low-anxiety-related behavior, LAB rats) (231). Given that genotype specific differences in anxiety-related behavior between these breeding lines have been convincingly shown in locomotion-independent (i.e., ultrasound vocalization) anxiety tests, this indicates that reduced locomotion may be one characteristic of anxious animals [for review see (232)]. This is further indicated by the fact that locomotion in the open field has been used not only as an index of general locomotor activity or exploratory behavior but also as index of anxiety [as referenced in (233)].

Assessment of homecage locomotion before, immediately after, and 1 week after CSC stressor exposure (70) confirmed the expected increase in locomotor activity at the beginning of the dark phase in both SHC and CSC mice prior to stress. While this increase is not seen immediately following CSC exposure, it is even more pronounced in CSC versus SHC mice 1 week later, indicating a long-lasting induction of dark phase hyperlocomotion/hyperactivity. Thus, the CSC paradigm poses one of the few animal models, which might help unraveling the mechanisms underlying stress-promoted hyperactivity.

State anxiety. Chronic psychosocial stressors have also been shown to reliably increase state anxiety in rodents (66, 67, 188, 200, 228, 229, 234) and to be a risk factor for anxiety disorders in humans [for review see (58)]. In keeping, CSC results in a profound and robust increase in state anxiety, which has been confirmed in at least five independent behavioral tests. In detail, exposure to 19 days of CSC reduces the time spent on the open arms of an EPM (130, 131, 173), specifically their distal parts during open arm exposure (135), as well as the time spent in the lit compartment of a LDB (131, 175). Moreover, CSC mice enter the central zone of an OF arena less often, explore novel objects less intensely during a novel object test (46, 70), and spend less time in the outer zone of a platform during EPF exposure (142). Importantly, in a recent study, we further described that CSC mice spent less time on the open arms of an EPM 4 and 8 days after stressor termination, indicating that the stressor-induced change in emotionality is a long-lasting phenomenon (70). With regard to the potential influence of the CSC-induced hyperlocomotion on the interpretation of these tests, they were performed in the early light phase, when home-cage locomotion is not affected. Moreover, no difference in locomotion parameters, such as closed arm entries or distance traveled, were observed between SHC and CSC mice. Therefore, the anxiogenic effect of CSC is robust and long lasting.

Given the anxiogenic effect of CSC exposure and that individuals vary in their response to chronic stressor exposure (234–237), in a recent study, we tested whether the genetic predisposition for high versus low anxiety-related behavior determines the vulnerability to CSC. Interestingly, and in line with our hypothesis, HAB CD1 mice and CD1 mice not selected for anxiety-related behavior (NAB) are equally vulnerable to the CSC-induced behavioral, physiological, neuroendocrine, and immunological effects, whereas CD1 mice bred for low-anxiety-related behavior (LAB) are found to be stress resilient (41). The latter is indicated by the fact that all stress-related parameters, including anxiety-related behavior, are comparable between CSC and SHC mice in the LAB group. In contrast, in both HAB and NAB genotypes, CSC results in an increased adrenal weight, a reduced adrenal *in vitro* ACTH responsiveness substantiated by a lower plasma CORT:ACTH

ratio, and an enhanced pro-inflammatory cytokine secretion from isolated and stimulated mesenteric lymph node cells compared with respective SHC mice. Notably, the CSC-induced increase in anxiety described before in C57BL/6 mice (as referenced above) was only detectable in the NAB group, probably due to a ceiling effect in the anxious HAB line.

Social anxiety. Social anxiety disorder with a lifetime prevalence of 12.1% (238), is the "persistent fear of one or more situations in which an individual is exposed to unfamiliar people or possible scrutiny by others." People suffering from social anxiety disorder attempt to avoid social situations that they fear, which only lead to a persistence of the disorder (239). CSC does not appear to result in social anxiety, despite the profound increase in state anxiety. In more detail, CSC mice spend a similar time investigating a novel object (empty cage) and a social contact (cage with an unknown conspecific) during the SPAT on day 20 of CSC, indicating if anything a lack of social preference (70). Interestingly, they show less investigation in both contexts, suggesting that anxiety of the novel environment may be, at least in part, involved. However, unlike following chronic social defeat, CSC mice do not show active social avoidance, i.e., less time investigating the social context than the non-social context (234, 240–242). Furthermore, when assessed 1 week after stressor termination, despite their still anxious-like phenotype (less time investigating the empty cage), CSC mice prefer to explore the novel conspecific (70). It is important to note that these findings were obtained with non-familiar conspecifics, and it is possible, if indeed not likely, that CSC mice exposed to one of the residents that they faced during the CSC paradigm, would show active social avoidance. For example, acute social defeat has repeatedly been shown to lead to social avoidance, but only to the aggressor [(243, 244); for review see (245)]. In agreement with Kalueff and coworkers (246), the initial lack of social preference following CSC is likely to reflect a temporary social deficit rather than depressive-like behavior (234, 240–242), particularly as CSC mice do not display depression-related behavior in the other tests (70).

Depressive-like behavior. In the literature, the majority of social stress paradigms lead to both increased depression- and anxiety-related behavior (67, 200, 228, 234, 240). This is perhaps not surprising, as there is high co-morbidity between the two disorders (247–250). However, in order to really dissect the mechanisms underlying anxiety or depression, animal models are sorely warranted, which specifically induce one phenotype. Importantly, the CSC paradigm seems to represent such a model, given that it does not lead to deficits in anhedonia (saccharine-preference) or depressive-like behaviour in the FST or TST for at least 1 week following stressor-termination. The use of more than one behavioral test is important, as, for example, $GABA_B$ receptor knockout mice were shown to display depression-related behavior in the FST, but not TST (251). Similarly, exposure to 10 days of social defeat did not alter FST or TST behavior, but lead to an anhedonic phenotype as assessed using the SPAT (234). Therefore, together with the SDR stress paradigm (252) and the social defeat/overcrowding stress paradigm (70, 199), CSC represents one of the few animal models that increase levels of anxiety without simultaneously increasing depression-related behavior. Of note, other depressive-like symptoms, such as cognitive dysfunction (63), have not yet been assessed following CSC.

Substance abuse disorders. Chronic psychosocial stress also represents a strong risk factor for the development of substance abuse disorders, such as alcoholism. Moreover, since CSC exposure reliably increases anxiety-related behavior (as referenced above), a known risk factor for developing ethanol- (EtOH) dependence in humans (253), we assessed whether CSC mice voluntarily consume more EtOH. Here, we could show that 14-day CSC exposure increases EtOH intake, as well as preference, without affecting taste preference or total fluid intake (175). This increased consumption is shown at all EtOH concentrations tested (2–8%), underlining the potency of CSC as a chronic stressor. This is in line with human studies, demonstrating a strong correlation between stressor exposure and the amount of EtOH consumed. It was, for instance, shown that individuals with increased numbers of stressful life events consume more EtOH and exhibit more indicators of EtOH dependence (254). In contrast, data gathered from rodent studies are less consistent. While, for instance, 5 min of daily social defeat over five consecutive days has the potential to increase EtOH consumption in male Long–Evans rats (255) and male C57BL/6 mice (256), there are also studies failing to detect a link between social stressor exposure and increased EtOH consumption [(257); for review see (258)]. These inconsistencies led us to consider our CSC model to be more relevant for the human situation, as it reliably induces an increase in EtOH consumption for a wide range of EtOH concentrations. At present, it is unclear whether CSC leads to abuse of other substances, such as cocaine or nicotine, remains to be determined in future studies.

Central mechanisms underlying the behavioral consequences of CSC. With respect to the central mechanisms underlying these CSC-induced behavioral consequences, we tested a possible involvement of the brain AVP, CRH, and OXT systems (130, 131, 155). These neuropeptides have all been linked with anxiety and substance abuse [(259–261); for review see (262)] and, thus, represent potential mediators of the CSC phenotype. While the expression patterns of hypothalamic OXT mRNA, generally known as an anxiolytic neuropeptide (38, 263–268), as well as the anxiogenic neuropeptide CRH (100) are not altered during CSC, the mRNA expression of the anxiogenic neuropeptide AVP (71, 269) is even reduced in the PVN following 20 days of CSC (131). Similarly, OXT mRNA expression in the PVN and supra optic nucleus is not affected on day 15 of CSC exposure (270). Immunohistochemistry further revealed unaffected numbers of AVP expressing parvo- and magnocellular PVN neurons in SHC and CSC mice (155), altogether making a substantial contribution of CRH, AVP, and OXT in CSC-induced anxiogenesis and EtOH preference, at least at the first glance, rather unlikely.

Recent findings strongly argue for a role of at least the oxytocinergic system in CSC-induced anxiety. Chronic central infusion of OXT (1 ng/h) via an osmotic minipump during 19-day CSC exposure – besides thymus atrophy, adrenal hypertrophy, and decreased adrenal *in vitro* ACTH sensitivity – further prevents CSC-induced anxiogenesis (270). The fact that chronic central OXT infusion

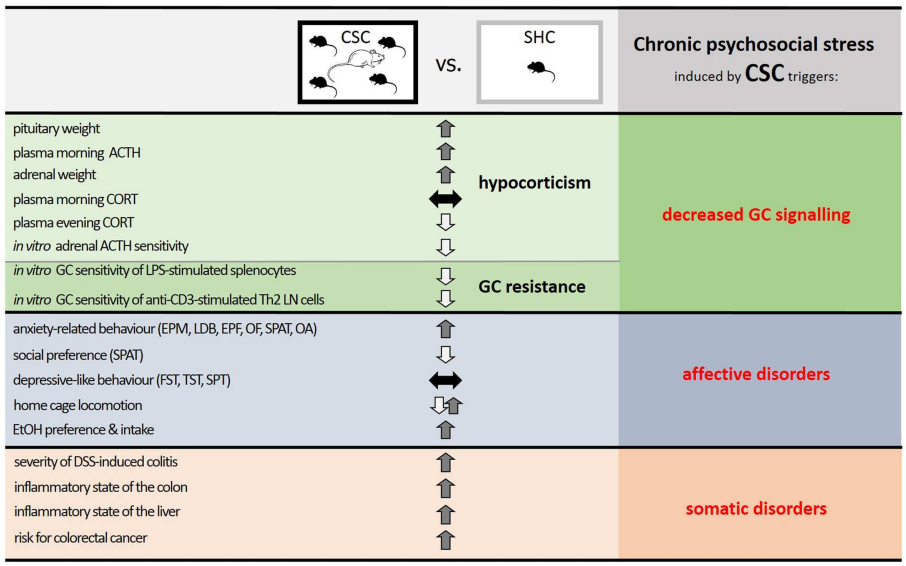

FIGURE 3 | Summary of the effects of chronic psychosocial stress in male mice induced by 19 days of chronic subordinate colony housing (CSC) on physiological, immunological, and behavioral parameters. Compared with single-housed controls (SHC), CSC mice develop a decreased glucocorticoid (GC) signaling, induced by a combination of hypocorticism and GC resistance, and phenotypes characteristic of affective and somatic disorders. Given that stressors that can lead to somatic and affective disorders in humans are mainly chronic and psychosocial in nature, and result in a decreased GC signaling, the CSC paradigm represents a promising animal model to mimic stress-related pathologies in humans and to unravel the underlying mechanisms. Abbreviations: ACTH, adrenocorticotropic hormone; CORT, corticosterone; LPS, lipopolysaccharide; Th2, T helper 2; LN, lymph node; EPM, elevated plus-maze; LDB, light–dark box; EPF, elevated platform; OF, open field; SPAT, social preference/avoidance test; OA, open arm exposure; FST, forced swim test; TST, tail suspension test; SPT, saccharine preference test; EtOH, ethanol; DSS, dextran-sulfate sodium.

additionally prevents the CSC-induced reduction in OXT receptor binding in the median raphe nucleus (270), a region in which OXT signaling has been recently implicated in serotonin release and subsequent anxiolytic effects (271), suggests a main role of the OXT system in this midbrain region in CSC-induced anxiety. Moreover, we have recent data showing that the same central OXT infusion procedure attenuates CSC-induced EtOH preference (Peters et al., unpublished observations). Since the raphe is hypothesized to be an important component of the circuitry involved in the reinforcing properties of drugs of abuse, including EtOH (272), and OXT can reduce drug intake and withdrawal symptoms (273), this region may be, at least in part, involved in CSC-induced heightened EtOH preference.

Besides these local changes in OXT-R binding, CSC and SHC mice show a different neuronal activity within various brain regions implicated in anxiety – under both basal and acute novel environment exposure conditions (135). For example, increased basal neuronal activation in the nucleus accumbens, as seen in CSC versus SHC, was shown in mice exposed to predator odor, which displayed increased anxiety-related behavior in the LDB (274). Furthermore, a decreased activation of the ventral and intermediate parts of the lateral septum, as seen in CSC mice following open arm exposure (135), has been described after acute stress in rats exposed to a learned-helplessness paradigm (275). Similarly, increasing the activity of lateral septum neurons was found to reduce feelings of fear and anxiety (276). Moreover, an increased activation of the dorsomedial part of the periaqueductal grey, as seen in open arm-exposed CSC versus SHC mice (135), has been reported in HAB rats after acute air jet exposure (277). Although acute stress-induced neuronal activation of the ventral hippocampus, a region well known to promote certain aspects of anxiety [for review see (278)], is not affected by CSC, given that cFOS activation in the hippocampal CA3 region is reduced in CSC versus SHC mice following open arm exposure (135). The latter may be explained by the well characterized effects of stress on retraction of dendritic spines (279), which is mainly restricted to this subfield (280). Of note, many of these changes occur in regions that form part of the reward circuitry, but whether acute administration of drugs of abuse (i.e., EtOH) would lead to differential activation between SHC versus CSC mice remains to be determined.

Together, these findings indicate that although the CSC-induced anxiety and substance abuse phenotype is pronounced, reliable, and long lasting, the detailed mechanisms behind are still poorly understood and await further investigation. Moreover, it remains to be seen whether traditional antidepressants or anxiolytics can reverse the CSC-induced behavioral and/or physiological phenotype. Furthermore, and more akin to the clinical situation, it will be interesting to assess whether post-CSC treatment of the mice can reverse the long-lasting behavioral and physiological consequences of stressor exposure.

CONCLUSION

In this review, we have highlighted the fact that numerous somatic and affective disorders, for which chronic psychosocial stress is an accepted risk factor, are characterized by insufficient GC signaling. Thus, hypocorticism and/or GC resistance are observed in

many disorders and following chronic psychosocial stress. Consequently, animal stress models, utilizing a chronic psychosocial component, which result in a decreased GC signaling and concomitant somatic and affective pathologies are likely to hold more translational relevance than other stress models. Indeed, chronic psychosocial stress in mice induced by the CSC paradigm results in both an anxiogenic and substance abuse phenotype, resembling affective disorders, and an overall pro-inflammatory- and cancer-prone phenotype, akin to somatic disorders. CSC further causes basal evening hypocorticism and GC resistance, resembling decreased GC signaling (see **Figure 3**).

Therefore, we are convinced that the CSC paradigm represents an appropriate animal model for studying stress-related disorders in which altered GC signaling is a core feature. Such detailed knowledge will provide further insight into how such stress-related HPA axis changes ultimately lead to somatic and affective disorders, at both behavioral and mechanistic level. Such detailed knowledge, in turn, will allow us to identify novel targets for the treatment of a wide variety of somatic and affective disorders.

REFERENCES

1. Cannon WB. *The Wisdom of the Body*. New York, NY: WW Norton (1939).
2. Selye H. A syndrome produced by diverse nocuous agents. *Nature* (1936) **138**:32. doi:10.1038/138032a0
3. Selye H. Confusion and controversy in the stress field. *J Human Stress* (1975) **1**(2):37–44. doi:10.1080/0097840X.1975.9940406
4. Dhabhar FS, McEwen BS. Stress-induced enhancement of antigen-specific cell-mediated immunity. *J Immunol* (1996) **156**:2608–15.
5. McEwen BS. Protection and damage from acute and chronic stress: allostasis and allostatic overload and relevance to the pathophysiology of psychiatric disorders. *Ann N Y Acad Sci* (2004) **1032**:1–7. doi:10.1196/annals.1314.001
6. Viswanathan K, Dhabhar FS. Stress-induced enhancement of leukocyte trafficking into sites of surgery or immune activation. *Proc Natl Acad Sci U S A* (2005) **102**:5808–13. doi:10.1073/pnas.0501650102
7. Dhabhar FS. Stress-induced augmentation of immune function – the role of stress hormones, leukocyte trafficking, and cytokines. *Brain Behav Immun* (2002) **16**:785–98. doi:10.1016/S0889-1591(02)00036-3
8. McEwen BS, Seeman T. Protective and damaging effects of mediators of stress. Elaborating and testing the concepts of allostasis and allostatic load. *Ann N Y Acad Sci* (1999) **896**:30–47. doi:10.1111/j.1749-6632.1999.tb08103.x
9. Sapolsky RM. The influence of social hierarchy on primate health. *Science* (2005) **308**:648–52. doi:10.1126/science.1106477
10. Goldstein DS, Kopin IJ. Evolution of concepts of stress. *Stress* (2007) **10**:109–20. doi:10.1080/10253890701288935
11. Dhabhar FS, McEwen BS. Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: a potential role for leukocyte trafficking. *Brain Behav Immun* (1997) **11**:286–306. doi:10.1006/brbi.1997.0508
12. Saul AN, Oberyszyn TM, Daugherty C, Kusewitt D, Jones S, Jewell S, et al. Chronic stress and susceptibility to skin cancer. *J Natl Cancer Inst* (2005) **97**:1760–7. doi:10.1093/jnci/dji401
13. Bernstein CN, Singh S, Graff LA, Walker JR, Miller N, Cheang M. A prospective population-based study of triggers of symptomatic flares in IBD. *Am J Gastroenterol* (2010) **105**:1994–2002. doi:10.1038/ajg.2010.140
14. Bitton A, Sewitch MJ, Peppercorn MA, Edwardes MD, Shah S, Ransil B, et al. Psychosocial determinants of relapse in ulcerative colitis: a longitudinal study. *Am J Gastroenterol* (2003) **98**:2203–8. doi:10.1111/j.1572-0241.2003.07717.x
15. Danese A, Pariante CM, Caspi A, Taylor A, Poulton R. Childhood maltreatment predicts adult inflammation in a life-course study. *Proc Natl Acad Sci U S A* (2007) **104**:1319–24. doi:10.1073/pnas.0610362104
16. Duffy LC, Zielezny MA, Marshall JR, Byers TE, Weiser MM, Phillips JF, et al. Relevance of major stress events as an indicator of disease activity prevalence in inflammatory bowel disease. *Behav Med* (1991) **17**:101–10. doi:10.1080/08964289.1991.9937553
17. Levav I, Kohn R, Iscovich J, Abramson JH, Tsai WY, Vigdorovich D. Cancer incidence and survival following bereavement. *Am J Public Health* (2000) **90**:1601–7. doi:10.2105/AJPH.90.10.1601
18. Levenstein S, Prantera C, Varvo V, Scribano ML, Andreoli A, Luzi C, et al. Stress and exacerbation in ulcerative colitis: a prospective study of patients enrolled in remission. *Am J Gastroenterol* (2000) **95**:1213–20. doi:10.1111/j.1572-0241.2000.02012.x
19. Pace TW, Mletzko TC, Alagbe O, Musselman DL, Nemeroff CB, Miller AH, et al. Increased stress-induced inflammatory responses in male patients with major depression and increased early life stress. *Am J Psychiatry* (2006) **163**:1630–3. doi:10.1176/appi.ajp.163.9.1630
20. Robertson DA, Ray J, Diamond I, Edwards JG. Personality profile and affective state of patients with inflammatory bowel disease. *Gut* (1989) **30**:623–6. doi:10.1136/gut.30.9.1300
21. Salem SN, Shubair KS. Non-specific ulcerative colitis in Bedouin Arabs. *Lancet* (1967) **1**:473–5. doi:10.1016/S0140-6736(67)91094-X
22. Bernstein CN. New insights into IBD epidemiology: are there any lessons for treatment? *Dig Dis* (2010) **28**:406–10. doi:10.1159/000320394
23. Heim C, Ehlert U, Hellhammer DH. The potential role of hypocortisolism in the pathophysiology of stress-related bodily disorders. *Psychoneuroendocrinology* (2000) **25**:1–35. doi:10.1016/S0306-4530(99)00035-9
24. Heim C, Nemeroff CB. The role of childhood trauma in the neurobiology of mood and anxiety disorders: preclinical and clinical studies. *Biol Psychiatry* (2001) **49**:1023–39. doi:10.1016/S0006-3223(01)01157-X
25. Mawdsley JE, Rampton DS. Psychological stress in IBD: new insights into pathogenic and therapeutic implications. *Gut* (2005) **54**:1481–91. doi:10.1136/gut.2005.064261
26. Mawdsley JE, Rampton DS. The role of psychological stress in inflammatory bowel disease. *Neuroimmunomodulation* (2006) **13**:327–36. doi:10.1159/000104861
27. Reiche EM, Nunes SO, Morimoto HK. Stress, depression, the immune system, and cancer. *Lancet Oncol* (2004) **5**:617–25. doi:10.1016/S1470-2045(04)01597-9
28. Bartolomucci A. Social stress, immune functions and disease in rodents. *Front Neuroendocrinol* (2007) **28**:28–49. doi:10.1016/j.yfrne.2007.02.001
29. Stratakis CA, Chrousos GP. Neuroendocrinology and pathophysiology of the stress system. *Ann N Y Acad Sci* (1995) **771**:1–18. doi:10.1111/j.1749-6632.1995.tb44666.x
30. Reul JM, de Kloet ER. Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. *Endocrinology* (1985) **117**:2505–11. doi:10.1210/endo-117-6-2505
31. Bowers SL, Bilbo SD, Dhabhar FS, Nelson RJ. Stressor-specific alterations in corticosterone and immune responses in mice. *Brain Behav Immun* (2008) **22**:105–13. doi:10.1016/j.bbi.2007.07.012
32. Koolhaas JM, Bartolomucci A, Buwalda B, de Boer SF, Flugge G, Korte SM, et al. Stress revisited: a critical evaluation of the stress concept. *Neurosci Biobehav Rev* (2011) **35**(5):1291–301. doi:10.1016/j.neurobiorev.2011.02.003
33. Bartlang MS, Neumann ID, Slattery DA, Uschold-Schmidt N, Kraus D, Helfrich-Förster C, et al. Time matters: pathological effects of repeated psychosocial stress during the active, but not inactive, phase of male mice. *J Endocrinol* (2012) **215**:425–37. doi:10.1530/joe-12-0267
34. Kalsbeek A, Ruiter M, La Fleur SE, Van Heijningen C, Buijs RM. The diurnal modulation of hormonal responses in the rat varies with different stimuli. *J Neuroendocrinol* (2003) **15**:1144–55. doi:10.1111/j.1365-2826.2003.01112.x
35. Perez-Cruz C, Simon M, Flügge G, Fuchs E, Czéh B. Diurnal rhythm and stress regulate dendritic architecture and spine density of pyramidal neurons in the rat infralimbic cortex. *Behav Brain Res* (2009) **205**:406–13. doi:10.1016/j.bbr.2009.07.021
36. Meijer OC, Topic B, Steenbergen PJ, Jocham G, Huston JP, Oitzl MS. Correlations between hypothalamus-pituitary-adrenal axis parameters depend on age and learning capacity. *Endocrinology* (2005) **146**(3):1372–81.
37. Sapolsky RM, Krey LC, McEwen BS. The adrenocortical axis in the aged rat: impaired sensitivity to both fast and delayed feedback inhibition. *Neurobiol Aging* (1986) **7**:331–5. doi:10.1016/0197-4580(86)90159-4
38. Neumann ID, Torner L, Wigger A. Brain oxytocin: differential inhibition of neuroendocrine stress responses and anxiety-related behaviour in virgin, pregnant and lactating rats. *Neuroscience* (2000) **95**:567–75. doi:10.1016/S0306-4522(99)00433-9

39. Torner L, Neumann ID. The brain prolactin system: involvement in stress response adaptations in lactation. *Stress* (2002) **5**:249–57. doi:10.1080/1025389021000048638
40. Torner L, Toschi N, Nava G, Clapp C, Neumann ID. Increased hypothalamic expression of prolactin in lactation: involvement in behavioural and neuroendocrine stress responses. *Eur J Neurosci* (2002) **15**:1381–9. doi:10.1046/j.1460-9568.2002.01965.x
41. Füchsl AM, Neumann ID, Reber SO. Stress resilience: a low-anxiety genotype protects male mice from the consequences of chronic psychosocial stress. *Endocrinology* (2014) **155**:117–26. doi:10.1210/en.2013-1742
42. Landgraf R, Wigger A, Holsboer F, Neumann ID. Hyper-reactive hypothalamo-pituitary-adrenocortical axis in rats bred for high anxiety-related behaviour. *J Neuroendocrinol* (1999) **11**:405–7. doi:10.1046/j.1365-2826.1999.00342.x
43. Ohta R, Shirota M, Adachi T, Tohei A, Taya K. Plasma ACTH levels during early, two-way avoidance acquisition in high- and low-avoidance rats (Hatano strains). *Behav Genet* (1999) **29**:137–44. doi:10.1023/A:1021616723969
44. Sternberg EM, Hill JM, Chrousos GP, Kamilaris T, Listwak SJ, Gold PW, et al. Inflammatory mediator-induced hypothalamic-pituitary-adrenal axis activation is defective in streptococcal cell wall arthritis-susceptible Lewis rats. *Proc Natl Acad Sci U S A* (1989) **86**:2374–8. doi:10.1073/pnas.86.7.2374
45. Touma C, Bunck M, Glasl L, Nussbaumer M, Palme R, Stein H, et al. Mice selected for high versus low stress reactivity: a new animal model for affective disorders. *Psychoneuroendocrinology* (2008) **33**:839–62. doi:10.1016/j.psyneuen.2008.03.013
46. Veenema AH, Reber SO, Selch S, Obermeier F, Neumann ID. Early life stress enhances the vulnerability to chronic psychosocial stress and experimental colitis in adult mice. *Endocrinology* (2008) **144**:2727–36. doi:10.1210/en.2007-1469
47. Wigger A, Neumann ID. Periodic maternal deprivation induces gender-dependent alterations in behavioral and neuroendocrine responses to emotional stress in adult rats. *Physiol Behav* (1999) **66**:293–302. doi:10.1016/S0031-9384(98)00300-X
48. Campeau S, Watson SJ. Neuroendocrine and behavioral responses and brain pattern of c-fos induction associated with audiogenic stress. *J Neuroendocrinol* (1997) **9**:577–88.
49. Cullinan WE, Herman JP, Battaglia DF, Akil H, Watson SJ. Pattern and time course of immediate early gene expression in rat brain following acute stress. *Neuroscience* (1995) **64**:477–505. doi:10.1016/0306-4522(94)00355-9
50. Cullinan WE, Helmreich DL, Watson SJ. Fos expression in forebrain afferents to the hypothalamic paraventricular nucleus following swim stress. *J Comp Neurol* (1996) **368**:88–99. doi:10.1002/(SICI)1096-9861(19960422)368:1<88::AID-CNE6>3.0.CO;2-G
51. Reis DG, Scopinho AA, Guimaraes FS, Correa FM, Resstel LB. Behavioral and autonomic responses to acute restraint stress are segregated within the lateral septal area of rats. *PLoS One* (2011) **6**:e23171. doi:10.1371/journal.pone.0023171
52. Balodis IM, Wynne-Edwards KE, Olmstead MC. The stress-response-dampening effects of placebo. *Horm Behav* (2011) **59**:465–72. doi:10.1016/j.yhbeh.2011.01.004
53. Elzinga BM, Spinhoven P, Berretty E, de Jong P, Roelofs K. The role of childhood abuse in HPA-axis reactivity in social anxiety disorder: a pilot study. *Biol Psychol* (2010) **83**:1–6. doi:10.1016/j.biopsycho.2009.09.006
54. Heinrichs M, Baumgartner T, Kirschbaum C, Ehlert U. Social support and oxytocin interact to suppress cortisol and subjective responses to psychosocial stress. *Biol Psychiatry* (2003) **54**:1389–98. doi:10.1016/S0006-3223(03)00465-7
55. Buske-Kirschbaum A, Geiben A, Hollig H, Morschhauser E, Hellhammer D. Altered responsiveness of the hypothalamus-pituitary-adrenal axis and the sympathetic adrenomedullary system to stress in patients with atopic dermatitis. *J Clin Endocrinol Metab* (2002) **87**:4245–51. doi:10.1210/jc.2001-010872
56. Buske-Kirschbaum A, Gierens A, Hollig H, Hellhammer DH. Stress-induced immunomodulation is altered in patients with atopic dermatitis. *J Neuroimmunol* (2002) **129**:161–7. doi:10.1016/S0165-5728(02)00168-6
57. Buske-Kirschbaum A, Ebrecht M, Hellhammer DH. Blunted HPA axis responsiveness to stress in atopic patients is associated with the acuity and severeness of allergic inflammation. *Brain Behav Immun* (2010) **24**:1347–53. doi:10.1016/j.bbi.2010.06.013
58. Cryan JF, Holmes A. The ascent of mouse: advances in modelling human depression and anxiety. *Nat Rev Drug Discov* (2005) **4**:775–90. doi:10.1038/nrd1825
59. Cryan JF, Mombereau C, Vassout A. The tail suspension test as a model for assessing antidepressant activity: review of pharmacological and genetic studies in mice. *Neurosci Biobehav Rev* (2005) **29**:571–625. doi:10.1016/j.neubiorev.2005.03.009
60. Cryan JF, Slattery DA. Animal models of mood disorders: recent developments. *Curr Opin Psychiatry* (2007) **20**:1–7. doi:10.1097/YCO.0b013e3280117733
61. Fuchs E. Social stress in tree shrews as an animal model of depression: an example of a behavioral model of a CNS disorder. *CNS Spectr* (2005) **10**:182–90. doi:10.1017/S1092852900010038
62. Gould TD, Gottesman II. Psychiatric endophenotypes and the development of valid animal models. *Genes Brain Behav* (2006) **5**:113–9. doi:10.1111/j.1601-183X.2005.00186.x
63. Hales CA, Stuart SA, Anderson MH, Robinson ES. Modelling cognitive affective biases in major depressive disorder using rodents. *Br J Pharmacol* (2014) **171**(20):4524–38. doi:10.1111/bph.12603
64. Harro J. Animal models of depression vulnerability. *Curr Top Behav Neurosci* (2013) **14**:29–54. doi:10.1007/7854_2012_221
65. Henn FA, Vollmayr B. Stress models of depression: forming genetically vulnerable strains. *Neurosci Biobehav Rev* (2005) **29**:799–804. doi:10.1016/j.neubiorev.2005.03.019
66. Avgustinovich DF, Gorbach OV, Kudryavtseva NN. Comparative analysis of anxiety-like behavior in partition and plus-maze tests after agonistic interactions in mice. *Physiol Behav* (1997) **61**:37–43. doi:10.1016/S0031-9384(96)00303-4
67. Keeney AJ, Hogg S. Behavioural consequences of repeated social defeat in the mouse: preliminary evaluation of a potential animal model of depression. *Behav Pharmacol* (1999) **10**:753–64. doi:10.1097/00008877-199912000-00007
68. Keeney AJ, Jessop DS, Harbuz MS, Marsden CA, Hogg S, Blackburn-Munro RE. Differential effects of acute and chronic social defeat stress on hypothalamic-pituitary-adrenal axis function and hippocampal serotonin release in mice. *J Neuroendocrinol* (2006) **18**:330–8. doi:10.1111/j.1365-2826.2006.01422.x
69. Savignac HM, Finger BC, Pizzo RC, O'Leary OF, Dinan TG, Cryan JF. Increased sensitivity to the effects of chronic social defeat stress in an innately anxious mouse strain. *Neuroscience* (2011) **192**:524–36. doi:10.1016/j.neuroscience.2011.04.054
70. Slattery DA, Uschold N, Magoni M, Bar J, Popoli M, Neumann ID, et al. Behavioural consequences of two chronic psychosocial stress paradigms: anxiety without depression. *Psychoneuroendocrinology* (2012) **37**(5):702–14. doi:10.1016/j.psyneuen.2011.09.002
71. Bosch OJ, Krömer SA, Neumann ID. Prenatal stress: opposite effects on anxiety and hypothalamic expression of vasopressin and corticotropin-releasing hormone in rats selectively bred for high and low anxiety. *Eur J Neurosci* (2006) **23**:541–51. doi:10.1111/j.1460-9568.2005.04576.x
72. Neumann ID, Wigger A, Krömer S, Frank E, Landgraf R, Bosch OJ. Differential effects of periodic maternal separation on adult stress coping in a rat model of extremes in trait anxiety. *Neuroscience* (2005) **132**:867–77. doi:10.1016/j.neuroscience.2005.01.034
73. Neumann ID, Krömer SA, Bosch OJ. Effects of psycho-social stress during pregnancy on neuroendocrine and behavioural parameters in lactation depend on the genetically determined stress vulnerability. *Psychoneuroendocrinology* (2005) **30**:791–806. doi:10.1016/j.psyneuen.2005.03.008
74. Veenema AH, Meijer OC, de Kloet ER, Koolhaas JM. Genetic selection for coping style predicts stressor susceptibility. *J Neuroendocrinol* (2003) **15**:256–67. doi:10.1046/j.1365-2826.2003.00986.x
75. Buckley T, Stannard A, Bartrop R, McKinley S, Ward C, Mihailidou AS, et al. Effect of early bereavement on heart rate and heart rate variability. *Am J Cardiol* (2012) **110**:1378–83. doi:10.1016/j.amjcard.2012.06.045
76. Dimsdale JE. Psychological stress and cardiovascular disease. *J Am Coll Cardiol* (2008) **51**:1237–46. doi:10.1016/j.jacc.2007.12.024
77. Kivimaki M, Nyberg ST, Batty GD, Fransson EI, Heikkila K, Alfredsson L, et al. Job strain as a risk factor for coronary heart disease: a collaborative meta-analysis of individual participant data. *Lancet* (2012) **380**:1491–7. doi:10.1016/S0140-6736(12)60994-5
78. Mausbach BT, Chattillion E, Roepke SK, Ziegler MG, Milic M, von Kanel R, et al. A longitudinal analysis of the relations among stress, depressive symptoms, leisure satisfaction, and endothelial function in caregivers. *Health Psychol* (2012) **31**:433–40. doi:10.1037/a0027783

79. Unden AL, Orth-Gomer K, Elofsson S. Cardiovascular effects of social support in the work place: twenty-four-hour ECG monitoring of men and women. *Psychosom Med* (1991) **53**:50–60. doi:10.1097/00006842-199101000-00005
80. Buckley T, Bartrop R, McKinley S, Ward C, Bramwell M, Roche D, et al. Prospective study of early bereavement on psychological and behavioural cardiac risk factors. *Intern Med J* (2009) **39**:370–8. doi:10.1111/j.1445-5994.2009.01879.x
81. Heim C, Nater UM, Maloney E, Boneva R, Jones JF, Reeves WC. Childhood trauma and risk for chronic fatigue syndrome: association with neuroendocrine dysfunction. *Arch Gen Psychiatry* (2009) **66**:72–80. doi:10.1001/archgenpsychiatry.2008.508
82. Almansa C, Rey E, Sanchez RG, Sanchez AA, Diaz-Rubio M. Prevalence of functional gastrointestinal disorders in patients with fibromyalgia and the role of psychologic distress. *Clin Gastroenterol Hepatol* (2009) **7**:438–45. doi:10.1016/j.cgh.2008.12.005
83. Wright RJ, Finn P, Contreras JP, Cohen S, Wright RO, Staudenmayer J, et al. Chronic caregiver stress and IgE expression, allergen-induced proliferation, and cytokine profiles in a birth cohort predisposed to atopy. *J Allergy Clin Immunol* (2004) **113**:1051–7. doi:10.1016/j.jaci.2004.03.032
84. Wright RJ, Mitchell H, Visness CM, Cohen S, Stout J, Evans R, et al. Community violence and asthma morbidity: the Inner-City Asthma Study. *Am J Public Health* (2004) **94**:625–32. doi:10.2105/AJPH.94.4.625
85. Buske-Kirschbaum A, Geiben A, Hellhammer D. Psychobiological aspects of atopic dermatitis: an overview. *Psychother Psychosom* (2001) **70**:6–16. doi:10.1159/000056219
86. Coker AL, Smith PH, Bethea L, King MR, McKeown RE. Physical health consequences of physical and psychological intimate partner violence. *Arch Fam Med* (2000) **9**:451–7. doi:10.1001/archfami.9.5.451
87. Herrmann M, Scholmerich J, Straub RH. Stress and rheumatic diseases. *Rheum Dis Clin North Am* (2000) **26**:737–763, viii. doi:10.1016/S0889-857X(05)70167-8
88. Campbell J, Jones AS, Dienemann J, Kub J, Schollenberger J, O'Campo P, et al. Intimate partner violence and physical health consequences. *Arch Intern Med* (2002) **162**:1157–63. doi:10.1001/archinte.162.10.1157
89. Cohen S, Tyrrell DA, Smith AP. Psychological stress and susceptibility to the common cold. *N Engl J Med* (1991) **325**:606–12. doi:10.1056/NEJM199108293250903
90. Kiecolt-Glaser JK, Glaser R, Gravenstein S, Malarkey WB, Sheridan J. Chronic stress alters the immune response to influenza virus vaccine in older adults. *Proc Natl Acad Sci U S A* (1996) **93**:3043–7. doi:10.1073/pnas.93.7.3043
91. Kiecolt-Glaser JK, Marucha PT, Malarkey WB, Mercado AM, Glaser R. Slowing of wound healing by psychological stress. *Lancet* (1995) **346**:1194–6. doi:10.1016/S0140-6736(95)92899-5
92. Kiecolt-Glaser JK, Page GG, Marucha PT, MacCallum RC, Glaser R. Psychological influences on surgical recovery. Perspectives from psychoneuroimmunology. *Am Psychol* (1998) **53**:1209–18. doi:10.1037/0003-066X.53.11.1209
93. Marucha PT, Kiecolt-Glaser JK, Favagehi M. Mucosal wound healing is impaired by examination stress. *Psychosom Med* (1998) **60**:362–5. doi:10.1097/00006842-199805000-00025
94. Kiecolt-Glaser JK, Glaser R. Psychoneuroimmunology and health consequences: data and shared mechanisms. *Psychosom Med* (1995) **57**:269–74. doi:10.1097/00006842-199505000-00008
95. Agid O, Shapira B, Zislin J, Ritsner M, Hanin B, Murad H, et al. Environment and vulnerability to major psychiatric illness: a case control study of early parental loss in major depression, bipolar disorder and schizophrenia. *Mol Psychiatry* (1999) **4**:163–72. doi:10.1038/sj.mp.4000473
96. de Kloet ER, Joels M, Holsboer F. Stress and the brain: from adaptation to disease. *Nat Rev Neurosci* (2005) **6**:463–75. doi:10.1038/nrn1683
97. Virtanen M, Stansfeld SA, Fuhrer R, Ferrie JE, Kivimaki M. Overtime work as a predictor of major depressive episode: a 5-year follow-up of the Whitehall II study. *PLoS One* (2012) **7**:e30719. doi:10.1371/journal.pone.0030719
98. Virtanen M, Kivimaki M. Saved by the bell: does working too much increase the likelihood of depression? *Expert Rev Neurother* (2012) **12**:497–9. doi:10.1586/ern.12.29
99. Hammen C. Stress and depression. *Annu Rev Clin Psychol* (2005) **1**:293–319. doi:10.1146/annurev.clinpsy.1.102803.143938
100. Holsboer F. Stress, hypercortisolism and corticosteroid receptors in depression: implications for therapy. *J Affect Disord* (2001) **62**:77–91. doi:10.1016/S0165-0327(00)00352-9
101. Raison CL, Miller AH. When not enough is too much: the role of insufficient glucocorticoid signaling in the pathophysiology of stress-related disorders. *Am J Psychiatry* (2003) **160**:1554–65. doi:10.1176/appi.ajp.160.9.1554
102. Friedman SB, Mason JW, Hamburg DA. Urinary 17-hydroxycorticosteroid levels in parents of children with neoplastic disease: a study of chronic psychological stress. *Psychosom Med* (1963) **25**:364–76. doi:10.1097/00006842-196307000-00007
103. Caplan RD, Cobb S, French JR Jr. White collar work load and cortisol: disruption of a circadian rhythm by job stress? *J Psychosom Res* (1979) **23**:181–92. doi:10.1016/0022-3999(79)90003-5
104. Yehuda R. Sensitization of the hypothalamic-pituitary-adrenal axis in posttraumatic stress disorder. *Ann N Y Acad Sci* (1997) **821**:57–75. doi:10.1111/j.1749-6632.1997.tb48269.x
105. Penninx BW, Beekman AT, Bandinelli S, Corsi AM, Bremmer M, Hoogendijk WJ, et al. Late-life depressive symptoms are associated with both hyperactivity and hypoactivity of the hypothalamo-pituitary-adrenal axis. *Am J Geriatr Psychiatry* (2007) **15**:522–9. doi:10.1097/JGP.0b013e318033ed80
106. Holsboer F. The corticosteroid receptor hypothesis of depression. *Neuropsychopharmacology* (2000) **23**:477–501. doi:10.1016/S0893-133X(00)00159-7
107. Pariante CM, Miller AH. Glucocorticoid receptors in major depression: relevance to pathophysiology and treatment. *Biol Psychiatry* (2001) **49**:391–404. doi:10.1016/S0006-3223(00)01088-X
108. Heim C, Newport DJ, Heit S, Graham YP, Wilcox M, Bonsall R, et al. Pituitary-adrenal and autonomic responses to stress in women after sexual and physical abuse in childhood. *JAMA* (2000) **284**:592–7. doi:10.1001/jama.284.5.592
109. Heim C, Newport DJ, Bonsall R, Miller AH, Nemeroff CB. Altered pituitary-adrenal axis responses to provocative challenge tests in adult survivors of childhood abuse. *Am J Psychiatry* (2001) **158**:575–81. doi:10.1176/appi.ajp.158.4.575
110. Crofford LJ, Pillemer SR, Kalogeras KT, Cash JM, Michelson D, Kling MA, et al. Hypothalamic-pituitary-adrenal axis perturbations in patients with fibromyalgia. *Arthritis Rheum* (1994) **37**:1583–92. doi:10.1002/art.1780371105
111. Demitrack MA, Dale JK, Straus SE, Laue L, Listwak SJ, Kruesi MJ, et al. Evidence for impaired activation of the hypothalamic-pituitary-adrenal axis in patients with chronic fatigue syndrome. *J Clin Endocrinol Metab* (1991) **73**:1224–34. doi:10.1210/jcem-73-6-1224
112. Heim C, Ehlert U, Hanker JP, Hellhammer DH. Abuse-related posttraumatic stress disorder and alterations of the hypothalamic-pituitary-adrenal axis in women with chronic pelvic pain. *Psychosom Med* (1998) **60**:309–18. doi:10.1097/00006842-199805000-00017
113. Straub RH, Buttgereit F, Cutolo M. Alterations of the hypothalamic-pituitary-adrenal axis in systemic immune diseases – a role for misguided energy regulation. *Clin Exp Rheumatol* (2011) **29**:S23–31.
114. Kruger U, Spiecker H. Diagnosis of adrenal cortex insufficiency in steroid-dependent bronchial asthma – the CRH test in comparison with diurnal cortisol profile in serum and cortisol in 24-hour urine. *Pneumologie* (1994) **48**:793–8.
115. Papanicolaou DA, Tsigos C, Oldfield EH, Chrousos GP. Acute glucocorticoid deficiency is associated with plasma elevations of interleukin-6: does the latter participate in the symptomatology of the steroid withdrawal syndrome and adrenal insufficiency? *J Clin Endocrinol Metab* (1996) **81**:2303–6. doi:10.1210/jc.81.6.2303
116. Stirrat L, O'Reilly J, Riley S, Howie A, Smith R, Walker B, et al. PPO.21 Altered maternal hypothalamic-pituitary-adrenal axis activity in obese pregnancy: a potential mechanism underlying macrosomia and prolonged pregnancy. *Arch Dis Child Fetal Neonatal Ed* (2014) **99**:A157. doi:10.1136/archdischild-2014-306576.461
117. Mayer EA. The neurobiology of stress and gastrointestinal disease. *Gut* (2000) **47**:861–9. doi:10.1136/gut.47.6.861
118. Reber SO. Stress and animal models of inflammatory bowel disease – an update on the role of the hypothalamo-pituitary-adrenal axis. *Psychoneuroendocrinology* (2012) **37**:1–19. doi:10.1016/j.psyneuen.2011.05.014
119. Reinshagen M, Haefele T, Holicki S, Adler G, Klaus J, Dahlbender R, et al. The ultra low dose ACTH-test Identifies a subgroup of patients with Crohn's disease and an impaired hypothalamo-pituitary-adrenal (HPA) axis. *Gastroenterology* (2002) **122**:S1384.

120. Minderhoud IM, Oldenburg B, van Dam PS, van Berge Henegouwen GP. High prevalence of fatigue in quiescent inflammatory bowel disease is not related to adrenocortical insufficiency. *Am J Gastroenterol* (2003) **98**:1088–93. doi:10.1111/j.1572-0241.2003.07414.x
121. Garcia Rodriguez LA, Ruigomez A, Wallander MA, Johansson S, Olbe L. Detection of colorectal tumor and inflammatory bowel disease during follow-up of patients with initial diagnosis of irritable bowel syndrome. *Scand J Gastroenterol* (2000) **35**:306–11. doi:10.1080/003655200750024191
122. Miller GE, Cohen S, Ritchey AK. Chronic psychological stress and the regulation of pro-inflammatory cytokines: a glucocorticoid-resistance model. *Health Psychol* (2002) **21**:531–41. doi:10.1037/0278-6133.21.6.531
123. Holsboer F. The dexamethasone suppression test in depressed patients: clinical and biochemical aspects. *J Steroid Biochem* (1983) **19**:251–7. doi:10.1016/S0022-4731(83)80033-8
124. Myers B, McKlveen JM, Herman JP. Glucocorticoid actions on synapses, circuits, and behavior: implications for the energetics of stress. *Front Neuroendocrinol* (2014) **35**:180–96. doi:10.1016/j.yfrne.2013.12.003
125. Uchoa ET, Aguilera G, Herman JP, Fiedler JL, Deak T, de Sousa MB. Novel aspects of glucocorticoid actions. *J Neuroendocrinol* (2014) **26**:557–72. doi:10.1111/jne.12157
126. Faubion WA Jr, Loftus EV Jr, Harmsen WS, Zinsmeister AR, Sandborn WJ. The natural history of corticosteroid therapy for inflammatory bowel disease: a population-based study. *Gastroenterology* (2001) **121**:255–60. doi:10.1053/gast.2001.26279
127. Creed TJ, Probert CS. Review article: steroid resistance in inflammatory bowel disease – mechanisms and therapeutic strategies. *Aliment Pharmacol Ther* (2007) **25**:111–22. doi:10.1111/j.1365-2036.2006.03156.x
128. Harbuz M, Korendowych E, Jessop D, Crown A, Li Pdfan S, Kirwan JR. Hypothalamo-pituitary-adrenal axis dysregulation in patients with rheumatoid arthritis after the dexamethasone/corticotrophin releasing factor test. *J Endocrinol* (2003) **178**:55–60. doi:10.1677/joe.0.1780055
129. Selander RK. Behavior and genetic variation in natural populations. *Am Zool* (1970) **10**:53–66.
130. Reber SO, Birkeneder L, Veenema AH, Obermeier F, Falk W, Straub RH, et al. Adrenal insufficiency and colonic inflammation after a novel chronic psychosocial stress paradigm in mice: implications and mechanisms. *Endocrinology* (2007) **148**:670–82. doi:10.1210/en.2006-0983
131. Reber SO, Neumann ID. Defensive behavioral strategies and enhanced state anxiety during chronic subordinate colony housing are accompanied by reduced hypothalamic vasopressin, but not oxytocin, expression. *Ann N Y Acad Sci* (2008) **1148**:184–95. doi:10.1196/annals.1410.003
132. Forkwa TK, Neumann ID, Tamm ER, Ohlmann A, Reber SO. Short-term psychosocial stress protects photoreceptors from damage via corticosterone-mediated activation of the AKT pathway. *Exp Neurol* (2014) **252**:28–36. doi:10.1016/j.expneurol.2013.11.016
133. Füchsl AM, Uschold-Schmidt N, Reber SO. Chronic psychosocial stress in male mice causes an up-regulation of scavenger receptor class B type 1 protein in the adrenal glands. *Stress* (2013) **16**:461–8. doi:10.3109/10253890.2013.793303
134. Uschold-Schmidt N, Peterlik D, Füchsl AM, Reber SO. HPA axis changes during the initial phase of psychosocial stressor exposure in male mice. *J Endocrinol* (2013) **218**:193–203. doi:10.1530/joe-13-0027
135. Singewald GM, Nguyen NK, Neumann ID, Singewald N, Reber SO. Effect of chronic psychosocial stress-induced by subordinate colony (CSC) housing on brain neuronal activity patterns in mice. *Stress* (2009) **12**:58–69. doi:10.1080/10253890802042082
136. Ohl F, Toschi N, Wigger A, Henniger MS, Landgraf R. Dimensions of emotionality in a rat model of innate anxiety. *Behav Neurosci* (2001) **115**:429–36. doi:10.1037/0735-7044.115.2.429
137. Salome N, Landgraf R, Viltart O. Confinement to the open arm of the elevated-plus maze as anxiety paradigm: behavioral validation. *Behav Neurosci* (2006) **120**:719–23. doi:10.1037/0735-7044.120.3.719
138. Bartolomucci A, Palanza P, Sacerdote P, Ceresini G, Chirieleison A, Panerai AE, et al. Individual housing induces altered immuno-endocrine responses to psychological stress in male mice. *Psychoneuroendocrinology* (2003) **28**:540–58. doi:10.1016/S0306-4530(02)00039-2
139. Gasparotto OC, Lopes DM, Carobrez SG. Pair housing affects anxiety-like behaviors induced by a social but not by a physiological stressor in male Swiss mice. *Physiol Behav* (2005) **85**:603–12. doi:10.1016/j.physbeh.2005.06.014
140. Blanchard RJ, McKittrick CR, Blanchard DC. Animal models of social stress: effects on behavior and brain neurochemical systems. *Physiol Behav* (2001) **73**:261–71. doi:10.1016/S0031-9384(01)00449-8
141. Palanza P. Animal models of anxiety and depression: how are females different? *Neurosci Biobehav Rev* (2001) **25**:219–33. doi:10.1016/S0149-7634(01)00010-0
142. Uschold-Schmidt N, Nyuyki KD, Füchsl AM, Neumann ID, Reber SO. Chronic psychosocial stress results in sensitization of the HPA axis to acute heterotypic stressors despite a reduction of adrenal in vitro ACTH responsiveness. *Psychoneuroendocrinology* (2012) **37**:1676–87. doi:10.1016/j.psyneuen.2012.02.015
143. Kraemer FB. Adrenal cholesterol utilization. *Mol Cell Endocrinol* (2007) **26**(5–266):42–5. doi:10.1016/j.mce.2006.12.001
144. Rodrigueza WV, Thuahnai ST, Temel RE, Lund-Katz S, Phillips MC, Williams DL. Mechanism of scavenger receptor class B type I-mediated selective uptake of cholesteryl esters from high density lipoprotein to adrenal cells. *J Biol Chem* (1999) **274**:20344–50. doi:10.1074/jbc.274.29.20344
145. Reaven E, Tsai L, Azhar S. Intracellular events in the "selective" transport of lipoprotein-derived cholesteryl esters. *J Biol Chem* (1996) **271**:16208–17. doi:10.1074/jbc.271.27.16208
146. Gwynne JT, Strauss JF. The role of lipoproteins in steroidogenesis and cholesterol metabolism in steroidogenic glands. *Endocr Rev* (1982) **3**:299–329. doi:10.1210/edrv-3-3-299
147. Krieger M. Charting the fate of the "good cholesterol": identification and characterization of the high-density lipoprotein receptor SR-BI. *Annu Rev Biochem* (1999) **68**:523–58. doi:10.1146/annurev.biochem.68.1.523
148. Williams DL, Connelly MA, Temel RE, Swarnakar S, Phillips MC, de la Llera-Moya M, et al. Scavenger receptor Bl and cholesterol trafficking. *Curr Opin Lipidol* (1999) **10**:329–40. doi:10.1097/00041433-199908000-00007
149. Kovanen PT, Faust JR, Brown MS, Goldstein JL. Low density lipoprotein receptors in bovine adrenal cortex. I. Receptor-mediated uptake of low density lipoprotein and utilization of its cholesterol for steroid synthesis in cultured adrenocortical cells. *Endocrinology* (1979) **104**:599–609. doi:10.1210/endo-104-3-599
150. Rosol TJ, Yarrington JT, Latendresse J, Capen CC. Adrenal gland: structure, function, and mechanisms of toxicity. *Toxicol Pathol.* (2001) **29**(1):41–8.
151. Biason-Lauber A. Molecular medicine of steroid hormone biosynthesis. *Mol Aspects Med* (1998) **19**:155–220. doi:10.1016/S0098-2997(98)00004-1
152. Miller WL. Molecular biology of steroid hormone synthesis. *Endocr Rev* (1988) **9**:295–318. doi:10.1210/edrv-9-3-295
153. Sewer MB, Waterman MR. ACTH modulation of transcription factors responsible for steroid hydroxylase gene expression in the adrenal cortex. *Microsc Res Tech* (2003) **61**:300–7. doi:10.1002/jemt.10339
154. Sewer MB, Dammer EB, Jagarlapudi S. Transcriptional regulation of adrenocortical steroidogenic gene expression. *Drug Metab Rev* (2007) **39**:371–88. doi:10.1080/03602530701498828
155. Füchsl AM, Langgartner D, Reber SO. Mechanisms underlying the increased plasma ACTH levels in chronic psychosocially stressed male mice. *PLoS One* (2013) **8**:e84161. doi:10.1371/journal.pone.0084161
156. Bhatnagar S, Vining C. Facilitation of hypothalamic-pituitary-adrenal responses to novel stress following repeated social stress using the resident/intruder paradigm. *Horm Behav* (2003) **43**:158–65. doi:10.1016/S0018-506X(02)00011-9
157. Aguilera G. Regulation of pituitary ACTH secretion during chronic stress. *Front Neuroendocrinol* (1994) **15**:321–50. doi:10.1006/frne.1994.1013
158. Reber SO, Peters S, Slattery DA, Hofmann C, Schölmerich J, Neumann ID, et al. Mucosal immunosuppression and epithelial barrier defects are key events in murine psychosocial stress-induced colitis. *Brain Behav Immun* (2011) **25**:1153–61. doi:10.1016/j.bbi.2011.03.004
159. Baranyi J, Bakos N, Haller J. Social instability in female rats: the relationship between stress-related and anxiety-like consequences. *Physiol Behav* (2005) **84**:511–8. doi:10.1016/j.physbeh.2005.01.005
160. Schwartz MW, Strack AM, Dallman MF. Evidence that elevated plasma corticosterone levels are the cause of reduced hypothalamic corticotrophin-releasing hormone gene expression in diabetes. *Regul Pept* (1997) **72**:105–12. doi:10.1016/S0167-0115(97)01043-4
161. Chiodini I, Scillitani A. Role of cortisol hypersecretion in the pathogenesis of osteoporosis. *Recenti Prog Med* (2008) **99**:309–13.

162. Chrousos GP. Stress and disorders of the stress system. *Nat Rev Endocrinol* (2009) **5**:374–81. doi:10.1038/nrendo.2009.106
163. McEwen BS. Protective and damaging effects of stress mediators. *N Engl J Med* (1998) **338**:171–9. doi:10.1056/NEJM199801153380307
164. McEwen BS. Allostasis, allostatic load, and the aging nervous system: role of excitatory amino acids and excitotoxicity. *Neurochem Res* (2000) **25**:1219–31. doi:10.1023/A:1007687911139
165. Donner NC, Montoya CD, Lukkes JL, Lowry CA. Chronic non-invasive corticosterone administration abolishes the diurnal pattern of tph2 expression. *Psychoneuroendocrinology* (2011) **37**:645–61. doi:10.1016/j.psyneuen.2011.08.008
166. Karatsoreos IN, Bhagat SM, Bowles NP, Weil ZM, Pfaff DW, McEwen BS. Endocrine and physiological changes in response to chronic corticosterone: a potential model of the metabolic syndrome in mouse. *Endocrinology* (2010) **151**(5):2117–27. doi:10.1210/en.2009-1436
167. Lechner O, Dietrich H, Oliveira dos Santos A, Wiegers GJ, Schwarz S, Harbutz M, et al. Altered circadian rhythms of the stress hormone and melatonin response in lupus-prone MRL/MP-fasIpr mice. *J Autoimmun* (2000) **14**:325–33. doi:10.1006/jaut.2000.0375
168. Miller AH, Spencer RL, Pearce BD, Pisell TL, Tanapat P, Leung JJ, et al. Effects of viral infection on corticosterone secretion and glucocorticoid receptor binding in immune tissues. *Psychoneuroendocrinology* (1997) **22**:455–74. doi:10.1016/S0306-4530(97)00028-0
169. Schmidt D, Reber SO, Botteron C, Barth T, Peterlik D, Uschold N, et al. Chronic psychosocial stress promotes systemic immune activation and the development of inflammatory Th cell responses. *Brain Behav Immun* (2010) **24**:1097–104. doi:10.1016/j.bbi.2010.04.014
170. Rook GA, Raison CL, Lowry CA. Can we vaccinate against depression? *Drug Discov Today* (2012) **17**:451–8. doi:10.1016/j.drudis.2012.03.018
171. Rook GA, Lowry CA, Raison CL. Microbial 'old friends', immunoregulation and stress resilience. *Evol Med Public Health* (2013) **2013**:46–64. doi:10.1093/emph/eot004
172. Rook GA, Raison CL, Lowry CA. Childhood microbial experience, immunoregulation, inflammation and adult susceptibility to psychosocial stressors and depression in rich and poor countries. *Evol Med Public Health* (2013) **2013**:14–7. doi:10.1093/emph/eos005
173. Reber SO, Obermeier F, Straub RH, Veenema AH, Neumann ID. Aggravation of DSS-induced colitis after chronic subordinate colony (CSC) housing is partially mediated by adrenal mechanisms. *Stress* (2008) **11**:225–34. doi:10.1080/10253890701733351
174. Czech B, Neumann ID, Muller M, Reber SO, Hellerbrand C. Effect of chronic psychosocial stress on nonalcoholic steatohepatitis in mice. *Int J Clin Exp Pathol* (2013) **6**:1585–93.
175. Peters S, Slattery DA, Flor PJ, Neumann ID, Reber SO. Differential effects of baclofen and oxytocin on the increased ethanol consumption following chronic psychosocial stress in mice. *Addict Biol* (2013) **18**:66–77. doi:10.1111/adb.12001
176. Peters S, Grunwald N, Rummele P, Endlicher E, Lechner A, Neumann ID, et al. Chronic psychosocial stress increases the risk for inflammation-related colon carcinogenesis in male mice. *Stress* (2012) **15**:403–15. doi:10.3109/10253890.2011.631232
177. Edwards AV, Jones CT. The effect of splanchnic nerve section on the sensitivity of the adrenal cortex to adrenocorticotrophin in the calf. *J Physiol* (1987) **390**:23–31. doi:10.1113/jphysiol.1987.sp016683
178. Edwards AV, Jones CT. The effect of splanchnic nerve stimulation on adrenocortical activity in conscious calves. *J Physiol* (1987) **382**:385–96. doi:10.1113/jphysiol.1987.sp016373
179. Ulrich-Lai YM, Arnhold MM, Engeland WC. Adrenal splanchnic innervation contributes to the diurnal rhythm of plasma corticosterone in rats by modulating adrenal sensitivity to ACTH. *Am J Physiol Regul Integr Comp Physiol* (2006) **290**:R1128–35. doi:10.1152/ajpregu.00042.2003
180. Ehrhart-Bornstein M, Hinson JP, Bornstein SR, Scherbaum WA, Vinson GP. Intraadrenal interactions in the regulation of adrenocortical steroidogenesis. *Endocr Rev* (1998) **19**(2):101–43. doi:10.1210/edrv.19.2.0326
181. Ehrhart-Bornstein M, Bornstein SR. Cross-talk between adrenal medulla and adrenal cortex in stress. *Ann N Y Acad Sci* (2008) **1148**:112–7. doi:10.1196/annals.1410.053
182. Wotjak CT, Ganster J, Kohl G, Holsboer F, Landgraf R, Engelmann M. Dissociated central and peripheral release of vasopressin, but not oxytocin, in response to repeated swim stress: new insights into the secretory capacities of peptidergic neurons. *Neuroscience* (1998) **85**:1209–22. doi:10.1016/S0306-4522(97)00683-0
183. Landgraf R, Neumann I, Schwarzberg H. Central and peripheral release of vasopressin and oxytocin in the conscious rat after osmotic stimulation. *Brain Res* (1988) **457**:219–25. doi:10.1016/0006-8993(88)90689-0
184. Jaroenporn S, Nagaoka K, Ohta R, Watanabe G, Taya K. Direct effects of prolactin on adrenal steroid release in male hatano high-avoidance (HAA) rats may be mediated through Janus kinase 2 (Jak2) activity. *J Reprod Dev* (2007) **53**:887–93. doi:10.1262/jrd.18174
185. Jaroenporn S, Nagaoka K, Ohta R, Shirota M, Watanabe G, Taya K. Differences in adrenocortical secretory and gene expression responses to stimulation *in vitro* by ACTH or prolactin between high- and low-avoidance Hatano rats. *Informa Healthcare* (2009) **12**:22–9. doi:10.1080/10253890801976652
186. Stachowiak A, Macchi C, Nussdorfer G, Malendowicz L. Effects of oxytocin on the function and morphology of the rat adrenal cortex: in vitro and in vivo investigations. *Res Exp Med* (1995) **195**:265–74. doi:10.1007/BF02576797
187. Straub RH, Herfarth H, Falk W, Andus T, Scholmerich J. Uncoupling of the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis in inflammatory bowel disease? *J Neuroimmunol* (2002) **126**:116–25. doi:10.1016/S0165-5728(02)00047-4
188. Berton O, Aguerre S, Sarrieau A, Mormede P, Chaouloff F. Differential effects of social stress on central serotonergic activity and emotional reactivity in Lewis and spontaneously hypertensive rats. *Neuroscience* (1998) **82**:147–59. doi:10.1016/S0306-4522(97)00282-0
189. Engler H, Stefanski V. Social stress and T cell maturation in male rats: transient and persistent alterations in thymic function. *Psychoneuroendocrinology* (2003) **28**:951–69. doi:10.1016/S0306-4530(02)00117-8
190. Stefanski V. Social stress in loser rats: opposite immunological effects in submissive and subdominant males. *Physiol Behav* (1998) **63**:605–13. doi:10.1016/S0031-9384(97)00492-7
191. Zelena D, Haller J, Halasz J, Makara GB. Social stress of variable intensity: physiological and behavioral consequences. *Brain Res Bull* (1999) **48**:297–302. doi:10.1016/S0361-9230(98)00176-2
192. Choi DC, Nguyen MM, Tamashiro KL, Ma LY, Sakai RR, Herman JP. Chronic social stress in the visible burrow system modulates stress-related gene expression in the bed nucleus of the stria terminalis. *Physiol Behav* (2006) **89**:301–10. doi:10.1016/j.physbeh.2006.05.046
193. Lucas LR, Celen Z, Tamashiro KL, Blanchard RJ, Blanchard DC, Markham C, et al. Repeated exposure to social stress has long-term effects on indirect markers of dopaminergic activity in brain regions associated with motivated behavior. *Neuroscience* (2004) **124**:449–57. doi:10.1016/j.neuroscience.2003.12.009
194. Razzoli M, Carboni L, Andreoli M, Ballottari A, Arban R. Different susceptibility to social defeat stress of BalbC and C57BL6/J mice. *Behav Brain Res* (2011) **216**:100–8. doi:10.1016/j.bbr.2010.07.014
195. Savignac HM, Hyland NP, Dinan TG, Cryan JF. The effects of repeated social interaction stress on behavioural and physiological parameters in a stress-sensitive mouse strain. *Behav Brain Res* (2011) **216**:576–84. doi:10.1016/j.bbr.2010.08.049
196. Melhorn SJ, Krause EG, Scott KA, Mooney MR, Johnson JD, Woods SC, et al. Meal patterns and hypothalamic NPY expression during chronic social stress and recovery. *Am J Physiol Regul Integr Comp Physiol* (2010) **299**:R813–22. doi:10.1152/ajpregu.00820.2009
197. Blanchard DC, Spencer RL, Weiss SM, Blanchard RJ, McEwen B, Sakai RR. Visible burrow system as a model of chronic social stress: behavioral and neuroendocrine correlates. *Psychoneuroendocrinology* (1995) **20**:117–34. doi:10.1016/0306-4530(94)E0045-B
198. Engler H, Engler A, Bailey MT, Sheridan JF. Tissue-specific alterations in the glucocorticoid sensitivity of immune cells following repeated social defeat in mice. *J Neuroimmunol* (2005) **163**:110–9. doi:10.1016/j.jneuroim.2005.03.002
199. Reber SO, Obermeier F, Straub RH, Falk W, Neumann ID. Chronic intermittent psychosocial stress (social defeat/overcrowding) in mice increases the severity of an acute DSS-induced colitis and impairs regeneration. *Endocrinology* (2006) **147**:4968–76. doi:10.1210/en.2006-0347
200. Schmidt MV, Sterlemann V, Ganea K, Liebl C, Alam S, Harbich D, et al. Persistent neuroendocrine and behavioral effects of a novel, etiologically relevant mouse paradigm for chronic social stress during adolescence. *Psychoneuroendocrinology* (2007) **32**:417–29. doi:10.1016/j.psyneuen.2007.02.011

201. Stefanski V, Peschel A, Reber S. Social stress affects migration of blood T cells into lymphoid organs. *J Neuroimmunol* (2003) **138**:17–24. doi:10.1016/S0165-5728(03)00076-6
202. Avitsur R, Stark JL, Sheridan JF. Social stress induces glucocorticoid resistance in subordinate animals. *Horm Behav* (2001) **39**:247–57. doi:10.1006/hbeh.2001.1653
203. Engler H, Bailey MT, Engler A, Stiner-Jones LM, Quan N, Sheridan JF. Interleukin-1 receptor type 1-deficient mice fail to develop social stress-associated glucocorticoid resistance in the spleen. *Psychoneuroendocrinology* (2008) **33**:108–17. doi:10.1016/j.psyneuen.2007.10.007
204. Bailey MT, Engler H, Powell ND, Padgett DA, Sheridan JF. Repeated social defeat increases the bactericidal activity of splenic macrophages through a toll-like receptor-dependent pathway. *Am J Physiol Regul Integr Comp Physiol* (2007) **293**:R1180–90. doi:10.1152/ajpregu.00307.2007
205. Quan N, Avitsur R, Stark JL, He L, Shah M, Caligiuri M, et al. Social stress increases the susceptibility to endotoxic shock. *J Neuroimmunol* (2001) **115**:36–45. doi:10.1016/S0165-5728(01)00273-9
206. Bailey MT, Engler H, Sheridan JF. Stress induces the translocation of cutaneous and gastrointestinal microflora to secondary lymphoid organs of C57BL/6 mice. *J Neuroimmunol* (2006) **171**:29–37. doi:10.1016/j.jneuroim.2005.09.008
207. van Hogezand RA, Eichhorn RF, Choudry A, Veenendaal RA, Lamers CB. Malignancies in inflammatory bowel disease: fact or fiction? *Scand J Gastroenterol Suppl* (2002) **236**:48–53. doi:10.1080/003655202320621454
208. Eaden JA, Mayberry JF. Colorectal cancer complicating ulcerative colitis: a review. *Am J Gastroenterol* (2000) **95**:2710–9. doi:10.1111/j.1572-0241.2000.02297.x
209. Eaden JA, Abrams KR, Mayberry JF. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut* (2001) **48**:526–35. doi:10.1136/gut.48.4.526
210. Tanaka T, Kohno H, Suzuki R, Yamada Y, Sugie S, Mori H. A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. *Cancer Sci* (2003) **94**:965–73. doi:10.1111/j.1349-7006.2003.tb01386.x
211. Konishi F, Morson BC. Pathology of colorectal adenomas: a colonoscopic survey. *J Clin Pathol* (1982) **35**:830–41. doi:10.1136/jcp.35.8.830
212. Tanaka T. Colorectal carcinogenesis: review of human and experimental animal studies. *J Carcinog* (2009) **8**:5–24. doi:10.4103/1477-3163.49014
213. Botrugno OA, Fayard E, Annicotte JS, Haby C, Brennan T, Wendling O, et al. Synergy between LRH-1 and beta-catenin induces G1 cyclin-mediated cell proliferation. *Mol Cell* (2004) **15**:499–509. doi:10.1016/j.molcel.2004.07.009
214. Moon RT, Bowerman B, Boutros M, Perrimon N. The promise and perils of Wnt signaling through beta-catenin. *Science* (2002) **296**:1644–6. doi:10.1126/science.1071549
215. Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. *Cell* (1996) **87**:159–70. doi:10.1016/S0092-8674(00)81333-1
216. Oshima M, Oshima H, Kitagawa K, Kobayashi M, Itakura C, Taketo M. Loss of Apc heterozygosity and abnormal tissue building in nascent intestinal polyps in mice carrying a truncated Apc gene. *Proc Natl Acad Sci U S A* (1995) **92**:4482–6. doi:10.1073/pnas.92.10.4482
217. Schoonjans K, Dubuquoy L, Mebis J, Fayard E, Wendling O, Haby CL, et al. Liver receptor homolog 1 contributes to intestinal tumor formation through effects on cell cycle and inflammation. *Proc Natl Acad Sci U S A* (2005) **102**:2058–62. doi:10.1073/pnas.0409756102
218. McConnell BB, Yang VW. The role of inflammation in the pathogenesis of colorectal cancer. *Curr Colorectal Cancer Rep* (2009) **5**:69–74. doi:10.1007/s11888-009-0011-z
219. Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology* (1994) **107**:1183–8.
220. Oguma K, Oshima H, Aoki M, Uchio R, Naka K, Nakamura S, et al. Activated macrophages promote Wnt signalling through tumour necrosis factor-alpha in gastric tumour cells. *EMBO J* (2008) **27**:1671–81. doi:10.1038/emboj.2008.105
221. Martin F, Ladoire S, Mignot G, Apetoh L, Ghiringhelli F. Human FOXP3 and cancer. *Oncogene* (2010) **29**:4121–9. doi:10.1038/onc.2010.174
222. Yehuda R. Post-traumatic stress disorder. *N Engl J Med* (2002) **346**:108–14. doi:10.1056/NEJMra012941
223. Mulder EJH, Robles de Medina PG, Huizink AC, Van den Bergh BRH, Buitelaar JK, Visser GHA. Prenatal maternal stress: effects on pregnancy and the (unborn) child. *Early Hum Dev* (2002) **70**:3–14. doi:10.1016/S0378-3782(02)00075-0
224. Cherkasova M, Sulla EM, Dalena KL, Ponde MP, Hechtman L. Developmental course of attention deficit hyperactivity disorder and its predictors. *J Can Acad Child Adolesc Psychiatry* (2013) **22**:47–54.
225. Slattery DA, Cryan JF. The ups and downs of modelling mood disorders in rodents. *ILAR J* (2014) **55**:297–309. doi:10.1093/ilar/ilu026
226. Young JW, Henry BL, Geyer MA. Predictive animal models of mania: hits, misses and future directions. *Br J Pharmacol* (2011) **164**:1263–84. doi:10.1111/j.1476-5381.2011.01318.x
227. Gorka Z, Moryl E, Papp M. Effect of chronic mild stress on circadian rhythms in the locomotor activity in rats. *Pharmacol Biochem Behav* (1996) **54**:229–34. doi:10.1016/0091-3057(95)02173-6
228. Blanchard RJ, Yudko E, Dulloog L, Blanchard DC. Defense changes in stress nonresponsive subordinate males in a visible burrow system. *Physiol Behav* (2001) **72**:635–42. doi:10.1016/S0031-9384(00)00449-2
229. Sterlemann V, Ganea K, Liebl C, Harbich D, Alam S, Holsboer F, et al. Long-term behavioral and neuroendocrine alterations following chronic social stress in mice: implications for stress-related disorders. *Horm Behav* (2008) **53**:386–94. doi:10.1016/j.yhbeh.2007.11.001
230. Adamec RE, Shallow T. Lasting effects on rodent anxiety of a single exposure to a cat. *Physiol Behav* (1993) **54**:101–9. doi:10.1016/0031-9384(93)90050-P
231. Henniger MSH, Ohl F, Holter SM, Weienbacher P, Toschi N, Lorscher P, et al. Unconditioned anxiety and social behaviour in two rat lines selectively bred for high and low anxiety-related behaviour. *Behav Brain Res* (2000) **111**:153–63. doi:10.1016/S0166-4328(00)00151-0
232. Landgraf R, Wigger A. High vs low anxiety-related behavior rats: an animal model of extremes in trait anxiety. *Behav Genet* (2002) **32**:301–14. doi:10.1023/A:1020258104318
233. Tang X, Sanford LD. Home cage activity and activity-based measures of anxiety in 129P3/J, 129X1/SvJ and C57BL/6J mice. *Physiol Behav* (2005) **84**:105–15. doi:10.1016/j.physbeh.2004.10.017
234. Krishnan V, Han M-H, Graham DL, Berton O, Renthal W, Russo SJ, et al. Molecular adaptations underlying susceptibility and resistance to social defeat in brain reward regions. *Cell* (2007) **131**:391–404. doi:10.1016/j.cell.2007.09.018
235. Castro JE, Diessler S, Varea E, Marquez C, Larsen MH, Cordero MI, et al. Personality traits in rats predict vulnerability and resilience to developing stress-induced depression-like behaviors, HPA axis hyper-reactivity and brain changes in pERK1/2 activity. *Psychoneuroendocrinology* (2012) **37**:1209–23. doi:10.1016/j.psyneuen.2011.12.014
236. Krishnan V, Han MH, Mazei-Robison M, Iniguez SD, Ables JL, Vialou V, et al. AKT signaling within the ventral tegmental area regulates cellular and behavioral responses to stressful stimuli. *Biol Psychiatry* (2008) **64**:691–700. doi:10.1016/j.biopsych.2008.06.003
237. Schmidt MV, Scharf SH, Sterlemann V, Ganea K, Liebl C, Holsboer F, et al. High susceptibility to chronic social stress is associated with a depression-like phenotype. *Psychoneuroendocrinology* (2010) **35**:635–43. doi:10.1016/j.psyneuen.2009.10.002
238. Kessler RC, Berglund P, Demler O, Jin R, Merikangas KR, Walters EE. Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the national comorbidity survey replication. *Arch Gen Psychiatry* (2005) **62**:593–602. doi:10.1001/archpsyc.62.6.593
239. American-Psychiatric-Association. *Diagnostic and Statistical Manual of Mental Disorders*. 4th ed. Washington, DC: American Psychiatric Association Press (1994).
240. Berton O, McClung CA, DiLeone RJ, Krishnan V, Renthal W, Russo SJ, et al. Essential role of BDNF in the mesolimbic dopamine pathway in social defeat stress. *Science* (2006) **311**:864–8. doi:10.1126/science.1120972
241. Covington HE III, Maze I, LaPlant QC, Vialou VF, Ohnishi YN, Berton O, et al. Antidepressant actions of histone deacetylase inhibitors. *J Neurosci* (2009) **29**:11451–60. doi:10.1523/jneurosci.1758-09.2009
242. Krishnan V, Nestler EJ. The molecular neurobiology of depression. *Nature* (2008) **455**:894–902. doi:10.1038/nature07455
243. Lukas M, Toth I, Reber SO, Slattery DA, Veenema AH, Neumann ID. The neuropeptide oxytocin facilitates pro-social behavior and prevents social avoidance in rats and mice. *Neuropsychopharmacology* (2011) **36**:2159–68. doi:10.1038/npp.2011.95
244. Toth I, Neumann ID, Slattery DA. Social fear conditioning: a novel and specific animal model to study social anxiety disorder. *Neuropsychopharmacology* (2012) **37**:1433–43. doi:10.1038/npp.2011.329

245. Toth I, Neumann ID, Slattery DA. Social fear conditioning as an animal model of social anxiety disorder. *Curr Protoc Neurosci* (2013) **Chapter 9**:Unit 9.42. doi:10.1002/0471142301.ns0942s63
246. Kalueff AV, Avgustinovich DF, Kudryavtseva NN, Murphy DL. BDNF in anxiety and depression. *Science* (2006) **312**:1598–9. doi:10.1126/science.312.5780.1598
247. Hirschfeld RM. The comorbidity of major depression and anxiety disorders: recognition and management in primary care. *Prim Care Companion J Clin Psychiatry* (2001) **3**:244–54. doi:10.4088/PCC.v03n0609
248. Kennedy SH. Core symptoms of major depressive disorder: relevance to diagnosis and treatment. *Dialogues Clin Neurosci* (2008) **10**:271–7.
249. Kessler RC, Coccaro EF, Fava M, Jaeger S, Jin R, Walters E. The prevalence and correlates of DSM-IV intermittent explosive disorder in the national comorbidity survey replication. *Arch Gen Psychiatry* (2006) **63**:669–78. doi:10.1001/archpsyc.63.6.669
250. Mackenzie CS, Reynolds K, Chou KL, Pagura J, Sareen J. Prevalence and correlates of generalized anxiety disorder in a national sample of older adults. *Am J Geriatr Psychiatry* (2011) **19**:305–15. doi:10.1097/JGP.0b013e318202bc62
251. Cryan JF, Slattery DA. GABAB receptors and depression. Current status. *Adv Pharmacol* (2010) **58**:427–51. doi:10.1016/S1054-3589(10)58016-5
252. Kinsey SG, Bailey MT, Sheridan JF, Padgett DA, Avitsur R. Repeated social defeat causes increased anxiety-like behavior and alters splenocyte function in C57BL/6 and CD-1 mice. *Brain Behav Immun* (2007) **21**:458–66. doi:10.1016/j.bbi.2006.11.001
253. Cappell H, Herman CP. Alcohol and tension reduction. A review. *Q J Stud Alcohol* (1972) **33**:33–64.
254. Linsky AS, Straus MA, Colby JP Jr. Stressful events, stressful conditions and alcohol problems in the United States: a partial test of Bales's theory. *J Stud Alcohol* (1985) **46**:72–80.
255. Caldwell EE, Riccio DC. Alcohol self-administration in rats: modulation by temporal parameters related to repeated mild social defeat stress. *Alcohol* (2010) **44**:265–74. doi:10.1016/j.alcohol.2010.02.012
256. Croft AP, Brooks SP, Cole J, Little HJ. Social defeat increases alcohol preference of C57BL/10 strain mice; effect prevented by a CCKB antagonist. *Psychopharmacology (Berl)* (2005) **183**:163–70. doi:10.1007/s00213-005-0165-6
257. van Erp AM, Miczek KA. Persistent suppression of ethanol self-administration by brief social stress in rats and increased startle response as index of withdrawal. *Physiol Behav* (2001) **73**:301–11. doi:10.1016/S0031-9384(01)00458-9
258. Becker HC, Lopez MF, Doremus-Fitzwater TL. Effects of stress on alcohol drinking: a review of animal studies. *Psychopharmacology (Berl)* (2011) **218**:131–56. doi:10.1007/s00213-011-2443-9
259. Cippitelli A, Damadzic R, Singley E, Thorsell A, Ciccocioppo R, Eskay RL, et al. Pharmacological blockade of corticotropin-releasing hormone receptor 1 (CRH1R) reduces voluntary consumption of high alcohol concentrations in non-dependent Wistar rats. *Pharmacol Biochem Behav* (2012) **100**:522–9. doi:10.1016/j.pbb.2011.10.016
260. Molander A, Vengeliene V, Heilig M, Wurst W, Deussing JM, Spanagel R. Brain-specific inactivation of the Crhr1 gene inhibits post-dependent and stress-induced alcohol intake, but does not affect relapse-like drinking. *Neuropsychopharmacology* (2012) **37**:1047–56. doi:10.1038/npp.2011.297
261. Slattery DA, Neumann ID. Chronic icv oxytocin attenuates the pathological high anxiety state of selectively bred Wistar rats. *Neuropharmacology* (2010) **58**:56–61. doi:10.1016/j.neuropharm.2009.06.038
262. Slattery DA, Neumann ID. Oxytocin and major depressive disorder: experimental and clinical evidence for links to aetiology and possible treatment. *Pharmaceuticals* (2010) **3**:702–24. doi:10.3390/ph3030702
263. Blume A, Bosch OJ, Miklos S, Torner L, Wales L, Waldherr M, et al. Oxytocin reduces anxiety via ERK1/2 activation: local effect within the rat hypothalamic paraventricular nucleus. *Eur J Neurosci* (2008) **27**:1947–56. doi:10.1111/j.1460-9568.2008.06184.x
264. Caspi A, Sugden K, Moffitt TE, Taylor A, Craig IW, Harrington H, et al. Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. *Science* (2003) **301**:386–9. doi:10.1126/science.1083968
265. McCarthy MM, McDonald CH, Brooks PJ, Goldman D. An anxiolytic action of oxytocin is enhanced by estrogen in the mouse. *Physiol Behav* (1997) **60**:1209–15. doi:10.1016/S0031-9384(96)00212-0
266. Waldherr M, Neumann ID. Centrally released oxytocin mediates mating-induced anxiolysis in male rats. *Proc Natl Acad Sci U S A* (2007) **104**:16681–4. doi:10.1073/pnas.0705860104
267. Windle RJ, Shanks N, Lightman SL, Ingram CD. Central Oxytocin administration reduces stress-induced corticosterone release and anxiety behavior in rats. *Endocrinology* (1997) **138**:2829–34. doi:10.1210/en.138.7.2829
268. Windle RJ, Kershaw YM, Shanks N, Wood SA, Lightman SL, Ingram CD. Oxytocin attenuates stress-induced c-fos mRNA expression in specific forebrain regions associated with modulation of hypothalamo-pituitary-adrenal activity. *J Neurosci* (2004) **24**:2974–82. doi:10.1523/JNEUROSCI.3432-03.2004
269. Landgraf R, Neumann ID. Vasopressin and oxytocin release within the brain: a dynamic concept of multiple and variable modes of neuropeptide communication. *Front Neuroendocrinol* (2004) **25**:150–76. doi:10.1016/j.yfrne.2004.05.001
270. Peters S, Slattery DA, Uschold-Schmidt N, Reber SO, Neumann ID. Dose-dependent effects of chronic central infusion of oxytocin on anxiety, oxytocin receptor binding and stress-related parameters in mice. *Psychoneuroendocrinology* (2014) **42**:225–36. doi:10.1016/j.psyneuen.2014.01.021
271. Yoshida M, Takayanagi Y, Inoue K, Kimura T, Young LJ, Onaka T, et al. Evidence that oxytocin exerts anxiolytic effects via oxytocin receptor expressed in serotonergic neurons in mice. *J Neurosci* (2009) **29**:2259–71. doi:10.1523/jneurosci.5593-08.2009
272. Kirby LG, Zeeb FD, Winstanley CA. Contributions of serotonin in addiction vulnerability. *Neuropharmacology* (2011) **61**:421–32. doi:10.1016/j.neuropharm.2011.03.022
273. McGregor IS, Callaghan PD, Hunt GE. From ultrasocial to antisocial: a role for oxytocin in the acute reinforcing effects and long-term adverse consequences of drug use? *Br J Pharmacol* (2008) **154**:358–68. doi:10.1038/bjp.2008.132
274. Hebb ALO, Zacharko RM, Gauthier M, Trudel F, Laforest S, Drolet G. Brief exposure to predator odor and resultant anxiety enhances mesocorticolimbic activity and enkephalin expression in CD-1 mice. *Eur J Neurosci* (2004) **20**:2415–29. doi:10.1111/j.1460-9568.2004.03704.x
275. Steciuk M, Kram M, Kramer GL, Petty F. Decrease in stress-induced c-Fos-like immunoreactivity in the lateral septal nucleus of learned helpless rats. *Brain Res* (1999) **822**:256–9. doi:10.1016/S0006-8993(99)01134-8
276. Yadin E, Thomas E, Grishkat HL, Strickland CE. The role of the lateral septum in anxiolysis. *Physiol Behav* (1993) **53**:1077–83. doi:10.1016/0031-9384(93)90362-J
277. Salchner P, Sartori SB, Sinner C, Wigger A, Frank E, Landgraf R, et al. Air-jet and FG-7142-induced Fos expression differs in rats selectively bred for high and low anxiety-related behavior. *Neuropharmacology* (2006) **50**:1048–58. doi:10.1016/j.neuropharm.2006.02.008
278. Bannerman DM, Rawlins JN, McHugh SB, Deacon RM, Yee BK, Bast T, et al. Regional dissociations within the hippocampus--memory and anxiety. *Neurosci Biobehav Rev* (2004) **28**:273–83. doi:10.1016/j.neubiorev.2004.03.004
279. Yoon SH, Kim BH, Ye SK, Kim MH. Chronic non-social stress affects depressive behaviors but not anxiety in mice. *Korean J Physiol Pharmacol* (2014) **18**:263–8. doi:10.4196/kjpp.2014.18.3.263
280. Kulesskaya N, Karpova NN, Ma L, Tian L, Voikar V. Mixed housing with DBA/2 mice induces stress in C57BL/6 mice: implications for interventions based on social enrichment. *Front Behav Neurosci* (2014) **8**:257. doi:10.3389/fnbeh.2014.00257

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Stress and the commensal microbiota: importance in parturition and infant neurodevelopment

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The body is colonized by an enormous array of microbes that are collectively called the microbiota. During quiescent periods, microbial communities within the gut are relatively resistant to change. However, several factors that disrupt homeostasis can also significantly change gut microbial community structure. One factor that has been shown to change the composition of the gut microbiota is exposure to psychological stressors. Studies demonstrate that the commensal microbiota are involved in stressor-induced immunomodulation, but other biological effects are not yet known. This review discusses emerging evidence that the microbiota can impact the brain and behavior and indicates that stressor-induced alterations in the composition of gut microbial communities contribute to stressor-induced behavioral changes. This review will also discuss the evidence that such effects are most evident early in life, where both stress and the microbiota have been linked to birth outcomes, such as prematurity, and neurodevelopment. When considered together, a paradigm emerges in which stressor-induced alterations in commensal microbial populations significantly impact parturition and infant neurodevelopment.

Keywords: microbiota, psychological stress, neurodevelopment, prematurity, neuroimmune, anxiety, depression

INTRODUCTION

The hypothesis that the origins of adult disease are developmental, beginning *in utero* is called the "Barker hypothesis" after one of its leading proponents and the author of a study demonstrating increased risk of cardiovascular disease in infants born underweight (1). It states that adverse influences early in development, such as poor nutrition or infection, result in permanent physiological changes and in increased disease risk in adulthood. This is an area of increasing research in many fields, including Psychiatry, Immunology, and Endocrinology, which are now exploring whether development of diseases such as asthma, diabetes, and anxiety contains a developmental, intrauterine element. Neurodevelopment is exquisitely sensitive to perturbations in the maternal milieu, including diet, infection, and stress, with potentially long-lasting behavioral consequences. Disorders such as schizophrenia, anxiety, depression, and autism have been found to be associated with *in utero* and early neonatal exposure to these stimuli (2). Infants exposed to antenatal stress demonstrate increased risk of developing a host of childhood and adult diseases. While alterations in the hypothalamic–pituitary–adrenal (HPA) axis and immune function have been the target of investigation as underlying mechanisms conferring increased risk, the microbiome is an emerging candidate as a potential mediator of stress-induced pathogenesis (**Figure 1**).

Every surface of the body naturally harbors unique microbial communities comprised of archea, protists, viruses, and bacteria. To date, bacteria residing on mucosal surfaces, including the oral cavity, reproductive tract, and gastrointestinal tract, are the best characterized. These bacteria form highly ordered microbial communities as a result of ecological successions that select microbes that are best adapted for their given niche. Although these microbial communities are relatively resistant to change, it is recognized that factors such as alterations in diet and the administration of antibiotics can result in modifications in microbial community structure. Studies from this laboratory, as well as others, have demonstrated that psychosocial stressors can also impact microbial community structure in the gut. This review will briefly describe studies that have linked stressor-induced alterations in gut microbial community structure to alterations in immune system activity and behavioral responses. The potential impact of these interactions on pregnancy outcome and on infant development will also be discussed.

NEUROENDOCRINE-BACTERIAL INTERACTIONS

The field of psychoneuroimmunology has amply demonstrated that the physiological response to different types of stressor significantly impacts immune system reactivity to antigenic challenge [reviewed in Ref. (3)]. Primary mediators of the stress response, including endogenous glucocorticoids such as cortisol in humans and corticosterone in rodents, can affect immune system reactivity by suppressing the expression and activity of key transcription factors, such as NF-kB (4). Likewise, stressor-induced

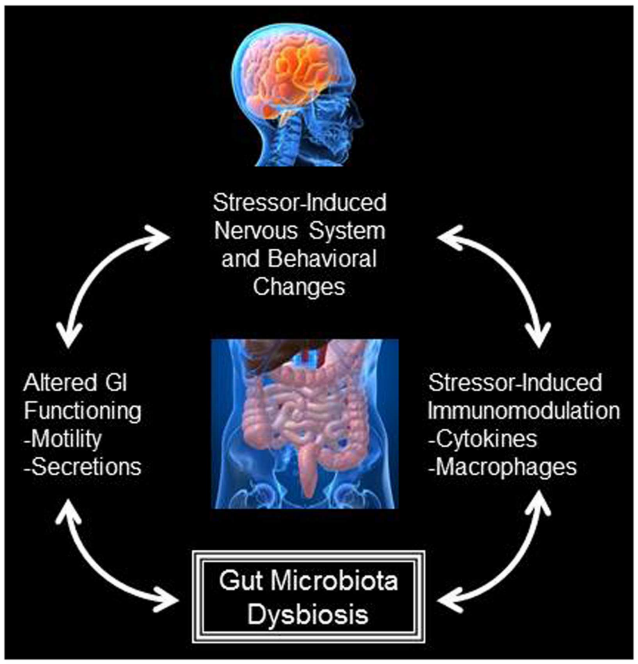

FIGURE 1 | Bidirectional interactions exist between the central nervous system and the gut microbiota. During exposure to stressful stimuli, the physiological stress response can lead to alterations in gastrointestinal functioning, such as alterations to gastrointestinal motility and the secretion of factors like gastric acid and bile. The physiological stress response is also known to impact immune system activity. Both alterations to gastrointestinal functioning and immune system activity can significantly change the composition of the gut microbiota. These alterations to the microbiota can feedback and impact gastrointestinal functioning, immune system activity, as well as the physiological stress response and stressor-induced behavioral states.

activation of the sympathetic nervous system, resulting in the release of endogenous catecholamine hormones (namely epinephrine from the adrenal medulla, and norepinephrine from adrenergic nerve terminals), can significantly increase or decrease immune cell activity depending on the leukocyte subset and the adrenergic receptor that is bound. As details of the importance of neuroendocrine mediators for leukocyte reactivity emerged (5), findings demonstrating that bacterial pathogens themselves also respond to neuroendocrine hormones began to emerge (6–12).

The growth of many types of bacteria, including both infectious and commensal organisms can be significantly impacted by neuroendocrine hormones. For example, the growth of commensal and of pathogenic *E. coli* can be increased over 10,000-fold by simply adding norepinephrine to a serum-based microbial medium (8, 10, 12–14). It is now recognized that a wide variety of neuroendocrine hormones can impact a vast array of bacteria in culture [reviewed in Ref. (10, 13)], however, demonstrating that direct neuroendocrine–bacterial interactions occur *in vivo* has been more challenging.

One of the first studies assessing the effects of neuroendocrine hormones on bacterial growth involved increasing norepinephrine levels *in vivo* using 6-hydroxydopamine, which lyses sympathetic nerve terminals. This resulted in an approximately 10,000-fold increase in commensal *E. coli* levels in the cecum of mice (11). The effects of norepinephrine on bacterial growth was also evident in an ileal loop model, where growing *Salmonella enterica* with norepinephrine prior to inoculation into the ileal loop significantly increased pathogen growth and associated disease (15). Findings that neuroendocrine mediators associated with the stress response could significantly impact bacterial growth led us to test whether stressor exposure could significantly change the levels of bacteria cultured from the intestines.

STRESSOR EXPOSURE AND THE COMMENSAL MICROBIOTA

Tannock and Savage demonstrated almost 40 years ago that moving mice into a cage lacking bedding, food, and water reduced the number of lactobacilli that could be cultured from the gastrointestinal tract (16). While this suggested that the stress response associated with new housing led to the differences, it was difficult to interpret these data due to the food and water deprivation. Thus, infant rhesus monkeys, with *ad libitum* access to food and water were exposed to a maternal separation stressor and the number of lactobacilli that could be cultured from the intestines was assessed. Exposure to the stressor significantly reduced lactobacilli levels, and the magnitude of the reduction was associated with stress-indicative behaviors. In general, monkeys which showed the greatest behavioral signs of distress also had the lowest levels of lactobacilli (17).

Stressor-induced reductions in lactobacilli have also been identified in humans during the stress of school examinations (18) and in murine studies utilizing prolonged restraint (19) or a short-lasting social stressor (19). The biological importance of stressor-induced alterations of the microbiota is not well understood. However, studies demonstrate that some aspects of stressor-induced increases in immune system reactivity are dependent upon the microbiota. For example, exposure to a social stressor, known as social disruption (SDR), significantly increases IL-6 levels in the blood and increases the reactivity of splenic macrophages to microbial stimulation. These effects, however, did not appear in germ-free (GF) mice or mice treated with antibiotics to reduce the microbiota (20). Similarly, exposing rats to repeat tail shock significantly increases cytokine levels in the blood; treatment with antibiotics to reduce the microbiota attenuated stressor-induced increases, but only for cytokines whose activation is dependent upon the inflammasome (21, 22). When considered together, these studies demonstrate the importance of the microbiota for stressor-induced immunopotentiation, and suggest that microbial products act as a danger signal to prime the immune system for enhanced reactivity (23).

STRESS, THE MICROBIOME, AND PRETERM BIRTH

While it is becoming increasing evident that stressor-induced alterations in the microbiota of adult animals can significantly impact host physiology, these effects are transient and return to baseline after termination of the stressor. However, the microbiota have exaggerated and prolonged effects when perturbed during gestation or early in infancy. Interest in the connection between stress and the human microbiome, and its impact specifically on female

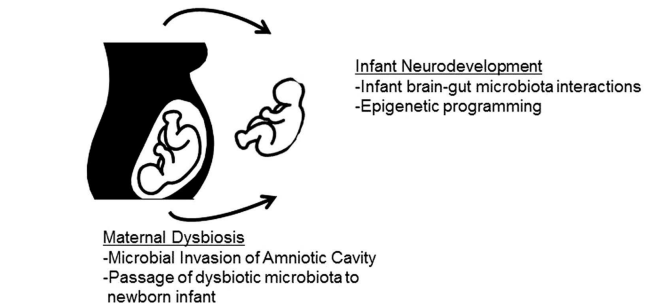

FIGURE 2 | Maternal stress can impact infant neurodevelopment through both microbiota-dependent and -independent pathways. The maternal physiological stress response can directly impact fetal development and may be involved with epigenetic changes in the offspring. In addition, the maternal physiological stress response may impact the commensal microbiota. Infants may become colonized with altered maternal microbes either *in utero*, through bacterial invasion of the amniotic cavity, or during/after birth when maternal microbes are passed to the infant. Either alterations in infant physiology (including epigenetic modifications) or colonization with dysbiotic microbial communities may lead to deficiencies in infant neurodevelopment.

reproduction and the developing fetus is evolving, but still in a nascent stage (**Figure 2**).

Preterm birth (PTB) is the first critical juncture to look at in the reproductive cycle when trying to discern the effects of stress and the microbiome on women, as stress is a well-known risk factor that affects the pillars of PTB: immune response/inflammation, and the HPA axis (24). There is considerable debate about stress, inflammation, cortisol, and PTB, and exact mechanisms are ripe for evaluation. Microbial invasion of the amniotic cavity and the associated host inflammatory response is a leading etiology of PTB (25–28). The mechanisms by which micro-organisms gain access to the decidua and amniotic cavity are not completely known, but are thought to involve invasion from microbes locally (e.g., lower gut or genital tract) or hematogenously (25). The ability of genital pathogens to invade the amnion and induce PTB has been well studied. However, the source of hematogenous microbes and their ability to invade the amniotic cavity remains unclear. Oral microbes have been proposed as a key source of microbes passed to the amniotic cavity via hematogenous transmission (29). This is based on recent evidence that the placenta harbors a commensal microbiome with a community structure that is more closely reflective of oral microbial communities than microbial communities found elsewhere in the body (30). In addition, periodontal diseases are often, but not always (31), related to increased risk of PTB (32). It is thought that during some cases of periodontal infection, oral pathogens, such as *Fusobacterium nucleatum* translocate from the oral cavity into circulation where they eventually reach and invade the pregnant uterus (32). Given the strong association between stress and PTB, as well as oral microbiota and PTB, it is important to consider the possibility that oral microbes play a critical role in linking the stress response to PTB. While it is known that stressor exposure can change the composition of the gut microbiota, and the ability of microbes to translocate from the lumen of the intestines to the interior of the body (19, 33–35), to date, it is not known whether stressor exposure also impacts the composition of the oral microbiota, or their ability to translocate to other organs. Moreover, the relationship between the various commensal microbial communities and the intrauterine environment is not well understood in part due to the lack of studies involving laboratory animal models, and this is certainly an important area of future studies.

STRESS, THE MICROBIOME, AND PARTURITION

With information suggesting that early-life exposures can be formative for neurodevelopmental, allergic, and immunological response later in life, attention can be turned to parturition for potential influencing factors specifically with the microbiome. Two studies suggest that the neonatal fecal microbiome is altered due to cesarean section at day 1 of life, and continues up to 6 weeks of age (36, 37). Meta-analysis suggests that a 20% increase in asthma rates exists among babies born with cesarean section, although substantial heterogeneity exists in the data (38). Breastfed babies have a different, more "beneficial" gut microbiota (39, 40), obtain different prebiotic human milk oligosaccharides (41), and acquire maternal intestinal bacteria from breast milk (42). In addition to having different routes of delivery, it is interesting to note that babies born via cesarean section are also born to mothers with higher levels of stress. Cesarean section, especially unplanned cesarean section, is perceived as severely stressful (43, 44) and can lead to significant increases in glucorticoid and catecholamine neuroendocrine hormones (45, 46). Thus, while the mode of delivery is undoubtedly important, delivery via cesarean section may have additional effects on the baby due to the high physiological stress response in the mother. For example, cesarean section is associated with a different human milk microbiome, potentially as a result of the stress of cesarean section. While never described before for stress, it is recognized that overall health of the mother can impact the milk microbiome; obese women's breast milk microbiome has been shown to be less diverse than healthy weight controls (47). Thus, it is likely that stress and hormonal alterations impact breast milk microbiome composition. Interestingly, early changes in the human gut microbiome with perinatal antibiotics were found to have an association with increased disease risks early in life (48).

NEONATAL MICROBIOME AND NEURODEVELOPMENT

There is accumulating evidence that the microbiome can influence behavior [Reviewed in Ref. (49)], supporting the concept of a microbiome–gut–brain axis. While specific mechanisms underlying this influence remain unknown, the presence of the microbiome in the placenta and amnion suggest proximity and capacity to influence the developing fetus. Studies examining the impact of microbiome on the developing central nervous system (CNS) have utilized GF mice that have decreased anxiety, as well as increased motor activity compared to conventional mice. These behavioral changes were accompanied by increased turnover of dopamine, norepinephrine, and serotonin in the striatum, though not, it

should be noted, in the hippocampus or frontal cortex. Levels of brain-derived neurotrophic factor (BDNF), a protein with a significant role in neurodevelopment, and the development of depression and anxiety, were found to be reduced in the hippocampus and amygdala. These are brain regions implicated in the pathogenesis of anxiety, suggesting a mechanistic substrate for the behavioral changes (50). Because BDNF has a significant role in neurodevelopment, these findings have tantalizing implications regarding the influence of stress, alterations in the microbiome, neonatal colonization, and neurodevelopmental disorders.

Not all aspects of neurodevelopment are influenced by the microbiota, and some components of microbial influences on neurodevelopment are gender-specific. For example, GF mice have also demonstrated significant deficits in social behavior (51), some of which, specifically social avoidance, were reversed with re-colonization post-weaning. However, social cognition did not improve with re-colonization, suggesting that social cognition is as amenable to microbial-based interventions.

The effects that the microbiota have on the developing CNS appear to be gender-dependent. GF male mice have increased levels of serotonin and its metabolites in their hippocampus, and these did not normalize with introduction of a regular microbiome following weaning. Immunological and neuroendocrine effects were also found, with GF mice demonstrating a blunted immune response, based on TNF-α production, with a larger effect in female mice. GF animals also had a stronger corticosterone response in relation to stress, with female mice showing a smaller response. Male GF mice showed decreased expression of BDNF; however, female mice did not (52).

When considered together, data from laboratory animals indicate that maternal microbes that are passed to the infant are necessary for normal neurodevelopment. Disrupting these microbial communities, or their passage to the infant, can in turn impact neurodevelopment. Moreover, there are significant sex differences in the impact of microbial colonization on neurodevelopment. While it is not yet clear how this occurs, sex difference mechanisms in mice may be due to estrous cycle hormones and the CNS serotonergic system, as the estrogen receptor (ER beta) has a role in the hippocampal serotonin concentration. It is unclear whether the estrogen and estrogen receptor have a larger impact, or whether the microbiome has a larger impact on serotonin, and further studies are needed.

NEONATAL MICROBIOME AND HEALTH OUTCOMES

Animal studies support the idea that alterations in neonatal life and gut microbiome can have substantial impact on long-lasting health. Rat offspring deprived of their mother showed increased permeability of the colonic mucosa and a 10- to 100-fold increase in bacterial adherence to colonic tissue and spleen translocation. Of note, these changes were prevented by injecting rat pups with a corticotropin-releasing hormone receptor antagonist daily during maternal separation, suggesting that the HPA axis mediated this effect (53). Further evidence of maternal influence on the offspring is provided by the finding that, in rats, maternal diet influenced offspring gut architecture (54) and high-fat diet in rat mothers led to decreases in maltase and sucrase in offspring, while *E. coli* introduced to rat mothers in pregnancy and lactation led to increased offspring intestinal permeability, systemic inflammation, and obesity for this next generation (55). Furthermore, *E coli* introduced in rat mothers led to changes in offspring weight and gut microbiota (56). Maternal administration of antibiotics in the prenatal period was associated with increased gut permeability and systemic inflammation (54) as well as increased visceral sensitivity (57). In rodents, maternal separation, a highly validated model of early-life stress, also lead to an increase in visceral sensitivity, with concomitant alterations in stress response and microbial community structure (58). A swine model for perinatal disturbance was adopted with oral antibiotics to sows. Short- and long-term changes were seen in paracellular permeability (59). Together, these studies suggest that altering either the maternal or perinatal flora has long-lasting implications on the gut of the offspring, as well as immunological repercussions. Mechanisms for this change are still being evaluated, but one potential route is that perinatal changes in bacterial colonization alter gastrointestinal heat shock protein expression with permanent implications on health (60).

HOW THE MICROBIOME MAY IMPACT NEURODEVELOPMENT AND FUTURE DIRECTIONS

While clear lines of evidence exist supporting an effect of stress on the microbiome, as well as the ability of stress to modulate neurodevelopment and behavioral changes, a delineated mechanism of action by the microbiome upon the CNS is an area of intense scrutiny and investigation (61–63). To date, the HPA axis and alterations in the immune response have accumulated the strongest evidence of associations with alterations in the microbiome.

The immune system, in addition to being directly influenced by stress, is a direct target of both microbiota and probiotic agents, and also has bidirectional communication with the CNS, making it an appealing candidate. The microbiome, through the innate immune system, alters levels of both pro- and anti-inflammatory cytokines, which are capable of directly impacting CNS function (64, 65). Stress also has a clear impact on the HPA axis, which is capable of regulating the inflammatory cascade, and growth of microbiota, as discussed above. However, directly tethering this phenomenon to the developing CNS is a more arduous task. While studies reviewed above demonstrate alterations in important players in CNS development, such as BDNF, with alterations (or lack of) microbiota, many steps in this process remain elusive.

Another potential mediator of the effect of stress on neurodevelopment, via the microbiome, is epigenetic regulation (66). This refers to heritable changes in gene expression not due to changes in DNA sequence, and epigenetic modification is a process known to be especially sensitive to early-life experiences. Thus, *in utero* and early-life alterations in microbiome could potentially utilize epigenetic processes to exert long-term behavioral changes, such as those described following psychosocial stressors. Indeed, a growing body of literature supports a major role of epigenetic modification in the neurobiology of psychiatric disorders (67). How might the microbiome influence epigenetics? Microbiotas are involved in the breakdown of nutrients, and in that process create metabolites with neuroactive properties, including amino acids and monoamines (68). Moreover, they are a key source of butyrate, which is a histone deacetylase (HDAC) inhibitor, a key

step in transcriptional regulation. However, whether these substances are able to make a substantial impact on CNS function itself, and how they would access the CNS from the periphery, is open to debate and requires further examination.

As a whole, the ability of the microbiome to impact the developing CNS, and participate in the effect of psychosocial stress is an exciting concept, because it is susceptible to targeting with pre-and probiotics, which has vast implications for both neurodevelopment and other health outcomes. While much work remains to be done to elucidate mechanisms, this daunting task is imperative to complete.

REFERENCES

1. Barker DJ, Osmond C. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *Lancet* (1986) **1**:1077–81. doi:10.1016/S0140-6736(86)91340-1
2. Bale TL, Baram TZ, Brown AS, Goldstein JM, Insel TR, McCarthy MM, et al. Early life programming and neurodevelopmental disorders. *Biol Psychiatry* (2010) **68**:314–9. doi:10.1016/j.biopsych.2010.05.028
3. Padgett DA, Glaser R. How stress influences the immune response. *Trends Immunol* (2003) **24**:444–8. doi:10.1016/S1471-4906(03)00173-X
4. Bellavance MA, Rivest S. The HPA – immune axis and the immunomodulatory actions of glucocorticoids in the brain. *Front Immunol* (2014) **5**:136. doi:10.3389/fimmu.2014.00136
5. Padro CJ, Sanders VM. Neuroendocrine regulation of inflammation. *Semin Immunol* (2014) **26**(5):357–68. doi:10.1016/j.smim.2014.01.003
6. Lyte M, Arulanandam BP, Frank CD. Production of shiga-like toxins by *Escherichia coli* O157:H7 can be influenced by the neuroendocrine hormone norepinephrine. *J Lab Clin Med* (1996) **128**:392–8. doi:10.1016/S0022-2143(96)80011-4
7. Lyte M. Induction of gram-negative bacterial growth by neurochemical containing banana (*Musa x paradisiaca*) extracts. *FEMS Microbiol Lett* (1997) **154**:245–50. doi:10.1111/j.1574-6968.1997.tb12651.x
8. Lyte M, Arulanandam B, Nguyen K, Frank C, Erickson A, Francis D. Norepinephrine induced growth and expression of virulence associated factors in enterotoxigenic and enterohemorrhagic strains of *Escherichia coli*. *Adv Exp Med Biol* (1997) **412**:331–9. doi:10.1007/978-1-4899-1828-4_54
9. Lyte M, Freestone PP, Neal CP, Olson BA, Haigh RD, Bayston R, et al. Stimulation of *Staphylococcus epidermidis* growth and biofilm formation by catecholamine inotropes. *Lancet* (2003) **361**:130–5. doi:10.1016/S0140-6736(03)12231-3
10. Lyte M. Microbial endocrinology and infectious disease in the 21st century. *Trends Microbiol* (2004) **12**:14–20. doi:10.1016/j.tim.2003.11.004
11. Lyte M, Bailey MT. Neuroendocrine-bacterial interactions in a neurotoxin-induced model of trauma. *J Surg Res* (1997) **70**:195–201. doi:10.1006/jsre.1997.5130
12. Lyte M, Nguyen KT. Alteration of *Escherichia coli* O157:H7 growth and molecular fingerprint by the neuroendocrine hormone noradrenaline. *Microbios* (1997) **89**:197–213.
13. Freestone PP, Sandrini SM, Haigh RD, Lyte M. Microbial endocrinology: how stress influences susceptibility to infection. *Trends Microbiol* (2008) **16**:55–64. doi:10.1016/j.tim.2007.11.005
14. Lyte M, Erickson AK, Arulanandam BP, Frank CD, Crawford MA, Francis DH. Norepinephrine-induced expression of the K99 pilus adhesin of enterotoxigenic *Escherichia coli*. *Biochem Biophys Res Commun* (1997) **232**:682–6. doi:10.1006/bbrc.1997.6356
15. Pullinger GD, Carnell SC, Sharaff FF, van Diemen PM, Dziva F, Morgan E, et al. Norepinephrine augments *Salmonella enterica*-induced enteritis in a manner associated with increased net replication but independent of the putative adrenergic sensor kinases QseC and QseE. *Infect Immun* (2010) **78**:372–80. doi:10.1128/IAI.01203-09
16. Tannock GW, Savage DC. Influences of dietary and environmental stress on microbial populations in the murine gastrointestinal tract. *Infect Immun* (1974) **9**:591–8.
17. Bailey MT, Coe CL. Maternal separation disrupts the integrity of the intestinal microflora in infant rhesus monkeys. *Dev Psychobiol* (1999) **35**:146–55. doi:10.1002/(SICI)1098-2302(199909)35:2<146::AID-DEV7>3.0.CO;2-G
18. Knowles SR, Nelson EA, Palombo EA. Investigating the role of perceived stress on bacterial flora activity and salivary cortisol secretion: a possible mechanism underlying susceptibility to illness. *Biol Psychol* (2008) **77**:132–7. doi:10.1016/j.biopsycho.2007.09.010
19. Galley JD, Nelson MC, Yu Z, Dowd SE, Walter J, Kumar PS, et al. Exposure to a social stressor disrupts the community structure of the colonic mucosa-associated microbiota. *BMC Microbiol* (2014) **14**:189. doi:10.1186/1471-2180-14-189
20. Bailey MT, Dowd SE, Galley JD, Hufnagle AR, Allen RG, Lyte M. Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. *Brain Behav Immun* (2011) **25**:397–407. doi:10.1016/j.bbi.2010.10.023
21. Maslanik T, Tannura K, Mahaffey L, Loughridge AB, Benninson L, Ursell L, et al. Commensal bacteria and MAMPs are necessary for stress-induced increases in IL-1beta and IL-18 but not IL-6, IL-10 or MCP-1. *PLoS One* (2012) **7**:e50636. doi:10.1371/journal.pone.0050636
22. Maslanik T, Mahaffey L, Tannura K, Beninson L, Greenwood BN, Fleshner M. The inflammasome and danger associated molecular patterns (DAMPs) are implicated in cytokine and chemokine responses following stressor exposure. *Brain Behav Immun* (2013) **28**:54–62. doi:10.1016/j.bbi.2012.10.014
23. Fleshner M. Stress-evoked sterile inflammation, danger associated molecular patterns (DAMPs), microbial associated molecular patterns (MAMPs) and the inflammasome. *Brain Behav Immun* (2013) **27**:1–7. doi:10.1016/j.bbi.2012.08.012
24. Shapiro GD, Fraser WD, Frasch MG, Seguin JR. Psychosocial stress in pregnancy and preterm birth: associations and mechanisms. *J Perinat Med* (2013) **41**:631–45. doi:10.1515/jpm-2012-0295
25. DiGiulio DB. Diversity of microbes in amniotic fluid. *Semin Fetal Neonatal Med* (2012) **17**:2–11. doi:10.1016/j.siny.2011.10.001
26. Goldenberg RL, Hauth JC, Andrews WW. Intrauterine infection and preterm delivery. *N Engl J Med* (2000) **342**:1500–7. doi:10.1056/NEJM200005183422007
27. Goncalves LF, Chaiworapongsa T, Romero R. Intrauterine infection and prematurity. *Ment Retard Dev Disabil Res Rev* (2002) **8**:3–13. doi:10.1002/mrdd.10008
28. Romero R, Gomez R, Chaiworapongsa T, Conoscenti G, Kim JC, Kim YM. The role of infection in preterm labour and delivery. *Paediatr Perinat Epidemiol* (2001) **15**(Suppl 2):41–56. doi:10.1046/j.1365-3016.2001.00007.x
29. Cetin I, Pileri P, Villa A, Calabrese S, Ottolenghi L, Abati S. Pathogenic mechanisms linking periodontal diseases with adverse pregnancy outcomes. *Reprod Sci* (2012) **19**:633–41. doi:10.1177/1933719111432871
30. Aagaard K, Ma J, Antony KM, Ganu R, Petrosino J, Versalovic J. The placenta harbors a unique microbiome. *Sci Transl Med* (2014) **6**:237ra65. doi:10.1126/scitranslmed.3008599
31. Abati S, Villa A, Cetin I, Dessole S, Luglie PF, Strohmenger L, et al. Lack of association between maternal periodontal status and adverse pregnancy outcomes: a multicentric epidemiologic study. *J Matern Fetal Neonatal Med* (2013) **26**:369–72. doi:10.3109/14767058.2012.733776
32. Madianos PN, Lieff S, Murtha AP, Boggess KA, Auten RL Jr, Beck JD, et al. Maternal periodontitis and prematurity. Part II: maternal infection and fetal exposure. *Ann Periodontol* (2001) **6**:175–82. doi:10.1902/annals.2001.6.1.175
33. Bailey MT, Engler H, Sheridan JF. Stress induces the translocation of cutaneous and gastrointestinal microflora to secondary lymphoid organs of C57BL/6 mice. *J Neuroimmunol* (2006) **171**:29–37. doi:10.1016/j.jneuroim.2005.09.008
34. Bailey MT, Dowd SE, Parry NM, Galley JD, Schauer DB, Lyte M. Stressor exposure disrupts commensal microbial populations in the intestines and leads to increased colonization by *Citrobacter rodentium*. *Infect Immun* (2010) **78**:1509–19. doi:10.1128/IAI.00862-09
35. Reber SO, Peters S, Slattery DA, Hofmann C, Scholmerich J, Neumann ID, et al. Mucosal immunosuppression and epithelial barrier defects are key events in murine psychosocial stress-induced colitis. *Brain Behav Immun* (2011) **25**:1153–61. doi:10.1016/j.bbi.2011.03.004
36. Biasucci G, Rubini M, Riboni S, Morelli L, Bessi E, Retetangos C. Mode of delivery affects the bacterial community in the newborn gut. *Early Hum Dev* (2010) **86**(Suppl 1):13–5. doi:10.1016/j.earlhumdev.2010.01.004
37. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, et al. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proc Natl Acad Sci U S A* (2010) **107**:11971–5. doi:10.1073/pnas.1002601107

38. Thavagnanam S, Fleming J, Bromley A, Shields MD, Cardwell CR. A meta-analysis of the association between caesarean section and childhood asthma. *Clin Exp Allergy* (2008) **38**:629–33. doi:10.1111/j.1365-2222.2007.02780.x
39. Fallani M, Young D, Scott J, Norin E, Amarri S, Adam R, et al. Intestinal microbiota of 6-week-old infants across Europe: geographic influence beyond delivery mode, breast-feeding, and antibiotics. *J Pediatr Gastroenterol Nutr* (2010) **51**:77–84. doi:10.1097/MPG.0b013e3181d1b11e
40. Roger LC, Costabile A, Holland DT, Hoyles L, McCartney AL. Examination of faecal *Bifidobacterium* populations in breast- and formula-fed infants during the first 18 months of life. *Microbiology* (2010) **156**:3329–41. doi:10.1099/mic.0.043224-0
41. Shen Q, Tuohy KM, Gibson GR, Ward RE. In vitro measurement of the impact of human milk oligosaccharides on the faecal microbiota of weaned formula-fed infants compared to a mixture of prebiotic fructooligosaccharides and galactooligosaccharides. *Lett Appl Microbiol* (2011) **52**:337–43. doi:10.1111/j.1472-765X.2011.03005.x
42. Albesharat R, Ehrmann MA, Korakli M, Yazaji S, Vogel RF. Phenotypic and genotypic analyses of lactic acid bacteria in local fermented food, breast milk and faeces of mothers and their babies. *Syst Appl Microbiol* (2011) **34**:148–55. doi:10.1016/j.syapm.2010.12.001
43. Ryding EL, Wijma K, Wijma B. Psychological impact of emergency cesarean section in comparison with elective cesarean section, instrumental and normal vaginal delivery. *J Psychosom Obstet Gynaecol* (1998) **19**:135–44. doi:10.3109/01674829809025691
44. Schindl M, Birner P, Reingrabner M, Joura E, Husslein P, Langer M. Elective cesarean section vs. spontaneous delivery: a comparative study of birth experience. *Acta Obstet Gynecol Scand* (2003) **82**:834–40. doi:10.1034/j.1600-0412.2003.00194.x
45. Loughran PG, Moore J, Dundee JW. Maternal stress response associated with caesarean delivery under general and epidural anaesthesia. *Br J Obstet Gynaecol* (1986) **93**:943–9. doi:10.1111/j.1471-0528.1986.tb08013.x
46. Scheinin M, Scheinin H, Ekblad U, Kanto J. Biological correlates of mental stress related to anticipated caesarean section. *Acta Anaesthesiol Scand* (1990) **34**:640–4. doi:10.1111/j.1399-6576.1990.tb03163.x
47. Cabrera-Rubio R, Collado MC, Laitinen K, Salminen S, Isolauri E, Mira A. The human milk microbiome changes over lactation and is shaped by maternal weight and mode of delivery. *Am J Clin Nutr* (2012) **96**:544–51. doi:10.3945/ajcn.112.037382
48. Bedford Russell AR, Murch SH. Could peripartum antibiotics have delayed health consequences for the infant? *BJOG* (2006) **113**:758–65. doi:10.1111/j.1471-0528.2006.00952.x
49. Dinan TG, Cryan JF. Melancholic microbes: a link between gut microbiota and depression? *Neurogastroenterol Motil* (2013) **25**:713–9. doi:10.1111/nmo.12198
50. Diaz HR, Wang S, Anuar F, Qian Y, Bjorkholm B, Samuelsson A, et al. Normal gut microbiota modulates brain development and behavior. *Proc Natl Acad Sci U S A* (2011) **108**:3047–52. doi:10.1073/pnas.1010529108
51. Desbonnet L, Clarke G, Shanahan F, Dinan TG, Cryan JF. Microbiota is essential for social development in the mouse. *Mol Psychiatry* (2014) **19**:146–8. doi:10.1038/mp.2013.65
52. Clarke G, Grenham S, Scully P, Fitzgerald P, Moloney RD, Shanahan F, et al. The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. *Mol Psychiatry* (2013) **18**:666–73. doi:10.1038/mp.2012.77
53. Gareau MG, Jury J, Yang PC, MacQueen G, Perdue MH. Neonatal maternal separation causes colonic dysfunction in rat pups including impaired host resistance. *Pediatr Res* (2006) **59**:83–8. doi:10.1203/01.pdr.0000190577.62426.45
54. Fak F, Ahrne S, Molin G, Jeppsson B, Westrom B. Microbial manipulation of the rat dam changes bacterial colonization and alters properties of the gut in her offspring. *Am J Physiol Gastrointest Liver Physiol* (2008) **294**:G148–54. doi:10.1152/ajpgi.00023.2007
55. Fak F, Karlsson CL, Ahrne S, Molin G, Westrom B. Effects of a high-fat diet during pregnancy and lactation are modulated by *E. coli* in rat offspring. *Int J Obes (Lond)* (2012) **36**:744–51. doi:10.1038/ijo.2011.118
56. Karlsson CL, Molin G, Fak F, Johansson Hagslatt ML, Jakesevic M, Hakansson A, et al. Effects on weight gain and gut microbiota in rats given bacterial supplements and a high-energy-dense diet from fetal life through to 6 months of age. *Br J Nutr* (2011) **106**:887–95. doi:10.1017/S0007114511001036
57. O'Mahony SM, Felice VD, Nally K, Savignac HM, Claesson MJ, Scully P, et al. Disturbance of the gut microbiota in early-life selectively affects visceral pain in adulthood without impacting cognitive or anxiety-related behaviors in male rats. *Neuroscience* (2014) **277**:885–901. doi:10.1016/j.neuroscience.2014.07.054
58. O'Mahony SM, Marchesi JR, Scully P, Codling C, Ceolho AM, Quigley EM, et al. Early life stress alters behavior, immunity, and microbiota in rats: implications for irritable bowel syndrome and psychiatric illnesses. *Biol Psychiatry* (2009) **65**(3):263–7. doi:10.1016/j.biopsych.2008.06.026
59. Boudry G, Morise A, Seve B, Huerou-Luron LE. Effect of milk formula protein content on intestinal barrier function in a porcine model of LBW neonates. *Pediatr Res* (2011) **69**:4–9. doi:10.1203/PDR.0b013e3181fc9d13
60. Lalles JP. Long term effects of pre- and early postnatal nutrition and environment on the gut. *J Anim Sci* (2012) **90**(Suppl 4):421–9. doi:10.2527/jas.53904
61. Funkhouser LJ, Bordenstein SR. Mom knows best: the universality of maternal microbial transmission. *PLoS Biol* (2013) **11**(8):e1001631. doi:10.1371/journal.pbio.1001631
62. Jašarević E, Rodgers AB,Tracy L. A novel role for maternal stress and microbial transmission in early life programming and neurodevelopment neurobiology of stress. *Neurobiol Stress* (2015) **1**:81–8.
63. Borre YE, O'Keeffe GW, Clarke G, Stanton C, Dinan TG, Cryan JF. Microbiota and neurodevelopmental windows: implications for brain disorders. *Trends Mol Med* (2014) **20**(9):509–18. doi:10.1016/j.molmed.2014.05.002
64. Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW. From inflammation to sickness and depression: when the immune system subjugates the brain. *Nat Rev Neurosci* (2008) **9**:46–56. doi:10.1038/nrn2297
65. Dantzer R, O'Connor JC, Lawson MA, Kelley KW. Inflammation-associated depression: from serotonin to kynurenine. *Psychoneuroendocrinology* (2011) **36**:426–36. doi:10.1016/j.psyneuen.2010.09.012
66. Stilling RM, Dinan TG, Cryan JF. Microbial genes, brain & behaviour – epigenetic regulation of the gut-brain axis. *Genes Brain Behav* (2014) **13**:69–86. doi:10.1111/gbb.12109
67. Tsankova N, Renthal W, Kumar A, Nestler EJ. Epigenetic regulation in psychiatric disorders. *Nat Rev Neurosci* (2007) **8**:355–67. doi:10.1038/nrn2132
68. Lyte M. Probiotics function mechanistically as delivery vehicles for neuroactive compounds: microbial endocrinology in the design and use of probiotics. *Bioessays* (2011) **33**:574–81. doi:10.1002/bies.201100024

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Stress-induced visceral pain: toward animal models of irritable-bowel syndrome and associated comorbidities

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Visceral pain is a global term used to describe pain originating from the internal organs, which is distinct from somatic pain. It is a hallmark of functional gastrointestinal disorders such as irritable-bowel syndrome (IBS). Currently, the treatment strategies targeting visceral pain are unsatisfactory, with development of novel therapeutics hindered by a lack of detailed knowledge of the underlying mechanisms. Stress has long been implicated in the pathophysiology of visceral pain in both preclinical and clinical studies. Here, we discuss the complex etiology of visceral pain reviewing our current understanding in the context of the role of stress, gender, gut microbiota alterations, and immune functioning. Furthermore, we review the role of glutamate, GABA, and epigenetic mechanisms as possible therapeutic strategies for the treatment of visceral pain for which there is an unmet medical need. Moreover, we discuss the most widely described rodent models used to model visceral pain in the preclinical setting. The theory behind, and application of, animal models is key for both the understanding of underlying mechanisms and design of future therapeutic interventions. Taken together, it is apparent that stress-induced visceral pain and its psychiatric comorbidities, as typified by IBS, has a multifaceted etiology. Moreover, treatment strategies still lag far behind when compared to other pain modalities. The development of novel, effective, and specific therapeutics for the treatment of visceral pain has never been more pertinent.

Keywords: visceral pain, stress, psychological, animal models, irritable-bowel syndrome, colorectal distension, microbiota–gut–brain axis

INTRODUCTION

Visceral pain is a severe form of pain that can be debilitating for the patient. Moreover, it affects a significant proportion of the population with up to 25% of people reporting visceral pain at any one time. Development of novel therapeutics is hindered by a lack of detailed knowledge of the underlying mechanisms, however progress is being made in this regard. The use of animal models has proved crucial in the advancement of our knowledge of what really is going on in visceral pain. This review aims to highlight the current state of play in the context of both preclinical and clinical research in the area of visceral pain. This review covers a broad range of research and as such in-depth details of studies is not included but is cited appropriately throughout. This review will summarize what is already known in the field and elude to future avenues yet to be explored in visceral pain research.

VISCERAL PAIN

Visceral pain is by definition, pain sensed as arising from the internal organs of the body (1). The pain may be described as sickening, deep, squeezing, and dull. Moreover, some organs are more sensitive to visceral pain than others (2). Diseases or disorders effecting certain organs such as the liver, lungs, or kidneys are commonly not associated with any overt symptoms of pain *per se* but mainly symptoms that are due to altered functioning of the organ itself. Conversely, other organs are far more sensitive to damage and can elicit excruciating pain. These organs include the stomach, bladder, and ureters (2, 3).

There are multiple etiologies for pain sensed in the internal organs, including: inflammation (acute and chronic), disruption of normal mechanical processes, neoplasms (benign or malignant), alterations in neurotransmission from the viscera, and ischemia (4–8).

Interestingly, visceral pain is intriguing in that pain is commonly felt in sites distant from the location of the organ itself. This referred pain, as it is known, is a key feature of visceral pain and is used by many clinicians in the diagnosis of certain diseases (1, 3). The pattern of pain sensation in referred pain can be similar across multiple organs and disease types, i.e., disorders of the gut, bladder, and other viscera are sensed as global abdominal pain, pelvic pain, or back pain, with specific localization very difficult to identify (3, 9, 10).

Visceral pain is the most common form of pain reported in the clinic and is the most common form of pain produced by disease (1). Although visceral pain is experienced by 25% of the population at any one time (11), in many cases it is insufficiently treated as it still remains to be considered as just a symptom of an underlying disease and not a disease in its own right. Over the last decades, the unsatisfactory treatment of visceral pain has led

to an immense economic and personal cost, with patients experiencing a reduced quality of life and increased work absenteeism with escalating healthcare costs (12, 13). However, more recent literature suggests that novel pharmaco-therapeutic targets such as linaclotide (14) and μ-opioid receptor agonists and antagonists, selective κ-opioid receptor agonists, anti-inflammatory drugs, serotonergic agents, bile acid modulators, and intestinal bile acid transporters are performing well in clinical trials (15). To build momentum on these advances in clinical treatments, we must strive to enhance our understanding of the underlying mechanisms of visceral pain to aid future development of novel therapeutics. To fully appreciate the complexity of visceral pain processing, we must first understand the characteristics and neurobiology of this pain modality.

CHARACTERISTICS OF VISCERAL PAIN

As mentioned earlier, visceral pain perception and psychological processing is divergent to that of somatic pain (1). Importantly, there are clear distinctions which set visceral pain aside from all other pain modalities. These clinical features are crucial for the understanding of this complex physiological process. The characteristics of visceral pain were first outlined by Cervero and Laird (1) and have advanced our appreciation of this complex phenomenon. These characteristics are summarized in **Table 1**.

TYPES OF VISCERAL PAIN

Visceral pain is the pain associated with a wide variety of disorders including gallstones, acute pancreatitis, acute appendicitis, diverticulitis, painful functional bowel syndromes such as irritable-bowel syndrome (IBS) and functional dyspepsia (FD), inflammatory bowel disease (IBD), gastroesophageal reflux disease (GERD), interstitial cystitis/bladder pain syndrome (IC/BPS), male chronic pelvic pain syndrome, and gynecological pain [endometriosis, vulvodynia, menstrual pain, polycystic ovary syndrome (PCOS)] (16–20). Moreover, and less commonly known is that visceral pain also encompasses chronic chest pain and colic (21, 22).

Appreciating this wide array of disorders has allowed us to understand the complex nature of the pathophysiology of visceral pain but have also induced considerable hurdles when aiming to fully understand the distinct subset of molecular mechanisms which underpin this phenomenon. For the remainder of this review, we will specifically discuss gastrointestinal (GI) visceral pain.

NEUROANATOMY OF VISCERAL PAIN

Knowing that the clinical characteristics of visceral pain are distinct from that of somatic pain, it implies that the neurobiology must also be distinct between these modalities. Indeed this appears to be the case with numerous groups reporting both anatomical and physiological differences (23–26).

Pathways for visceral sensation are diffusely organized both peripherally and centrally. Communication of sensory information from the GI tract to the central nervous system (CNS) occurs via vagal, pelvic, and splanchnic nerve pathways (27–29). Vagal afferents innervate the GI tract from the esophagus to the transverse colon (8). Pelvic nerves innervate the remaining parts of the colon and rectum (8). A smaller group of afferents called the splanchnic afferents whose cell bodies arise from the thoracolumbar region of the spinal cord innervate the whole GI tract (27), **Figure 1**.

More specifically, primary afferent nerve fibers innervating the viscera, project into the CNS via three pathways: (1) the vagus nerve and its branches; (2) sympathetic pathways; and (3) the pelvic nerve (parasympathetic pathways) and its branches (26). Primary afferents signaling to the CNS reside primarily in the vagal nodose ganglion, which project to the nucleus tractus solitarius (NTS) located within the medulla of the brainstem (8), and in the T2–L2 and S1–5 dorsal root ganglia (30).

Visceral primary afferents have been demonstrated to enter the spinal cord and form synapses with dorsal horn neurons ipsilateral and contralateral to the site of entry. The result is extensive, diffuse CNS activation (31, 32). These axons form the postsynaptic dorsal column pathway. Interestingly, the dorsal column in itself has been shown to relay visceral nociceptive information and is now

Table 1 | Characteristics of visceral pain [adapted from Cervero and Laird (1)].

Characteristics of visceral pain
1. Not all viscera have sensory innervation
2. It is not linked to visceral injury
3. It is referred to other locations
4. It is diffuse and poorly localized
5. It is accompanied by motor and autonomic reflexes

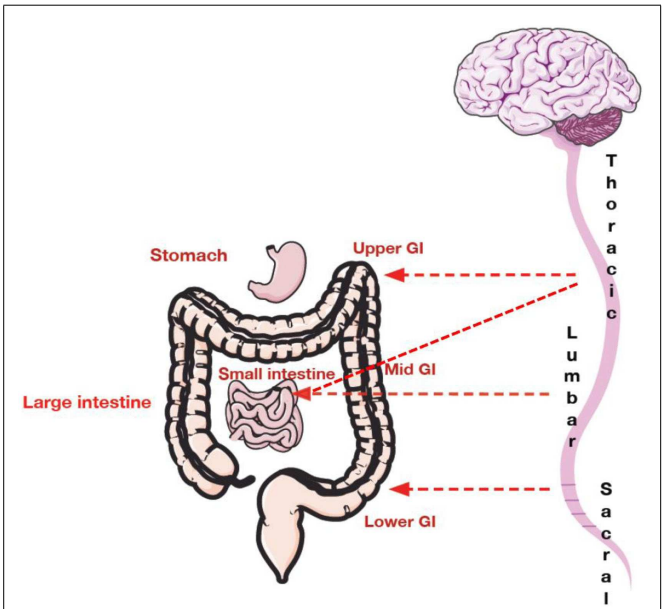

FIGURE 1 | Spinal innervation of the gastrointestinal tract. The upper GI tract (including esophagus and stomach) is innervated with thoracic and lumbar afferents. The mid GI tract composed of the small intestine is innervated by both thoracic and lumbar afferents. The mid to lower GI tract including the large intestine is innervated by lower lumbar afferents and upper sacral afferents. The pelvic region is innervated by sacral afferents.

thought of as a visceral pain pathway in its own right. Numerous clinical studies have shown that lesioning the fibers of the dorsal columns significantly relieves pain and decreases analgesic requirements in patients suffering from cancer originating in the visceral organs (33, 34).

Visceral afferents terminate in laminae I, $II_{(outer)}$, V–VII and X in the spinal cord. Laminae I and V form part of the spinothalamic tract, laminae VII and X constitute part of the dorsal column pathway (35, 36).

Second-order processing of visceral stimuli occurs at spinal segments and brainstem sites receiving primary afferent input. **Figure 2** depicts the principal visceral afferent pathways projecting to the spinal cord, and then ascending to the thalamus and midbrain. These are the spinothalamic, spinoreticular, and spinomesencephalic tracts (37, 38). The spinothalamic tract transmits sensory information from the spinal cord to the reticular formation in the brainstem and terminates in the thalamus (medial and posterior) at which point the thalamocortical fibers project to the primary somatosensory cortex (38). This tract is responsible for sensory discrimination and localization of painful stimuli and thus the reflexive, affective, and motivational properties of noxious stimulation (38, 39).

The brain regions innervated by these pathways, more commonly known as the pain matrix, which are activated in response to colorectal stimulation include the prefrontal cortex (dorsolateral), the insula, the thalamus, the amygdala, and the anterior cingulate cortex (ACC) (38). The ACC is a critical component in the pain matrix and is functionally divided into two discrete regions, the perigenual ACC and the midcingulate cortex (MCC), with the former involved in affect and the latter in behavioral response modification (38, 40). Moreover, the amygdala, in particular, the central nucleus of the amygdala (CeA), is a critical region in the limbic system and the pain matrix. The CeA integrates many signals for the processing of painful stimuli. It receives input from the brainstem, as well as more complex information from the thalamus and the cerebral cortex. Moreover, it has also been shown to have direct synaptic interactions with locus coeruleus (LC) neurons (41), highlighting a clear role of stress pathways in the development of visceral pain. Furthermore, the amygdala is also known to receive afferents from the spinal cord through the spino-(trigemino-)parabrachio-amygdaloid pathway (42). It is also involved in the descending pain pathway and is involved in the emotional, affective, and cognitive functions of pain processing. Numerous preclinical studies have shown the important role of the amygdala in pain processing (43–52).

This multicomponent integration of nociceptive information explains the variability in the experience and reporting of visceral pain (37, 53) and thus the difficulty in development of

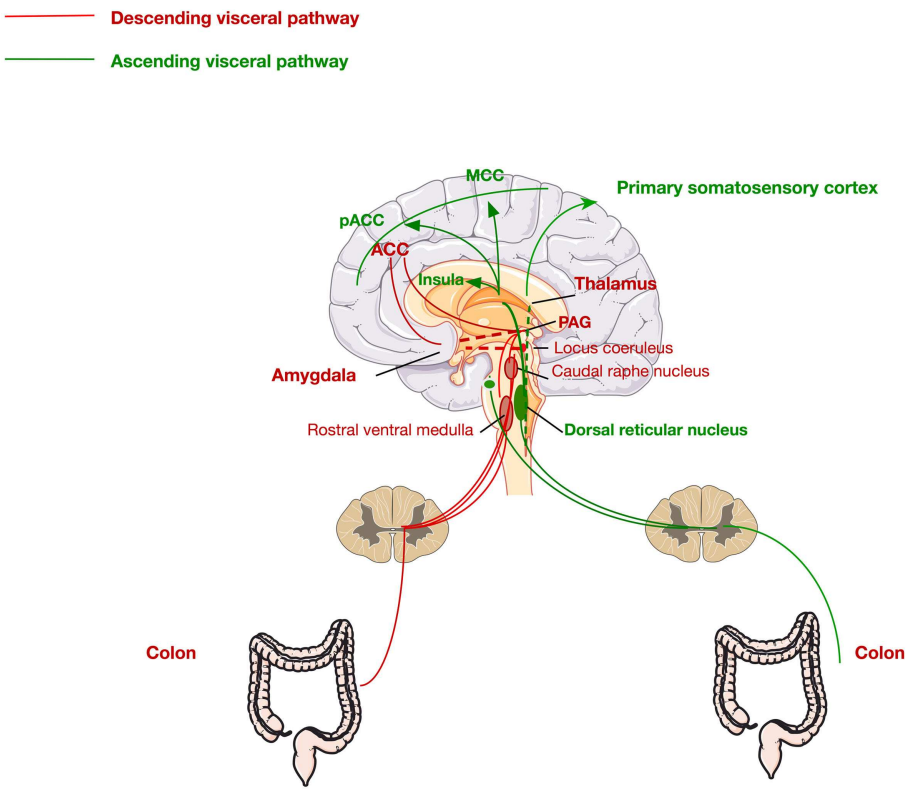

FIGURE 2 | Ascending and descending pathways mediating visceral pain sensation. The ascending pathway for visceral pain perception from the periphery through the dorsal root ganglia via the dorsal reticular nucleus to the primary somatosensory cortex, insula, pregenual anterior cingulate cortex (pACC), and the midcingulate cortex (MCC). The descending pathway is mediated via signals from the ACC, thalamus, and amygdala to the periaqueductal gray (PAG), locus coeruleus, and raphe nucleus, returning via the rostral ventral medulla to the colon.

effective pharmacological treatments. Interestingly, diffuse noxious inhibitory controls (DNIC) are a phenomenon more commonly referred to as "pain inhibiting pain" whereby a painful stimulus is applied to a part of the body, distant from the actual site of pain, thus inhibiting neurones within the dorsal horn of the spinal cord that are actively responding to chronic (unexplained) pain as seen in visceral hypersensitivity (54). DNIC is frequently used to quantify the central pain sensitization in chronic pain patients such as in the case of IBS (55–58). IBS patients consistently show a deficit in DNIC which correlates with symptom severity. These findings elude to the hypothesis that chronic pain patients are not only hyper-sensitive to pain but they also demonstrate reduced DNIC, possibly because of dysfunction of endogenous pain inhibition systems (55).

BIOCHEMICAL MEDIATORS OF VISCERAL PAIN

Neurotransmitters, cytokines, and other mediators such as peptides and neuropeptides are thought to mediate visceral nociceptive signals from the periphery to the central pathways. Indeed, mediators released during peripheral inflammation and injury, are thought to influence spinal nociceptors resulting in increased nociceptive activity and central sensitization (27). For example, the inflammation and irritation associated with bladder infections is believed to cause the release of glutamate that sensitizes visceral primary afferents (59). Moreover, glutamatergic signaling in particular, metabotropic glutamate (mGlu) receptors and glutamate reuptake are fast becoming attractive areas of research in the context of visceral pain (60, 61). However, there are a whole host of other mediators and receptors that are involved in visceral pain processing including; neurotransmitter receptors [acetylcholine nicotinic receptors (62), cannabinoid receptors (63), opioid receptors (64, 65), $GABA_A$, $GABA_B$, and $GABA_C$ receptors (66, 67), glutamate (ionotropic) receptors (68), glutamate (metabotropic) receptors (69, 70), glucocorticoid receptors (47, 71)], inflammatory receptors (bradykinin receptors, cholecystokinin receptors, cytokine receptors, leukotriene receptors, prostanoid receptors, tachykinin receptors, nitric oxide signaling, cyclooxygenase, lipoxygenase) (72, 73) and ion channel receptors [transient receptor potential vanilloid (TRPV) (74), purinergic (P2X) receptors (75), voltage-gated calcium channels (Ca_V), voltage-gated potassium (K_V) channels, voltage-gated sodium (Na_V) channels] (76). Due to the vast array of mediators and pathways, a clear pathophysiology of visceral pain remains to be elucidated thus hindering drug development.

TREATMENT OF VISCERAL PAIN

Although most visceral pain disorders are not life-threatening (non-cancer pain), they have a considerable negative impact on the quality of lives of patients with increased psychological distress, increased work absenteeism and both sleep and sexual dysfunction (77, 78). There are currently no pharmacological treatments on the market specifically for visceral pain. This leads to persistent bouts of discomfort and possible debilitation for the patient but also results in recurring visits to clinicians, associated with a significant economic burden on both the patient and healthcare services (12). Many patients are treated with multiple drug combinations to no avail. This void of effective analgesics in the context of visceral pain is frustrating for all parties involved. The drive to develop new analgesics has started right back to the basic molecular mechanisms of which very little is known (60, 79). Basic science and animal models have proved crucial in this effort for future developments of novel visceral analgesics.

In the clinical setting, treatment of visceral pain is extremely difficult. This is due to its complex nature, in that individuals can have many different triggering factors of their visceral pain and with no known cause, effective treatment strategies are difficult to identify. Treatment can differ from patient to patient and indeed treatment of the same patient over time may also change. As a result of this, a wide variety of pharmacological tools are used including a variety of analgesics [opioids (80), non-steroidal anti-inflammatory drugs (NSAIDS) (81), benzodiazepines (82)] and others (antibiotics, laxatives, serotonin modulators) (83, 84). Moreover, patients may also be treated with antispasmodics, particularly in the case of GI visceral pain and anti-depressants as well as others (84). Due to this heterogeneous pharmacological treatment profile, there are numerous and serious side effects including constipation, sedation, vomiting, tolerance, dependence, and addiction. However, none of the above are specific for the treatment of visceral pain *per se*, and mainly target some other features associated with chronic pain in general. However, in recent years, promising findings are emerging from both preclinical and clinical research which are nicely reviewed in Ref. (15, 83, 85, 86).

As with all chronic/severe pain disorders, opioids form the core of pain management for visceral pain conditions. However, as mentioned earlier, this class of analgesics are associated with the most serious side effects particularly over chronic use ranging from constipation (87) to analgesic tolerance (88–90) and nociceptive sensitization (91). More worryingly is the development of opioid-induced hyperalgesia after periods of prolonged opioid use (92–94).

Over the counter analgesics are routinely used by patients suffering with visceral pain. These include NSAIDS (aspirin, ibuprofen) and paracetamol. Again as with the case of most pharmacological agents used in the treatment of visceral pain, these agents do not specifically target visceral pain and only provide mild pain relief. Moreover, as these compounds are available over the counter they can be abused by consumers and carry a host of side effects not least GI bleeding, increased stomach acid, liver disease, liver failure, and even death in some cases (95).

Serotonergic compounds such as tegaserod (5-HT4 agonist) and alosetron (5-HT3 antagonist) (96–102) have been the main route of treatment for a range of visceral pain conditions, in particular IBS. However, tegaserod has since been removed from the market due to significant cardiovascular effects as outlined by the FDA. Alosetron was also withdrawn from the market in 2000 due to life-threatening adverse GI effects, however, in 2002, it was reintroduced but its availability and use were dramatically restricted.

Recent evidence from preclinical studies has pointed to a role of the transient receptor potential (TRP) channel family in the pathophysiology of visceral pain, which may lead to future development of novel therapeutics (103, 104). Moreover, both preclinical and clinical data have shown analgesic efficacy of pregabalin

and gabapentin in acute and chronic visceral pain conditions, acting both at spinal and supra-spinal levels in particular at the level of the rostral ventromedial medulla (RVM) (60, 105–108). Furthermore, the efficacy of mGlu receptors and also glial glutamate transporters have revealed themselves to be very promising targets (59, 61, 70, 109–115).

As mentioned previously, there is an unmet need for effective pharmacological therapeutics in the context of visceral pain. However, there are still major challenges to be met, not least in the fact that we still lack a clear understanding of the etiopathogenesis of visceral pain disorders.

COMORBIDITIES OF VISCERAL PAIN

Psychiatric disturbances are the most frequent comorbidity of visceral pain (116–125). In particular, anxiety and depression are the most commonly reported comorbidities. This complex link between visceral sensation and psychological perceptions are mediated via the brain–gut axis.

Moreover, stress-related changes in bowel habit can attest to the fact that the brain can influence gut function and sensation (126). Several clinical studies have suggested that psychosocial comorbidity is a major contributor to the severity and impact on quality of life of visceral pain disorders such as IBS and somatic pain disorders such as fibromyalgia (127–131). These findings are reinforced by a considerable volume of experimental research that links stress, anxiety, and depression to altered GI sensory and motor function as well as altered pain processing (8, 132–138). Indeed, successful management of patients with visceral pain disorders requires careful attention to these psychosocial factors, often in consultation with mental health professionals.

PATHOPHYSIOLOGY OF VISCERAL PAIN

The etiology of visceral pain is most likely multi-factorial involving biological, psychological, and social factors leading many to describe visceral pain as a biopsychosocial disorder. Due to the array of comorbidities associated with visceral pain, it is clear that both the brain and viscera play significant roles through the brain–gut axis (139–142). Moreover, the emerging role of the gut microbiota on brain signaling has now led to the concept of the microbiota–brain–gut axis (143–145). Numerous other pathways and systems feed into this complex network of communication, including the stress axis and immune system. The role of the amygdala in IBS has been extensively reviewed in the context of brain–gut axis communication (146) and numerous clinical and preclinical trials have also highlighted an important contribution of the amygdala to visceral pain processing and IBS (52, 147, 148). Here, we discuss the pathophysiology of visceral pain in terms of signaling along the microbiota–brain–gut axis, the hypothalamic–pituitary–adrenal (HPA) axis, and the immune system. Many other factors such as gender, genetics, and epigenetics are implicated in these pathways which not only exacerbate visceral hyperalgesia but may also be predisposing factors for the development of visceral hypersensitivity in later life. Furthermore, we also discuss possible future targets for visceral analgesia including the modulation of mGlu receptors in addition to glial glutamate transporters and histone deacetylation.

STRESS

Stress was first described by Hans Selye almost 80 years ago, and is defined as an acute threat to the homeostasis of an organism (149). Stressors can be in the form of physical threats such as an adverse event, or psychological stressor, such as anticipation of a threat. Exposure to these stressors will elicit a sequence of physiological, emotional, and behavioral reactions that allow one to cope adequately with the situation (150–152). Behavioral effects of the stress response include increased awareness, improved cognition, and altered pain sensitivity. Physiological adaptations include increased cardiovascular function, enhanced respiratory rate, and altered metabolism, along with inhibition of feeding, digestion, growth, reproduction, and immunity (153, 154).

The complexity of the sequence of responses to stress involves a range of systems including endocrine, nervous, and immune systems. The efficiency of this response ensures that the correct behavioral and physiological changes occur so that the individual responds appropriately to the stressor presented and improves the individual's chance of survival (155, 156). Understandably, due to the considerable complexity of the stress response, a host of regulatory mechanisms are at play to ensure the stress response is tightly regulated and does not become pathogenic to the host. These regulatory mechanisms are present at all levels of the stress response but particularly so at the neuronal and endocrine level which function to tightly regulate this adaptive process (157). However, the body can also elicit maladaptive changes in brain structure and function in response to chronic and uncontrollable stressors, thus, leaving long-lasting signatures on global wellbeing (158–160).

Dysregulation of the stress response has been associated with a plethora of disorders and diseases including chronic and visceral pain, autoimmune diseases, hypertension, affective disorders, and major depression (8, 161). Deciphering the pathogenesis of such disorders and their aberrant regulatory mechanisms will aid future therapeutic strategies for treatment and prophylaxis of stress-related disorders including stress-induced visceral hypersensitivity (154).

THE HYPOTHALAMIC–PITUITARY–ADRENAL AXIS

The HPA axis is the main stress axis in mammals and its anatomical structures are located both in the CNS and in the periphery. The major components of the stress axis are localized in the paraventricular nucleus (PVN) of the hypothalamus, the pituitary gland (anterior lobe), and the adrenal gland (154). In response to stress, corticotrophin-releasing hormone (CRH) is released and travels to the anterior pituitary gland where binding of CRH to CRH receptors (CRHR1 and CRHR2) leads to the release of adrenocorticotropic hormone (ACTH) into systemic circulation. ACTH targets the adrenal cortex to produce and secrete glucocorticoids (154). Glucocorticoids are the main effector molecules of the stress response and regulate the physiological adaptations through binding to their intracellular receptors (162, 163). Dysregulation of the HPA axis via inadequate or excessive activation, is thought to contribute to the development of a wide array of pathologies (154, 162, 164). Indeed CRH and its receptors have been extensively investigated in the context of stress and visceral pain (165–177).

Taken together, it is apparent that stringent regulation of the HPA axis is essential to a normal adaptive and efficient stress response.

STRESS AND IRRITABLE-BOWEL SYNDROME

The association between stress and psychiatric disorders, is well known, however, what exact molecular changes occur to underpin this increased vulnerability to disease is on-going (145, 178). Psychiatric disorders in addition to stressful life events are predisposing factors for the development of functional gastrointestinal disorders (FGIDs) such as IBS. IBS is one of the most common FGIDs with an estimated prevalence of 10–20% (179). Symptoms include abdominal pain, altered stool consistency and frequency, bloating, and distension. The pathophysiology of IBS has implicated stress as one the most significant risk factors for the development of the disorder (180–183). Stress at different stages throughout life, and especially early in life, can have deleterious effects on both psychological wellbeing and GI function of the host (**Figure 5**). **Figure 5** depicts the roles of stress (vulnerability, trigger, perpetuating) in IBS pathophysiology at critical points throughout life.

Over the last decade, there has been an abundance of reports implicating stress in the onset or exacerbation of symptoms of IBS (182, 184–186). Moreover, in a preclinical setting, animal models of IBS are predominantly stress-based models (187) aimed at elucidating biomarkers of this complex biopsychosocial disorder. If we look at stress throughout our life, some critical developmental windows such as early life and adolescence are associated with the development of a wide variety of disorders, not least visceral pain disorders such as IBS. Stress, particularly during early life can manifest in many different forms including physical trauma, loss of a parent, abuse (physical/sexual), all of which have been associated with an increased risk of developing FGIDs later in life (188, 189). Furthermore, acute stressors such as sexual abuse, rape, traumatic event (near fatal event), and warfare are also risk factors for the development of IBS (122). Individuals responses to stress vary, a phenomenon thought to be based both on genetic and epigenetic mechanisms (180). The area of stress susceptibility and stress resilience is of interest across all areas of psychiatry (190, 191) and also in the context of comorbidities such as chronic pain disorders (192). Chronic stress may alter individual's responses and play a strong role in symptom exacerbation. For example, psychosocial stressors in the form of sustained, threatening life events have been associated with onset and symptom exacerbation in IBS (181, 182, 193–195). Moreover, chronic stress has also been shown to induce

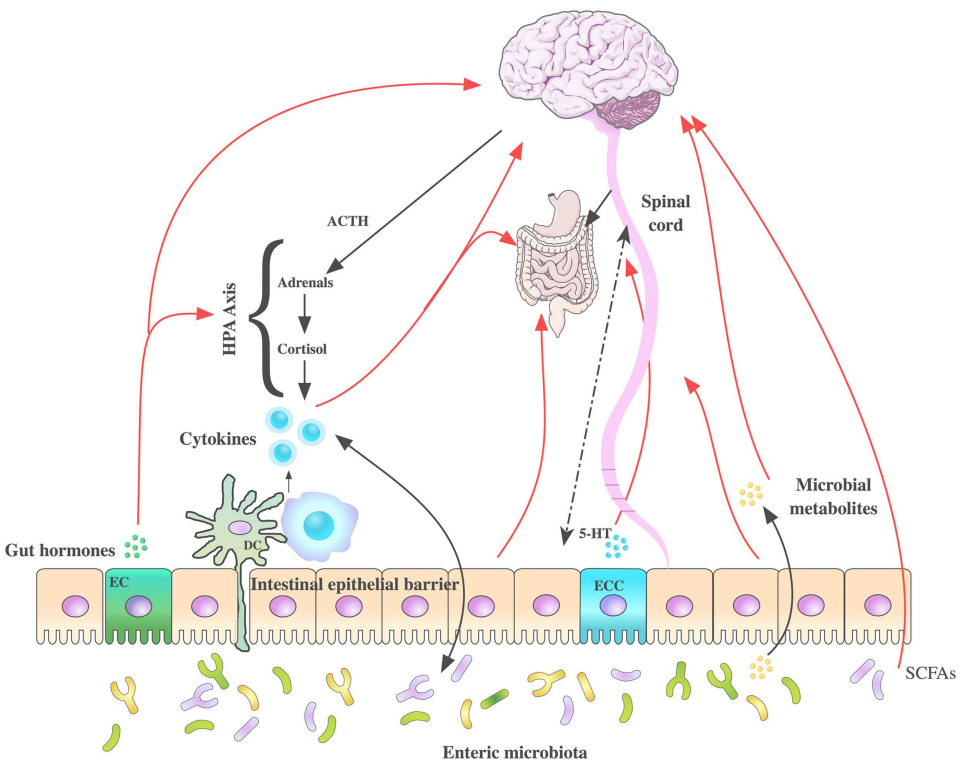

FIGURE 3 | Routes of communication along the microbiota–brain–gut axis. Several pathways have been proposed to understand the communication between the intestinal microbiota and brain function, some of which have been summarized in this figure. These include neuroendocrine (hypothalamic–pituitary–adrenal axis), immune system (neuromodulating cytokines), enteric nervous system, autonomic nervous systems (vagus nerve), and spinal afferents. 5-hydroxytryptamine (5-HT) is produced by enterochromaffin cells in the GI tract. Gut microbes produce tryptophan-related metabolites, gut hormones, short chain fatty acids (SCFAs), and neurometabolites GABA, noradrenaline, and dopamine potentially modulating CNS function. Stress can influence the microbial composition of the intestine through the release of stress hormones (corticosterone/cortisol) or sympathetic neurotransmitters that in turn can influence gut physiology and alter the microflora balance. DC, dendritic cell; EC, enteroendocrine cells; ECC, enterochromaffin cells.

adaptive changes in neuronal circuitry at the level of the CRH receptor 1 expression in the PVN of the rat hypothalamus, with reduced CRHR1 expression in comparison to acutely stressed rats after repeated homotypic stress exposures (196).

Differing coping strategies employed by patients, have also been shown to have a profound effect on symptom severity due in part to adaptation to stress (197–200). These studies highlight the high degree of catastrophizing or maladaptive coping within IBS groups. Indeed, positive coping strategies were shown to have positive effects on perceived stress levels and symptoms, however biomarkers such as cortisol remained unchanged. Furthermore, other functional somatic syndromes such as fibromyalgia are also known to exacerbate IBS symptoms (201, 202).

Psychological stressors are not the only risk factor for the development of IBS, but also physical stressors such as infection. Recent evidence from a systematic review and meta-analysis demonstrated that there was a sixfold increase in the risk of developing IBS after GI infection. Moreover, this enhanced risk remained elevated for at least 2–3 years post-infection (203, 204). The vicious cycle that stress plays in terms of vulnerability, trigger and perpetuation is most notable in patients who have developed IBS and associate particular situations, surroundings, or scenarios as provoking factors. This type of fear conditioning plays a key role in triggering stress responses to situations and contexts that by themselves are not threatening or stressful (205). In the context of IBS patients, this forward drive of conditioned fear-responses to both physical stimuli (infection) or contextual stimuli (location of stressful event) may be a significant factor in symptom chronicity (182). Using this knowledge and applying it to animal models will be discussed in later sections (206).

MICROBIOTA AND VISCERAL PAIN

THE MICROBIOTA–BRAIN–GUT AXIS

The bidirectional signaling network that exists between the GI tract and the brain is vital for maintaining homeostasis and is regulated at the neural [both central and enteric nervous systems (ENS)], hormonal, and immunological levels. Perturbation of these systems results in alterations in the stress response and overall behavior (143, 207). The high rate of psychiatric comorbidities with GI disorders and vice versa (208–210) are further evidence of the importance of this network of communication. The brain–gut axis is by no means a new discovery however its role in many disorders outside of gastroenterology has become an area of intense research. Moreover, advances in biomedical research have allowed us to elucidate the role of the gut microbiota community in signaling along this axis, which is now more commonly referred to as the microbiota–brain–gut axis (**Figure 3**). The impact of the microbiota–brain–gut axis has become a rapidly advancing research topic encompassing broad domains of biomedical research including neuroscience, psychiatry, gastroenterology, and microbiology.

The classical construct of the brain–gut axis, describes the way in which complex bidirectional signals can transmit between the CNS and the GI tract. This axis is critical for preserving gut homeostasis, dysregulation of which has been implicated in an array of disorders and disease states including gut inflammation, chronic abdominal pain syndromes, and eating disorders (143–145, 210–217). The brain–gut axis is responsible for some of our most basic functions such as facilitating the central regulation of digestive function. Indeed, the role of this axis is a well-developed concept in the area of food intake and satiety (218, 219) and more recently obesity (220, 221).

Moreover, the role of the microbiota–brain–gut axis in the stress response via modulation of the HPA axis has been investigated by numerous independent research groups (222–228). Furthermore, many forms of psychological stress have been shown to alter the gut microbiota fingerprint with many prebiotics and probiotics showing beneficial effects against the negative impact of stress. Thus, it is now acknowledged that the gut microbiota themselves are critical mediators of information along this bidirectional communication network (145, 207, 211, 213, 229, 230).

Indeed, in IBS patient cohorts, numerous independent research groups have shown distinct gut microbiota populations when compared to healthy controls (231–236). This was recently reviewed by Mayer and colleagues (237). Moreover, probiotic interventions appear to be beneficial to IBS patients (238).

Manipulation of the gut microbiota through the use of probiotic and prebiotics treatments have shown that by augmenting so-called "good bacteria" such as *Bifidobacteria* and *Lactobacillus*, in the gut, visceral hypersensitivity can be reversed in preclinical models. A mixture of eight probiotic bacteria strains (VSL#3) was shown to have protective effects against development of visceral hypersensitivity in the neonatal maternal separation model. Moreover, TPH1, tryptophan hydroxylase 1, the gene for the enzyme responsible for synthesizing serotonin, a key neurotransmitter involved in IBS treatment, was markedly up-regulated by neonatal maternal separation and this effect was reversed by VSL#3 intervention (239). Moreover, the same cocktail of probiotics was shown to prevent visceral hypersensitivity induced by inflammation via intra-colonic instillation of 4% acetic acid when given prophylactically (240). *Bifidobacterium* species particularly, *Bifidobacterium infantis* 35624 has been shown to be particularly effective at ameliorating visceral hyperalgesia in both stress-induced visceral hypersensitivity and colitis (241–243). Moreover, *Lactobacillus* species have also displayed efficacy in visceral pain models (244–247).

Furthermore, antibiotic-induced visceral hypersensitivity again underpins a role of the gut microbiota in the pathophysiology of visceral pain (244, 248). Interestingly, rifaximin, a semisynthetic, non-absorbable antibiotic that demonstrates no clinically relevant bacterial resistance has also shown positive effects in the treatment of IBS (249–257). These findings may seem contradictory, however, rifaximin is particularly efficacious in cases of small bowel bacterial overgrowth found in IBS patients. These findings add to the growing literature that microbiota dysfunction may be a key player in the pathophysiology of IBS and may lead to future novel therapeutic interventions.

IMMUNE SYSTEM AND VISCERAL PAIN

The immune system and thus inflammation have long been associated with psychiatric disorders, in particular, depression (258–260) and chronic pain disorders (261). Depression is a common comorbidity of visceral pain, as discussed earlier, so it is not

surprising that a common mechanism such as neuroinflammation may be at play. The immune system is a critical component of the microbiota–brain–gut axis and plays vital roles in maintaining homeostasis in the nervous systems and GI tract (262). Moreover, direct communication occurs between the immune system and the HPA axis, autonomic nervous system (ANS) and ENS (263–267). These integrated pathways are all known to be involved in the pathophysiology of visceral pain, and thus it is not unforeseen that the immune system plays a key role in the development of visceral hypersensitivity.

MICROGLIA AND VISCERAL PAIN

Microglia represent the first line of defense for the CNS, acting as a sensor for pathological events (268). The process of central sensitization and the subsequent changes in synaptic plasticity has long been thought to play a major role in nociceptive processes both in the context of chronic pain as well as acute, in both somatic and visceral modalities (269, 270). In the last decade, the role of microglia, both spinal and supra-spinal, has become an area of interest in the context of nociception (271–273). In animal models of both inflammatory and neuropathic pain, activation of microglia is a key step in the onset and maintenance of hypersensitivity and allodynia (274–279).

The role of spinal microglia in visceral pain has only recently been addressed and reviewed nicely by Lu (269). Saab and colleagues were first to report increased activated microglia in a rat model of chronic visceral hyperalgesia, namely the neonatal colon irritation model (280). Moreover, minocycline, a second-generation tetracycline compound known to interrupt microglia activation and its associated pro-inflammatory response, reversed the visceral hypersensitivity in adulthood (269, 280).

Furthermore, enhanced microglia activation was reported in the hippocampus, in a model of trinitrobenzene sulfonic acid (TNBS)-induced colitis, concomitant with increased tumor necrosis factor-α (281). However, this study did not assess nociceptive behavior, but one could predict visceral hyperalgesia as observed by others in the TNBS-model (282). More recently, it was shown that the activation of spinal microglia plays a critical role in the initiation and maintenance of visceral hypersensitivity in the TNBS-induced chronic pancreatitis (CP) rat model (283). Intrathecal injection of minocycline attenuated visceral hypersensitivity and phospho-p38 levels in CP rats.

In other non-inflammatory models of visceral hyperalgesia, it has also been shown that chronic psychological stress leads to microglia activation in the lumbar spinal cord (284). Moreover, this stress-induced increase in activated microglia could be blocked by SB203580, a p38 inhibitor or minocycline. Other compounds known to alter the microglia phenotype such as FKN, a microglia activator, was shown to induce visceral hypersensitivity in naïve rats when administered spinally, an effect which was blocked by minocycline (269), thus adding further evidence to support the role of spinal microglia in mediating stress-induced visceral hypersensitivity (269). Taken together, these studies suggest that microglia may play an important role in the pathogenesis of visceral pain.

However, it is also pertinent to note that exact role of inflammation and indeed micro-inflammation still remains controversial in the context of IBS. Indeed, no effect of anti-inflammatory drugs has been shown in IBS such as prednisolone (285).

TOLL-LIKE RECEPTORS AS NOVEL THERAPEUTIC TARGETS FOR VISCERAL ANALGESIA

Toll-like receptors (TLRs) are a family of pattern-recognition receptors of the innate immune system (**Figure 4**). There are 10 human TLRs and 12 mouse TLRs (286). TLR signaling consists of at least two distinct pathways: (1) the MyD88-dependent pathway, which leads to a pro-inflammatory phenotype, and (2) the MyD88-independent pathway, which leads to the production of interferon-β and the maturation of dendritic cells. The MyD88-dependent pathway is common to all TLRs, except TLR3 (287). TLRs are important players in the maintenance of mucosal and commensal homeostasis within the gut via innate host defense mechanisms. Intestinal dysbiosis and inflammation underlie several disease states affecting the GI tract such as IBS and IBD (288, 289). Indeed, reports have shown the involvement of peripheral TLR4 in patients suffering from IBS (290, 291) and in animal models of visceral pain (289, 292, 293). Moreover, the presence of TLR4 in the ENS and in the dorsal root ganglia indicate a role for TLR4 in the transmission of sensory information from the GI tract (294, 295). Furthermore, TLR4 is also expressed within the CNS, predominately in microglia (296), which have been discussed above and their role in visceral pain. Moreover, it is now emerging as a possible therapeutic target for neuropathic pain (297). Taken together, these findings suggest TLR4 as a promising novel target for the treatment of visceral pain.

Interaction of TLRs and Opioid Receptors

In recent years, it has been shown using *in vivo, in vitro*, and *in silico* techniques that members of each structural class of opioids (μ, κ, δ) activate TLR4 (298). Moreover, opioid antagonists such as naloxone and naltrexone non-stereoselectively block TLR4 signaling (299, 300). Modulation of TLR4 expression/function by acute blockade of TLR4, genetic knockout of TLR4, or blockade of TLR4 downstream signaling each lead to a potentiation of the magnitude and duration of opioid analgesia (301). These effects are thought to be mediated at both spinal and supra-spinal sites (299). Given the breadth of opioids now documented to interact with TLR4, many off-target opioid effects previously attributed to unilateral opioid action at classical neuronal opioid receptors might in fact result, at least in part, from the duality of opioid actions at TLR4 (301).

GENDER AND VISCERAL PAIN

Sex differences in pain sensitivity has been a topic of debate for many years and was recently reviewed by Mogil (302). More recently, gender differences in visceral pain and in particular IBS have been reviewed in Ref. (303) and indeed the contribution of sex hormones (304). Moreover, we can now appreciate that gender differences are also apparent in analgesic response (305). Many forms of visceral pain due to their nature are especially prevalent in women, i.e., pain associated with reproductive function (menstruation pains, pain of child birth, or postmenopausal pelvic pain). IBS is a disorder predominated by females (female to male ratio ~ 2:1) which is in parallel with women also being more

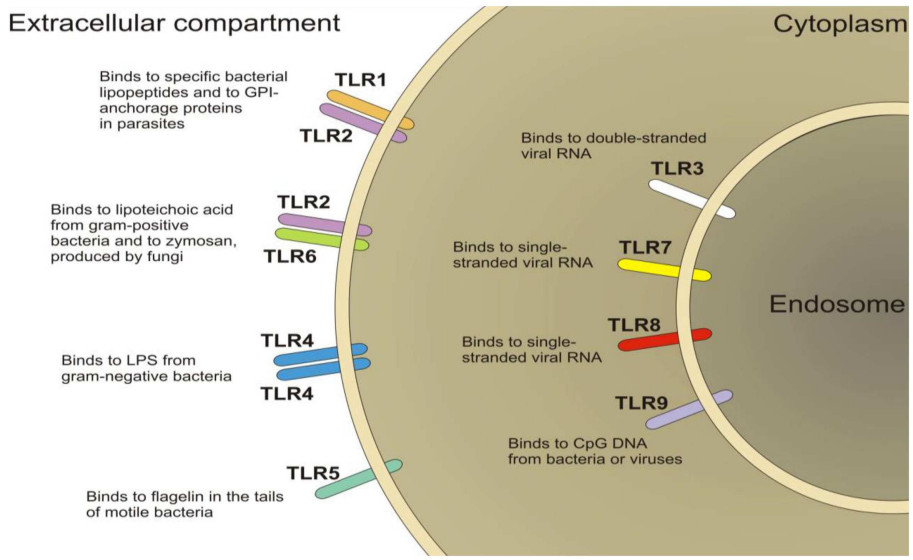

FIGURE 4 | Schematic of the localization of toll-like receptors (TLRs). TLRs are located on the plasma membrane (TLR1, TLR2, TLR4, TLR5, TLR6, TLR10) with the exception of TLR3, TLR7, TLR8, and TLR9, which are localized in the endosomal compartment.

susceptible to stress-related disorders (306, 307). Indeed, many studies have reported sex differences in the stress response itself and stress-induced pain modulation (8, 308). However, in the case of animal studies, the picture is not as clear. This is due in part to a bias for using male rodents in most preclinical studies addressing the role of stress modulation on visceral hypersensitivity (134, 135, 289, 309–311).

Sex/gender can influence the brain–gut axis, at an array of sites which logically will effect subsequent clinical outcomes including response to behavioral and drug therapies in patients suffering with visceral hypersensitivity (312). A plethora of factors including mood, stress, gender role, hormones, as well as inflammatory mediators modulate the brain–gut axis (313). As a result, the explanation for gender-related differences in visceral pain is likely multi-factorial involving environmental, psychological, and biological (sex) influences (312, 314, 315).

Studying gender differences in any context comes with significant difficulties, not least in the context of visceral pain disorders. Due to the greater number of women who are diagnosed with these disorders, there is often insufficient power to detect differences in the etiologic factors and treatment response by gender or sex. For example, several studies examining the effectiveness of cognitive behavioral therapy in reducing symptom distress have included only women (316, 317) or were disproportionately composed of women (318). In particular, the smaller number of men in drug trials leaves many studies underpowered to examine gender differences (313).

The variability in reproductive hormones such as estrogen and progesterone, across the menstrual cycle as well as significantly reduced ovarian function during and after menopause may explain changes in GI motility and visceral sensitivity (319). This has been reported in several studies documenting the impact of menstrual cycle on GI symptom reporting including visceral pain (320–323). The most consistent finding is that, many women with and without FGIDs experience an increase in GI symptoms including visceral pain during the late luteal and menses phases of the cycle relative to other cycle phases (313, 323–325). To add to the complexity, is the addition of pharmacological agents known to impact on the menstrual cycle and associated functions, such as oral contraceptive use and hormone replacement therapy. Moreover, the presence of other reproductive organ problems unique to women such as dysmenorrhea can also confound such studies.

It has been well established that adult females have higher basal and stress levels of ACTH and corticosterone than do males (326–330). Moreover, there is convergent lines of evidence reporting that gonadal hormones, specifically the estrogens, are important regulators of the HPA axis. Indeed, estrogen receptors α and β themselves have been shown to increase CRH expression (8, 331, 332). Females have higher CRH, ACTH, and corticosterone levels during proestrus (333), the phase of the cycle in which estradiol levels are highest, than during other phases of the estrous cycle (334–336). Fluctuations in gonadal hormones specifically, estradiol, during the menstrual cycle can also lead to changes in the neurotransmitter systems, in particular, the serotonergic and glutamatergic systems. Interactions between these systems have implications in the etiology and treatment of stress-related disorders and pain circuitry (142, 307, 337–340). Moreover, gonadal hormones have been shown to significantly affect visceral sensitivity in animal models, however some conflicting results have also shown no effect again highlighting the complexity of sex differences in pain processing both in a preclinical and clinical setting (173, 341–344). Interestingly, the gender of the experimenter was also shown to have a role in pain responses in preclinical models (345). Taken together, it is clear that the role of gender and/or sex in the pathophysiology of visceral pain remains a complex phenomenon which requires further investigation.

GLUTAMATE AND VISCERAL PAIN

IONOTROPIC GLUTAMATE RECEPTORS AND VISCERAL PAIN

N-Methyl-D-aspartate (NMDA) receptors are not only known for their important role in excitatory synaptic transmission but also for their role in pain (346). Glutamate found in vagal and spinal afferents contributes to nociceptive signaling via NMDA and non-NMDA receptors (347). Though evidence for direct links between glutamate and visceral nociception is lacking, NMDA receptor antagonists have been shown to reduce the response of vagal and pelvic afferents in the colon and other viscera to mechanical stimuli (348). A study by Coutinho and colleagues (349) investigated the role of glutamate receptors in the RVM in visceral hyperalgesia using a colonic irritation model. They found that activation of NMDA receptors by colonic inflammation facilitated visceral hyperalgesia while non-NMDA receptors mediate inhibitory effects (349). Moreover, NMDA receptors may also be involved in the transmission of visceral nociception in the non-inflamed gut (350). It was observed that activated peripheral NMDA receptors in the colon caused the release of pro-inflammatory peptides, calcitonin gene-related peptide (CGRP) and substance-P, which have significant roles in the mediation of chronic and severe pain (350).

METABOTROPIC GLUTAMATE RECEPTORS AND VISCERAL PAIN

Metabotropic glutamate receptors with the exception of mGlu6 receptor are expressed in all areas of the pain matrix from the spinal cord to supra-spinal sites. The action of mGlu receptors can be pro-nociceptive or anti-nociceptive depending on the subtype and site of action (351).

In the last decade, the role of mGlu receptors has gained attention in the realm of visceral pain. To our knowledge, the first study explicitly investigating the role of these receptors in visceral nociceptive processes was performed by Chen et al. (352). They found that antagonism of group 1 mGlu receptors with LY393053 reduced nociceptive behaviors in the mouse acetic acid writhing test. Furthermore, administration of a group 1 mGlu receptor agonist (S)-3,5-dihydroxyphenylglycine (DHPG) into the CeA by microdialysis increased the responses to innocuous visceral stimulation; an effect that was reversed by a reactive oxygen species (ROS) scavenger phenyl-*N*-*t*-butyl nitrone (PBN) and a superoxide dismutase mimetic (TEMPOL). In the same study, mGlu receptor 1 antagonist LY367385 was also found to decrease the responses to visceral stimulation (353).

Moreover, work by Lindstrom and colleagues investigated specifically the role of mGlu5 receptor in a rat model of visceral hypersensitivity (111). Here they found that mGlu5 receptor antagonism with MPEP [2-Methyl-6-(phenylethynyl)-pyridine] and MTEP [3-(2-Methyl-1,3-thiazol-4-yl)ethynyl]pyridine was sufficient to reduce the visceromotor response (VMR) in conscious Sprague-Dawley (SD) rats without altering colonic compliance. Moreover, mGlu5 receptor antagonism reduced colorectal distension (CRD)-evoked increases in heart rate and blood pressure (111). In this study, the effects seen could not be conclusively due to a peripheral or central site of action. Indeed, it has also been found that noxious colonic stimulation increases the number of Fos-positive neurons in the dorsal horn of the thoracic and lumbar spinal cord. Moreover, pre-treatment with MPEP significantly attenuated this (354).

Visceral pain originating from the bladder has also been shown to be mediated via mGlu receptors; specifically mGlu receptor 5 activation in the CeA induces bladder pain sensitization by increasing CeA output, an effect that was reversed by intra-amygdala MPEP treatment and lentivirus-mediated conditional disruption of mGlu5 receptor in the CeA (70).

GLUTAMATE TRANSPORTERS AND VISCERAL PAIN

Due to the negative side effects (psychomimetic activity) seen with modulation of ionotropic receptors, compounds which target these receptors are not suitable for long-term treatment of pain. To redress this and in the drive to develop better analgesics, other mechanisms such as glutamate reuptake may provide more effective treatments in controlling glutamate neurotransmission and thereby exert anti-nociceptive effects (109).

One of the first clear demonstrations that glutamate transport [via excitatory amino-acid transporters (EAATs)] may be implicated in pain processing was performed by Liaw and colleagues where they showed that selective inhibition of glutamate transporters with DL-threo-β-benzyloxyaspartate (TBOA) and dihydrokainate (DHK) produced a dose-dependent spontaneous nociceptive behavior response. These behaviors included licking, shaking, and caudally directed biting (355). Moreover, TBOA administered intrathecally was also found to induce visceral nociceptive behaviors in naïve rats (114). To further unravel the role of glutamate transport in visceral nociception, in particular stress-induced visceral hypersensitivity, the neuroprotective drug riluzole known to activate glutamate reuptake, was investigated to see whether it could attenuate visceral hypersensitivity induced by maternal separation of rats. It was found that riluzole reduces visceral hypersensitivity in stressed animals only, having no effect in non-separated animals. Moreover, EAAT-1 expression was also found to be reduced in the lumbar region of the spinal cord in hyper-sensitive animals. As mentioned previously, riluzole does not affect visceral sensitivity in naïve animals, further emphasizing the role of glutamate transport in pathological pain (114).

EAAT-2 is the main glial transporter for glutamate reuptake and its experimental over-expression in animal models has recently been found to be effective in reducing visceral pain (59, 112, 113). A study by Lin and colleagues (112) found the over-expression of EAAT-2 with the use of cephalosporin antibiotic ceftriaxone, in wildtype mice, attenuated the visceral nociceptive response to CRD. Moreover, similar effects were seen in the EAAT-2 overexpressing transgenic mouse (112). To further support these findings, systemic and intrathecal administration of DHK, a selective EAAT-2 inhibitor blocks the ceftriaxone-induced attenuation of visceral pain (112, 113). In subsequent studies performed by the same group assessing the efficacy of glutamate reuptake in animal models of colitis, it was shown that adeno-associated virus-mediated EAAT-2 over-expression was effective to mitigate VMR to 60 mmHg CRD (113). Interestingly, overexpression of EAAT-2 has been shown to reduce bladder nociception (59). However, colon irritation may affect afferents innervating the bladder thus giving a plausible mechanism for cross-organ sensitization (59). In the same study, it was also found that enhanced expression of EAAT-2 by ceftriaxone also reduced the VMR to bladder distension caused by colonic irritation (59).

As stated earlier, stress is one of the main predisposing factors for the development of visceral pain. Interestingly, glutamate transport has also been shown to be altered due to stress, with both early-life stress and stress in adulthood both showing significant alteration in EAAT expression (114, 115). Taken together, these findings provide evidence for an important role of glutamate transport in visceral pain states (271, 356). Moreover, glutamatergic signaling is critical to the process of central sensitization. This term describes the way in which excessive glutamatergic function within neurons and circuits, in particular pain circuits, leads to increases in membrane excitability and synaptic efficacy as well as reduced inhibition. These changes in synaptic function manifest as altered plasticity within the somatosensory nervous system in response to activity, inflammation, and neural injury (357).

GABA RECEPTORS AND VISCERAL PAIN

γ-Aminobutyric acid (GABA), the major inhibitory neurotransmitter in the CNS, plays an important role in anti-nociception. GABA is the key player acting at inhibitory synapses where it exerts its effect by binding to it respective receptors both pre- and post-synaptically. This binding causes conformational change and subsequent opening of the ion channel. The direction of the flow of ions (in/out) and their charge (+/−) result in a negative change in the transmembrane potential, causing hyperpolarization. To date, two classes of GABA receptors are known: (1) $GABA_A$ receptors are ligand-gated ion channels, and (2) $GABA_B$ receptors are metabotropic receptors, which are G protein-coupled receptors.

Pharmacological modulation of the $GABA_A$ receptor through the use of agonists and antagonists indicate that modulation of these circuits within the spinal cord has important implications for pain processing (358–360). However, these analgesic effects particularly seen with benzodiazepines may not be purely anti-nociceptive actions and maybe more non-specific centrally mediated effects.

GABA's anti-nociceptive effects are thought to be mediated by $GABA_B$ receptors, which are ideally located in both the brain and spinal cord with ubiquitous expression. Moreover, $GABA_B$ receptors have also been implicated in a whole host of GI functions, such as altering GI motility and visceral sensation (361). The mostly widely used $GABA_B$ receptor agonist, baclofen has been shown to produce anti-nociception in numerous rat models of visceral pain (67, 362–365). Moreover, subcutaneous injection of baclofen was shown to prevent behavioral responses to bladder pain (362). Likewise, intrathecal administration of baclofen increases the sensitivity threshold to CRD (363). Furthermore, intravenous administration of baclofen attenuated CRD-evoked increases in arterial blood pressure and heart rate (67, 364).

Intraperitoneal injection of baclofen was shown to alter the expression of early immediate genes such as c-fos in the lumbosacral spinal cord after intra-colonic mustard oil-induced inflammation (366, 367). Moreover, it has also been shown via electrophysiological examination that $GABA_B$ receptor agonists have the ability to modulate responses of vagal mucosal and muscle afferents innervating particular parts of the GI tract, specifically, the esophagus and proximal stomach (368–370). More recently, it has also been shown that baclofen dose-dependently attenuates responses of mechanosensitive pelvic nerve afferents to noxious CRD (367, 371). This data provide evidence that $GABA_B$ receptor agonists may exert their positive effects by acting at peripheral sites.

However, it is important to note the effects of $GABA_B$ receptor agonism, other than its anti-nociceptive effect, are undesirable centrally mediated effects, including sedation, respiratory depression, drug tolerance, and motor deficiency, thus, its potential therapeutic use could be significantly curtailed (367). Interestingly, GABA receptor pharmacology is complex with dimerization required for many subunits to form a functional receptor. Moreover, a number of splice variants of the $GABA_{B1}$ subunit ($GABA_{B1a}$, $GABA_{B1b}$, $GABA_{B1c}$, and $GABA_{B1d}$) have been identified both in rat and human tissues, and have been found to be differentially expressed depending on the tissue type (372, 373).

Indeed GABA analogs such as gabapentin and pregabalin have also shown efficacy in preclinical models of visceral hypersensitivity (105, 106, 108, 374–377) and moreover, pregabalin has undergone clinical trials for painful CP (107). However, these compounds may exert their effects indirectly in the GABAergic system, as their main mode of action is on α2δ subunits of Ca_V (375). The emergence of positive allosteric modulators of $GABA_B$ may provide a novel therapeutic target for treatment of visceral pain disorders (66).

GENETIC AND EPIGENETIC REGULATION OF VISCERAL PAIN

Over the last two decades, the field of pain genetics has explored the influence of genetic make-up on pain perception and processing. Recent work by Camilleri and others has described the role of genetics in IBS (378–384). Genome-wide association (GWA) studies amongst others have led to the elucidation of specific genetic alterations in IBS such as $Na_{v1.5}$ (385), GPBAR1 (G protein-coupled bile acid receptor 1) (386), KDELR2 (KDEL endoplasmic reticulum protein retention receptor-2) and GRID2IP [glutamate receptor, ionotropic, delta 2 (Grid2) interacting protein] (381), NXPH1 (neurexophilin 1), and CDC42 (cell division control protein 42 homolog) (387). These studies are illustrative examples of how research in the genetic area can contribute to the achievement of better general knowledge in the visceral pain field.

Strain differences have been shown in many behavioral phenotypes including anxiety and depression (388, 389). Moreover, differences due to background strain have also been shown for somatic nociception (390–392). However, there is a dearth of information regarding strain differences in basal visceral sensitivity as opposed to inflammatory-induced visceral sensitivity. To our knowledge, the Wistar Kyoto (WKY) rat strain and the Flinders Sensitive Line rat strain are the only well-validated models of genetic predisposition to visceral hypersensitivity (132, 393, 394). This highlights the need for more comprehensive testing in particular assessment of visceral pain in animal models and elucidating the underlying genetic mechanisms. Moreover, the field of pain epigenetics has progressed our simplistic view of one gene, one protein, one function, to a more complex view of gene–environment interactions.

The term epigenetics refers to processes that lead to stable and/or heritable changes in gene function without any concomitant DNA sequence changes (395). Examples include DNA methylation, histone modification, and chromatin remodeling. The vast

majority of work investigating epigenetic mechanisms in pain processing center around histone acetylation and DNA methylation. Pharmacological interference with the process of histone acetylation can affect pain behavior, with both systemic and intrathecal administration of histone deacetylase (HDAC) inhibitors having analgesic effects in models of inflammatory pain (396–398). In one study, this effect was shown to be mediated by expression changes of the mGlu2 receptor in both dorsal root ganglia (DRG) and spinal cord (399). Indeed, histone acetylation has been implicated in stress-induced visceral hypersensitivity and HDAC inhibitors have also shown efficacy in a model of visceral pain (69, 71, 400–402). The hypothesis that IBS could be transferred to future generations has recently been investigated (403) and discussed (404).

Similar influences could be shown in the case of DNA methylation and its reader molecule MeCP2. The methyl binding protein MeCP2 has been shown to promote abnormal up-regulation of a group of genes in inflammatory pain conditions. In rats, its usually repressive function appears to be curtailed through phosphorylation after injection of Complete Freund's adjuvant (CFA) into the ankle joint (405), an effect thought to be partly dependent on intact descending serotonergic input into the spinal dorsal horn (398, 406). Recently, it was shown that chronic stress was associated with increased methylation of the Nr3c1 (glucocorticoid nuclear receptor) promoter and reduced expression of this gene in L6–S2 region of the spinal cord which was associated with visceral hypersensitivity in rat model (401).

Finally, recent speculation has implicated that the mechanisms and indeed pathways by which the gut microbiota may communicate with the CNS may be due in part to epigenetic processes (407).

EXPERIMENTAL MODELS OF VISCERAL PAIN

COLORECTAL DISTENSION

Colorectal distension is the most widely used method to assess visceral sensation both preclinically and clinically. This technique involves insertion of a balloon into the colorectal cavity of the human subject or animal, and with the aid of a barostat, distending in a repeated or ascending phasic manner. Although the main premise of the technique is essentially identical, the way in which it is performed varies between laboratories and researchers. This can be due to numerous factors including the model employed; whether it be human, rat, or mouse, the parameter of interest; baseline visceral sensation, pain threshold, and tolerance to painful stimuli. CRD has been characterized in humans, rats, and mice with the bulk of the work being performed in rats due to ease of use and robust reproducibility (408).

MONITORING OF VISCERAL PAIN IN RODENTS

In 1988, Ness and Gebhart were the first to describe a technique used to assess visceral sensitivity in the preclinical setting. The technique was based on the assessment of pseudoaffective and behavioral responses to controlled isobaric distensions of the GI tract. This has now become the mainstay for assessing visceral pain both clinically and preclinically (409). CRD in rats induces an array of autonomic and behavioral outputs termed pseudoaffective reflexes. These reflexes include; alterations in blood pressure and heart rate, passive avoidance behaviors, and contraction of the abdominal musculature (8), the latter of which is more commonly referred to as the VMR. VMR is the most widely used parameter of the visceral pain response (8, 408).

In the last decade, the use of electromyographic (EMG) signals and its numerous applications have allowed many to assess visceral sensitivity in conscious animals. This procedure allows recording from electrodes which are implanted in the musculature and externalized through the skin, primarily on the abdomen or neck (410–412). They can also be connected to radiotelemetric implants in the abdominal cavity (413, 414). Moreover, the development of manometric recordings measuring changes in the pressure of the balloon inserted into the GI tract, have also allowed for VMR to be assessed in freely moving animals (134, 175, 311, 408, 415, 416). Other groups have implemented other indirect approaches such as manual scoring of the abdominal withdrawal reflex (408, 417), operant behavioral assays (409), and functional brain imaging tools such as functional MRI (44, 418).

ANIMAL MODELS OF STRESS-INDUCED VISCERAL PAIN

Stressful episodes during critical windows of development can have long-lasting effects on the host. Stress occurring during the perinatal period has been linked to the development of psychiatric disorders such as schizophrenia and autism spectrum disorders (419, 420). The early postnatal period is a stress hypo-responsive period during which time there is an intense phase of neuronal growth and myelination (421). Stress during this critical time point has been linked to the development of both somatic and psychiatric phenotypes in preclinical models, including IBS (135, 422, 423). The adolescent period is also a time of neuronal restructuring and is fundamental to precise development of the CNS (215). This developmental period is also the peak time for the onset of numerous psychiatric disorders including schizophrenia, substance abuse, and mood disorders (424). Stress in adulthood can have profound effects on its host. Both acute and chronic stressors can elicit detrimental impacts on physical as well as mental well-being. Taken together, it is clear that stress at critical points during our life time can have lifelong effects on the host. Overcoming the effects of these stressors is based on the individual's HPA axis ability to adapt and overcome such insults, however, in the genetically predisposed individual, this feat maybe too high. Aberrant development of the HPA axis can prove detrimental and exhibit itself in the form of both psychiatric and somatic disorders. The importance of animal models in the search for underlying molecular mechanisms and future development of novel therapeutics has never been more pertinent (8, 425–431). Here, we review the current stress-based models in the context of IBS (**Figure 5**).

Experimental models of stress and stressful events have been developed bearing in mind the critical windows for HPA axis development and maturation. Animal models have been specifically developed to target different periods throughout the lifespan to assess (1) vulnerability, (2) triggering, and (3) perpetuating influences of stress and future development of IBS (425). Early-life stressors in the form of maternal neglect (maternal separation model) or injury (colonic irritation) can increase an individual's risk to develop IBS and other disorders in adulthood (425). During adulthood, life-threatening stressors (rape, warfare), psychological

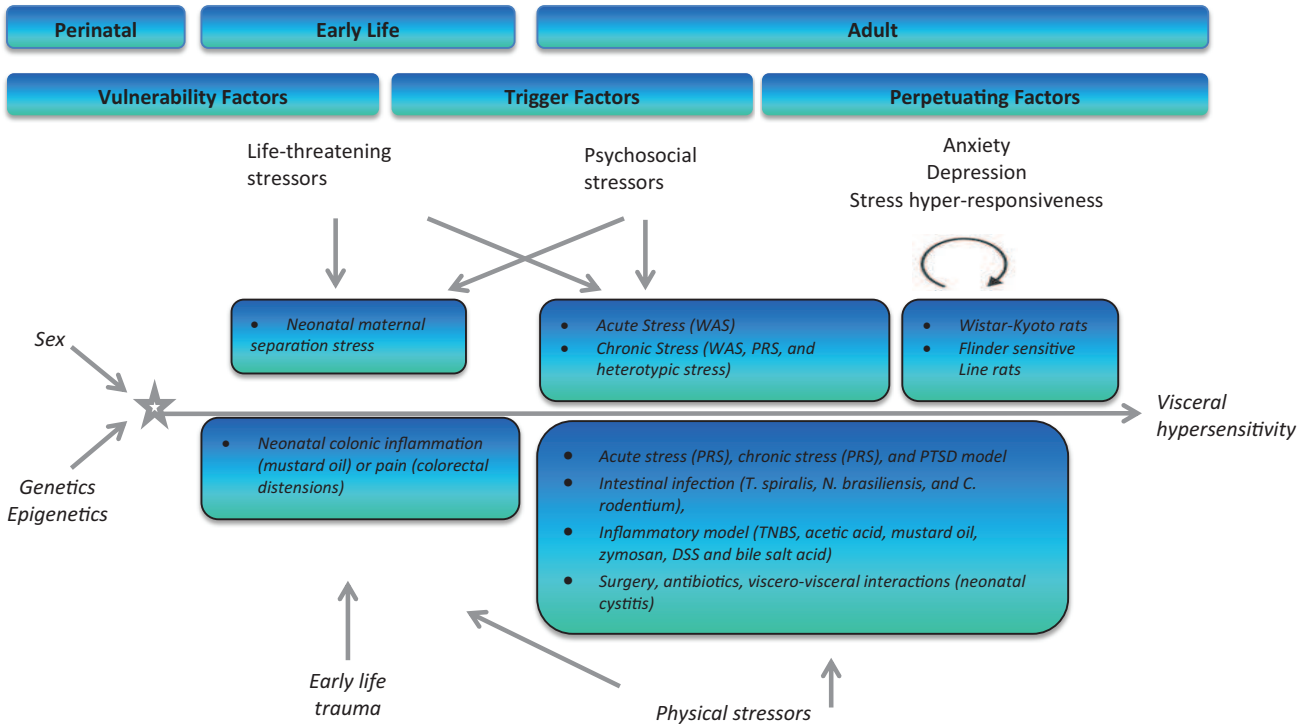

FIGURE 5 | Schematic representation of both physical and psychological stressors used in the generation of animal models for stress-induced visceral hypersensitivity. WAS, water-avoidance stress; PRS, partial restraint stress; TNBS, trinitro benzene sulfonic acid; DSS, dextran sodium sulfate.

stressors (acute and chronic stress), or physical stressors (in the form of intestinal dysbiosis due to infection, inflammation, antibiotic usage, and surgery) have all been described and documented as triggering factors to the development of an IBS-type phenotype in rats and mice (425). Finally, the ever expanding catalog of rodent strains and transgenic models has allowed us to use specific strains/knockouts known to exhibit various levels of stress responsivity (Wistar Kyoto and Flinders Sensitive Line) to mimic the influence of genetic and perpetuating factors on the development, severity and duration of IBS symptoms (425).

GENETIC STRESS MODELS OF VISCERAL PAIN

Mood disorders are commonly comorbid with IBS (432–434). Genetic predisposition to such disorders has been implicated in the development of IBS in later life. These genetic factors may not lead directly to the development of IBS *per se* but may indirectly, through heightened stress responsivity, cause altered GI function and IBS symptomatology. The availability of a catalog of rodent strains has led to the advent of genetic studies assessing the exact contribution of genetic factors to disease presentation and progression. Moreover, the use of transgenic rodent strains allows us to specifically investigate single genes implicated in disease pathology.

Using different rat strains of known levels of baseline anxiety: low-anxiety SD and Fisher-344 (F344), and high-anxiety WKY rats, Gunter and colleagues were able to demonstrate a link between anxiety and visceral hypersensitivity. Specifically, high-anxiety WKY animals had increased response to CRD compared to low-anxiety strains (132). Moreover, WKY rats exhibited an exaggerated response to acetic acid instillation into the colon, a peripheral sensitization model, compared with low-anxiety strains, SD and F344 (132).

The role of the stress response and the development of IBS was again shown to be intrinsically linked when CRHR1 ($CRHR1^{-/-}$) knockout animals were shown to have an altered VMR to CRD. VMR to CRD was only observed at the highest distension pressure (60 mmHg). Moreover, pharmacological CRHR1 antagonism decreased the VMR to CRD in $CRHR1^{+/+}$ mice (167).

Small interfering RNA (siRNA) technologies have now become one of the most widely used approaches to selectively suppress gene expression and is a powerful tool to assess gene function. This technology has been used extensively to suppress stress-related genes in specific brain areas and models are continuously being developed (435–440). Whilst genetically modified mice have been the mainstay of behavioral genetics to date there is a growing utility for genetically modified rats (441). Moreover, other genetic tools such as optogenetic and Designer Receptor Exclusively Activated by Designer Drugs (DREADD)-based manipulations allows for both species to be used in the future (442).

ANIMAL MODELS OF EARLY-LIFE STRESS-INDUCED VISCERAL PAIN

Maternal separation stress model

Stress in early life is a well-established risk factor for the development of psychiatric and somatic disorders in later life. The biopsychosocial model of IBS pathophysiology implicates adverse early-life events and childhood traumas such sexual abuse, neglect,

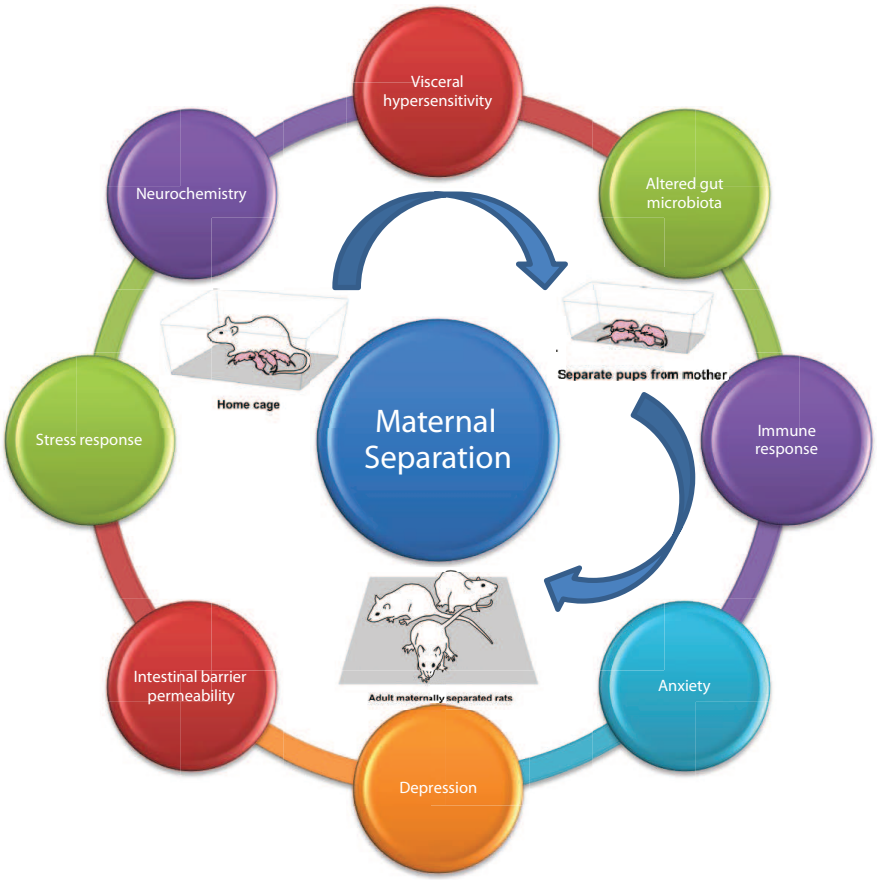

FIGURE 6 | Maternal separation model of brain–gut axis dysfunction. Adult rodents subjected to maternal separation in early life develop the characteristic MS phenotype; typified by altered; visceral sensation, microbiota, immune response, anxiety, depression, intestinal permeability, stress response, neurochemistry adapted from Ref. (108).

loss of a family member, or a life-threatening situation. These factors have been linked to enhanced vulnerability of individuals to develop stress-related affective disorders such as depression and anxiety. Moreover, these individuals are also at a higher risk for developing FGIDs such as IBS and visceral pain (181, 443).

To model these environmental factors in rodent models, the maternal separation model was constructed (**Figure 6**) (444). Briefly, this model involves removing new-born pups from the dam, during the critical HPA axis hypo-responsive phase in the early postnatal period. Most commonly, pups are removed for 2–3 h per day during the first 2 weeks of life from postnatal days 1–2 to postnatal day 12–14 (108, 188, 445, 446). This interruption in the normal maternal environment, leads to a stress response not only in the pups but also in the dams. As a result of this maternal stress response, the maternal care given to the pups is altered. In the last 20 years, the critical importance of maternal care has been researched extensively no more so in the area of epigenetics (447–449). Alterations in maternal care has been shown to have effects on the development and function of the HPA axis. Moreover, the impaired stress response is thought to underlie the deficits seen in different behavioral domains, in particular, both cognitive and emotional modalities (447). This disruption of the normal maternal environment and dam–offspring interaction can subsequently affect the quality of the maternal care received by the pups. Indeed, this model of early-life stress results in long-lasting changes to the offspring's CNS, at all levels including altered expression, neurochemistry, electrophysiology, and morphology (8, 450).

When animals are allowed to grow to maturity, a behavioral phenotype is present, characterized by visceral hypersensitivity, increased anxiety and depressive behaviors, altered stress responsivity, altered neurochemistry specifically serotonergic neurotransmission, enhanced immune response, altered gut microbiota profile, and disruption of the intestinal barrier (108). We have previously shown that adult male rats previously subjected to maternal separation exhibit visceral hypersensitivity to CRD (135). Moreover, the effects of MS are exacerbated by exposure to an acute stressor specifically 1 h of water-avoidance stress (WAS) (451, 452).

The maternal separation model is sensitive to many factors such as sex differences, with the abundance of studies being performed in male rodents, however clinically the preponderance of female to male IBS patients is 3:1. It is unclear from the literature that the MS procedure induces the same robust phenotype in female rodents.

Moreover, the protocol of separation itself has also proved crucial. Specifically, sex-dependent effects on VMR were evident when the separation was performed by the removal of an entire or half litter from the home cage (446). Males that were exposed to whole litter separation and females that were exposed to both whole litter and half litter separation developed visceral allodynia and hypersensitivity to CRD (446). Moreover, when males underwent an additional acute stress, this did not modify the CRD response. On the other hand, when females were exposed to an additional acute stress, they exhibited an exacerbated response to CRD (8, 446). The rat MS model is the most commonly used rodent model of IBS and has proved crucial in delineating the underlying mechanisms as well as testing novel therapeutic strategies.

Although the maternal separation model is well established and characterized in rats, its utility in mouse strains has proved difficult to replicate. Data from our lab and others suggest that maternal separation stress alone is insufficient to induce a robust, reproducible behavioral phenotype (453, 454). However, when maternal separation stress is combined with unpredictable maternal stress, a more overt behavioral phenotype is exhibited (310, 455).

Neonatal Irritation Models

Early-life stress not only in the form of psychological stress but also in the context of physical stress has been shown to be a valid preclinical model of IBS pathophysiology. The neonatal intestine when exposed to mechanical and chemical stressors results in a pro-inflammatory phenotype with mucosal inflammation and tissue irritation. Daily irritation of the neonatal colon by mechanical irritation (daily noxious CRD) or chemical irritation (daily intra-colonic injection of mustard oil) increases pain behaviors in response to CRD from adolescence to adulthood (417, 456). Moreover, this heightened pain response was accompanied with decreases in exploratory behavior, indicative of an anxiety-phenotype. The findings described in these studies implies that irritation of the neonatal gut, be it via mechanical or chemical means, can lead to the process of central sensitization due in part to sensitized peripheral afferents (8, 417, 456). These behavioral outputs are in parallel with IBS in the clinical scenario; visceral hypersensitivity with comorbid anxiety.

ANIMAL MODELS OF ADULT STRESS-INDUCED VISCERAL PAIN

Acute stress models

Acute stressors in adulthood can lead to an immediate behavioral phenotype. The most widely used acute stress paradigms to model IBS preclinically are WAS and restraint stress. The WAS paradigm was originally developed by Bonaz and Tache and Enck et al. (457, 458) to assess stress-related alterations in gut motility and motor function. Briefly, rodents are placed on a small platform raised slightly above water level. This stressor is based on the aversive environment of surrounding water. The WAS paradigm has been used extensively to assess the impact of psychological stress on visceral pain modulation (8, 311). Data from the literature show that 1 h WAS was sufficient to induce a delayed visceral hypersensitivity to CRD, in male Wistar rats (459). Moreover, others have shown that chronic WAS-induced effects are thought to be modulated at an epigenetic level, with HDAC inhibitors shown to reverse WAS-induced hyperalgesia (400).

Moreover, other forms of acute stress such as restraint stress for 2 h, was also found to induce heightened VMR to CRD in male (460) and female Wistar rats (446). The time spent in an acute stress situation has proven crucial to the development of the IBS phenotype (461). Acute stressors of longer duration >2 h tend to be susceptible to the animals habituating to the stressful environment and this can confound the behavioral outputs. Moreover, WAS was recently shown to induce both hyperalgesia and analgesia depending on the duration and number of stress sessions (311, 462). Transient stressors generally trigger adaptive responses and this type of model mimics the stress-related hypersensitivity to CRD as reported in IBS patients.

Chronic mild stress

Daily life stressors affect individuals in different ways. Persistent mild stressors can accumulate and potentiate the effects of stress on the host. Modeling chronic daily stress in rodents is achieved through the chronic mild stress (CMS) paradigm. Convergent lines of evidence suggests that high levels of chronic daily stress can impact on the intensity and severity of visceral pain symptoms (181, 463–466). In light of this, an array of rodent models involving unpredictable chronic and intermittent exposure to variety of stressors have recently been developed (8). Following on from the previous section, WAS was in fact one of the first chronic stress models to be adapted to the study of visceral hypersensitivity (467). Early data proved promising with studies showing that a 1 h daily WAS, for 10 consecutive day protocol was sufficient to induce visceral hypersensitivity to CRD in male Wistar rats (410, 468). This effect was long-lasting and persistent up to 30 days after the end of the stress. One of the confounds of this study was based on the methodology used to assess VMR. EMG recordings were performed however this involves surgical implantation of electrodes and ensuing single housing of animals, to avoid injury (410), which in itself could be described as a stressor. Indeed, when VMR was assessed using intraluminal colonic solid-state manometry, both male and female naïve Wistar rats exposed to WAS developed a reduced response to CRD referred to as visceral analgesia (311). This findings appear to be in direct contrast to each other and highlight the challenges of developing models of stress-induced visceral hypersensitivity.

Moreover, the picture becomes even more complex when we consider mouse models. Indeed, chronic WAS in C57Bl/6 mice has shown many varied effects including visceral hyperalgesia (311), visceral analgesia (311), or to have no influence on the VMR (469). Many other factors may be of course at play such as the conditions prior to CRD including surgery and housing environment, and these factors are dependent on the route of VMR assessment (311). Taken together, it becomes clear that the basal environment and state of the animals prior to stress exposure can impact their response to the stressor itself (8).

Furthermore, the nature or type of stressor is also crucial, with habituation known to occur when animals are repeatedly exposed to the same type of stressor, a phenomenon thought to be mediated via oxytocin (470) and endocannabinoid pathways (471). Moreover, these pathways are indeed themselves also important in pain processing (472, 473). Taking this knowledge on board, that homotypic stressors may/do lead to habituation, more recent models

employ stressors of different natures. These heterotypic stressors such as cold restraint stress, WAS, or forced swimming were found to induce immediate but not long-term visceral hyperalgesia as monitored by EMG recordings at 8 h, 24 h, and 7 days after the cessation of the stress paradigm in male Wistar rats (474). Developing the model further, with more multifaceted paradigms and longer lasting stress sessions (134, 475, 476) causes a behavioral phenotype in rodents that mimics aspects of depression. This more recently developed model may prove useful when assessing the mechanisms of chronic visceral pain comorbid with depression-like symptoms (432). Taken together, it is clear that many other factors outside of the stress model can have significant effects on the parameter of interest and thus limit the models utility (477). Factors such as the sex and strain of the animal model, housing conditions before, during and after the stress paradigm and diurnal variation are all known to alter sensitivity to stress (477). From the literature, what we have learned is that variety within the stress paradigm itself (time, type of stressor) appears crucial to inducing stress-induced effects and preventing habituation (470, 478–480). All of these facets could also in themselves potentially alter the influence of CMS paradigms on the visceral pain sensitivity, as recently assessed by Larauche et al. (311).

Chronic psychosocial stress

Daily stressful events are commonplace in modern society. However, when modeling stress in rodent models, the stressors most often occur in a novel environment and not in the animals' home cage. This has been a cause for critique with some of the most widely used stress models. The chronic social defeat and overcrowding paradigm was designed to overcome this obstacle and thus animals undergo stress scenarios in their home cage. The paradigm is based on the unpredictable nature of life's stressors. Sessions of resident intruder social defeat and cage overcrowding are randomized so as to occur at different times of the day, in an unpredictable manner and for a chronic period, 19 days in total. We have recently shown this model to be an effective preclinical model to mimic many of the key phenotypes in the IBS population, specifically a heightened response to CRD (134) and anxiety- and depression-related behaviors (481).

Moreover, Reber and colleagues (482) have shown that this model of chronic psychosocial stress robustly enhances GI dysfunction in a mouse model of colitis. Furthermore, other chronic psychosocial stressors such as chronic overcrowding when applied to rats also induces a heightened sensitivity to CRD concomitant with enhanced HPA axis activity and intestinal mucosal inflammation (483). Taken together, these studies demonstrate that this model of chronic psychosocial stress may have multiple effects across the brain–gut axis resulting in an IBS phenotype.

Conditioned fear-induced stress model

There is increasing evidence of augmented prevalence of GI symptoms, including visceral pain and IBS in patients suffering from post-traumatic stress disorder (PTSD) (122, 484–487). Indeed, it has been shown that in some individuals, the experience of stress can prime such individuals to respond differently to subsequent stressful events. Over the last decade, Stam and colleagues have investigated this in preclinical models, particularly rats. They have shown that short exposures to shocks or a social confrontation environment with a predator or aggressive conspecific animal induces long-lasting conditioned fear-responses to trauma-related cues (425). Moreover, these animals exhibit a generalized behavioral sensitization to novel stressful stimuli that is persistent and may intensify over time (488–491). Furthermore, this group have also shown that 2 weeks after a single session of foot shocks, repeated CRD causes increased cardiovascular reflexes in pre-shocked rats when compared to their non-shocked controls (489). Remarkably, female rats appear to exhibit an alternative pattern of sensitized behavioral responsiveness to the same challenge, again highlighted the strong role of sex differences in visceral pain processing (492). This specific rodent model mimics clinical features of a subset of IBS patients exhibiting stress-related visceral hypersensitivity due to PTSD. However, it is important to note that the findings presented above are mainly characterized by a single group of investigators, thus the reliability of the model needs further independent confirmation.

Physical stressors

Post-infectious models of visceral pain. A significant proportion of IBS cases occur after an illness particularly an infection of the GI tract. This is reported to be as much as 10% of patients with IBS (493). Interestingly, the proportion of people who suffer from an intestinal infection who will then go on to develop IBS ranges from 3 to 36% and appears to be dependent upon the infecting organism. Moreover, the psychological state of the individual at the time of infection appears to also play a critical role in the development of IBS symptoms (494). Infections of bacterial origin when compared to the short-lived effects of viral gastroenteritis appear to be more long-lasting and persistent (494). A transient *Trichinella spiralis* infection was shown to induce sustained visceral hypersensitivity in a mouse model (5, 495). Moreover, similar findings were found in a rat model of *Nippostrongylus brasiliensis* infection (496). Although the vast majority of human post-infectious hypersensitivity symptoms are observed after bacterial infection with *Salmonella, Escherichia coli*, *Shigella*, or *Campylobacter*, there has been limited animal models of this type of visceral hypersensitivity (497, 498).

Post-inflammatory models of visceral pain. Inflammation is one of the leading causes/mechanisms thought to underpin IBS and its associated symptomatology (499–502). However, this remains a controversial topic and others have reviewed this previously (503, 504). Moreover, this symptom set appears to be common in patients of inflammatory bowel disorders also such as those in remission from ulcerative colitis (505). Indeed, many clinical cases of IBD switch to IBS cases and this occurs in 30–50% of patients (506). Inflammatory pain models have been well developed in somatic pain assays and to some extinct in visceral pain models. Indeed, an array of chemical irritants have been used in preclinical models to induce colonic inflammation and resultant visceral hypersensitivity (8). In rats, mustard oil (507, 508), acetic acid (509), and zymosan (510, 511) have been shown to induce short-term visceral hypersensitivity due in part to colonic inflammation. Moreover, other compounds such as TNBS induce severe colonic inflammation and visceral hypersensitivity when

administered intra-colonically (512, 513). Interestingly, in some models of inflammatory-induced visceral hypersensitivity, the phenotype can re-emerge days or weeks after the initial inflammatory response. Moreover this re-emergence is not associated with any inflammatory biomarkers (512, 514). Importantly, the experimental design appears to play a major role in the application and effectiveness of such models. Models, such as the dextran sodium sulfate (DSS)-induced colitis model has provided us with some surprising findings where animals exhibited an increased response to CRD on day 5 or day 60 after the induction of colitis in male Balb/c mice. However, chronic DSS colitis was not associated with changes in VMR to CRD (515). However, contrasting findings have also been reported by other groups, whereby DSS colitis failed to induce any alterations in VMR or visceral sensitivity at all time points tested in either C57Bl/6 or Balb/c mice (516). One can speculate on the many possible reasons for these observed differences, however, what they do suggest is that inflammation on its own may not be sufficient to induce visceral hypersensitivity and that the nature and severity of the inflammatory stimulus and their combined effects may determine when/whether post-inflammatory hypersensitivity will result if at all (8, 512). Moreover, as is seen in post-infectious visceral hypersensitivity, psychological state may also play a role (517). Indeed this was investigated by Larsson et al. (469), who demonstrated that prior exposure of animals to a stressor, be it either psychological or psychosocial in origin, revealed an enhanced susceptibility to colitis and an aggravated colonic inflammatory response (482, 518, 519). Furthermore, this was also associated with a heightened susceptibility to recurrence of colonic inflammation even though the colitis had dissipated (520, 521). Similarly, a prior bout of colitis was sufficient to leave the colon in a state more susceptible to the negative effects of stress (522). However, the complex association between colitis, stress, and visceral pain response remains contentious as stress has also been found to both exacerbate or indeed to have no effect on post-inflammatory visceral sensitivity in rats (523) and in mice (8, 469).

FUTURE DIRECTIONS

MICROBIOTA MANIPULATIONS

The role of the microbiota–brain–gut axis on health and disease is an area of biomedicine that is receiving much media attention of late. The complex communication between the gut microbial population and the CNS has far reaching implications not least in the area of visceral pain and psychiatric comorbidities. Modulation of the gut microbiota via prebiotic/probiotic treatment has been shown to have positive effects on visceral pain behaviors as discussed earlier (222, 240–242). Antibiotic treatment has long been known to alter GI function, effects which are reversed upon probiotic treatment (524). Thus, it is logical that antibiotic-induced visceral pain may be a future model used to investigate the underlying mechanism of such a phenomenon. Indeed work from our own lab has recently demonstrated that early-life antibiotic-induced disruption of the colonizing microbiota is associated with visceral hypersensitivity and altered spinal signaling in adulthood. These results indicate that a temporary alteration in the composition of the GI microbiota during a crucial time-window in the neonatal rat has a long-lasting impact on nociceptive pathways and that the developing pain systems are subject to modulation by the microbiota (248).

Moreover, intriguing studies to assess the exact contribution of the gut microbiota to the development of visceral hypersensitivity and IBS can be achieved through fecal transplantation studies whereby fecal matter from donor IBS patients can be used to inoculate recipient animals and subsequent behaviors can be assessed. This paradigm has already shown its merit in transferring other behaviors specifically anxiety-related behaviors in a mouse model (525).

CONCLUSION

An effective stress response is critical to the survival of any living creature. The ability to sense changes in the environment and adapt accordingly is quintessence to survival. However, the host's reaction to perceived stress can sometimes become disturbed leading to an over-exaggerated response. Stress has been implicated in and associated with a plethora of somatic disorders; ulcers, migraine, hypertension, and psychiatric disorders; anxiety, depression, PTSD. Here, we specifically reviewed the literature in the context of stress as a major risk factor for the development of IBS and visceral pain. The mechanism by which stress impacts on normal physiology to cause such devastating disorders still remains unclear. Numerous mechanisms have been postulated including alterations in plasticity, neurogenesis, catecholaminergic neurotransmission, and more recently gut microbiota. Modern day life exposes us all to stressors of varying types (psychological/physical) and severities (acute/chronic) and as such the search for novel pharmaco-therapeutics has never been more pertinent.

Despite the ever growing body of literature, the exact mechanisms underlying visceral pain still remain less well understood than that of somatic pain. Central sensitization of primary sensory afferents is an important underlying mechanism for both somatic and visceral hypersensitivity and hyperalgesia. One of the hurdles in understanding the mechanisms of stress-induced visceral pain is that visceral pain response is experienced differentially depending on numerous factors, including the time at which stress was applied, duration of stress, sex differences, and genetic background amongst others. Psychological stress at any point during our lifetime can lead to permanent alterations of the HPA axis, the descending pain modulatory system, the immune system, and the gut microbiota, all of which can be manifested as chronic visceral hypersensitivity. Mechanisms by which physical stress such as infections, mediate visceral hypersensitivity are likely to be different to that of psychological stress and may involve altered immune system functioning. Considering the diverse mechanisms of visceral pain, the development of treatment strategies and therapeutic interventions will rely on good animal models, all of which have been reviewed here. It is clear that that the drive to develop clinically relevant models and thus design new novel therapeutics has never been more pertinent.

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REFERENCES

1. Cervero F, Laird JM. Visceral pain. *Lancet* (1999) **353**:2145–8. doi:10.1016/S0140-6736(99)01306-9
2. Robinson DR, Gebhart GF. Inside information: the unique features of visceral sensation. *Mol Interv* (2008) **8**:242–53. doi:10.1124/mi.8.5.9
3. Giamberardino MA. Recent and forgotten aspects of visceral pain. *Eur J Pain* (1999) **3**:77–92. doi:10.1053/eujp.1999.0117
4. Giamberardino MA. Visceral pain model, kidney stone pain. In: Schmidt RF, Willis WD, editors. *Encyclopedia of Pain*. Berlin: Springer (2007). p. 2626–9.
5. Bercik P, Wang L, Verdu EF, Mao YK, Blennerhassett P, Khan WI, et al. Visceral hyperalgesia and intestinal dysmotility in a mouse model of postinfective gut dysfunction. *Gastroenterology* (2004) **127**:179–87. doi:10.1053/j.gastro.2004.04.006
6. Caraceni A, Martini C, Zecca E, Portenoy RK. Breakthrough pain characteristics and syndromes in patients with cancer pain. An international survey. *Palliat Med* (2004) **18**:177–83. doi:10.1191/0269216304pm890oa
7. Stoney RJ, Reilly LM. Chronic visceral ischemia. An often overlooked cause of abdominal pain. *Postgrad Med* (1983) **74**:111–8.
8. Larauche M, Mulak A, Tache Y. Stress and visceral pain: from animal models to clinical therapies. *Exp Neurol* (2012) **233**:49–67. doi:10.1016/j.expneurol.2011.04.020
9. Giamberardino MA, Vecchiet L. Visceral pain, referred hyperalgesia and outcome: new concepts. *Eur J Anaesthesiol Suppl* (1995) **10**:61–6.
10. Laird JM, Martinez-Caro L, Garcia-Nicas E, Cervero F. A new model of visceral pain and referred hyperalgesia in the mouse. *Pain* (2001) **92**:335–42. doi:10.1016/S0304-3959(01)00275-5
11. Halder SL, Mcbeth J, Silman AJ, Thompson DG, Macfarlane GJ. Psychosocial risk factors for the onset of abdominal pain. Results from a large prospective population-based study. *Int J Epidemiol* (2002) **31**:1219–25. doi:10.1093/ije/31.6.1219
12. Hillila MT, Farkkila NJ, Farkkila MA. Societal costs for irritable bowel syndrome – a population based study. *Scand J Gastroenterol* (2010) **45**:582–91. doi:10.3109/00365521003637211
13. Faresjo A, Grodzinsky E, Hallert C, Timpka T. Patients with irritable bowel syndrome are more burdened by co-morbidity and worry about serious diseases than healthy controls – eight years follow-up of IBS patients in primary care. *BMC Public Health* (2013) **13**:832. doi:10.1186/1471-2458-13-832
14. Yu SW, Rao SS. Advances in the management of constipation-predominant irritable bowel syndrome: the role of linaclotide. *Therap Adv Gastroenterol* (2014) **7**:193–205. doi:10.1177/1756283X14537882
15. Barboza JL, Talley NJ, Moshiree B. Current and emerging pharmacotherapeutic options for irritable bowel syndrome. *Drugs* (2014) **74**:1849–70. doi:10.1007/s40265-014-0292-7
16. Vercellini P, Somigliana E, Vigano P, Abbiati A, Barbara G, Fedele L. Chronic pelvic pain in women: etiology, pathogenesis and diagnostic approach. *Gynecol Endocrinol* (2009) **25**:149–58. doi:10.1080/09513590802549858
17. Prakash Gyawali C. Esophageal hypersensitivity. *Gastroenterol Hepatol (N Y)* (2010) **6**:497–500.
18. Farmer AD, Aziz Q. Gut pain & visceral hypersensitivity. *Br J Pain* (2013) **7**:39–47. doi:10.1177/2049463713479229
19. Farmer AD, Aziz Q. Visceral pain hypersensitivity in functional gastrointestinal disorders. *Br Med Bull* (2009) **91**:123–36. doi:10.1093/bmb/ldp026
20. Handa M, Nukina H, Ando K, Kubo C. What does pain or discomfort in irritable bowel syndrome mean? *Dig Dis Sci* (2004) **49**:575–8. doi:10.1023/B:DDAS.0000026301.47638.92
21. Fass R, Achem SR. Noncardiac chest pain: epidemiology, natural course and pathogenesis. *J Neurogastroenterol Motil* (2011) **17**:110–23. doi:10.5056/jnm.2011.17.2.110
22. Giamberardino MA, Valente R, de Bigontina P, Vecchiet L. Artificial ureteral calculosis in rats: behavioural characterization of visceral pain episodes and their relationship with referred lumbar muscle hyperalgesia. *Pain* (1995) **61**:459–69. doi:10.1016/0304-3959(94)00208-V
23. Cervero F. Visceral versus somatic pain: similarities and differences. *Dig Dis* (2009) **27**(Suppl 1):3–10. doi:10.1159/000268115
24. Dunckley P, Wise RG, Fairhurst M, Hobden P, Aziz Q, Chang L, et al. A comparison of visceral and somatic pain processing in the human brainstem using functional magnetic resonance imaging. *J Neurosci* (2005) **25**:7333–41. doi:10.1523/JNEUROSCI.1100-05.2005
25. Dunckley P, Wise RG, Aziz Q, Painter D, Brooks J, Tracey I, et al. Cortical processing of visceral and somatic stimulation: differentiating pain intensity from unpleasantness. *Neuroscience* (2005) **133**:533–42. doi:10.1016/j.neuroscience.2005.02.041
26. Vermeulen W, de Man JG, Pelckmans PA, de Winter BY. Neuroanatomy of lower gastrointestinal pain disorders. *World J Gastroenterol* (2014) **20**:1005–20. doi:10.3748/wjg.v20.i4.1005
27. Grundy D. Neuroanatomy of visceral nociception: vagal and splanchnic afferent. *Gut* (2002) **51**(Suppl 1):i2–5. doi:10.1136/gut.51.suppl_1.i2
28. Anand P, Aziz Q, Willert R, van Oudenhove L. Peripheral and central mechanisms of visceral sensitization in man. *Neurogastroenterol Motil* (2007) **19**:29–46. doi:10.1111/j.1365-2982.2006.00873.x
29. Almeida TF, Roizenblatt S, Tufik S. Afferent pain pathways: a neuroanatomical review. *Brain Res* (2004) **1000**:40–56. doi:10.1016/j.brainres.2003.10.073
30. Johnson AC, Greenwood-van Meerveld B. Stress-induced pain: a target for the development of novel therapeutics. *J Pharmacol Exp Ther* (2014) **351**:327–35. doi:10.1124/jpet.114.218065
31. Ness TJ. Evidence for ascending visceral nociceptive information in the dorsal midline and lateral spinal cord. *Pain* (2000) **87**:83–8. doi:10.1016/S0304-3959(00)00272-4
32. Camilleri M, Coulie B, Tack JF. Visceral hypersensitivity: facts, speculations, and challenges. *Gut* (2001) **48**:125–31. doi:10.1136/gut.48.1.125
33. Palecek J. The role of dorsal columns pathway in visceral pain. *Physiol Res* (2004) **53**(Suppl 1):S125–30. doi:10.1016/S0304-3959(03)00075-7
34. Willis WD, Al-Chaer ED, Quast MJ, Westlund KN. A visceral pain pathway in the dorsal column of the spinal cord. *Proc Natl Acad Sci U S A* (1999) **96**:7675–9. doi:10.1073/pnas.96.14.7675
35. Rustioni A, Hayes NL, O'Neill S. Dorsal column nuclei and ascending spinal afferents in macaques. *Brain* (1979) **102**:95–125. doi:10.1093/brain/102.1.95
36. Bennett GJ, Seltzer Z, Lu GW, Nishikawa N, Dubner R. The cells of origin of the dorsal column postsynaptic projection in the lumbosacral enlargements of cats and monkeys. *Somatosens Res* (1983) **1**:131–49. doi:10.3109/07367228309144545
37. Drossman DA. Functional abdominal pain syndrome. *Clin Gastroenterol Hepatol* (2004) **2**:353–65. doi:10.1016/S1542-3565(04)00118-1
38. Sharma A, Lelic D, Brock C, Paine P, Aziz Q. New technologies to investigate the brain-gut axis. *World J Gastroenterol* (2009) **15**:182–91. doi:10.3748/wjg.15.182
39. Jones MP, Dilley JB, Drossman D, Crowell MD. Brain-gut connections in functional GI disorders: anatomic and physiologic relationships. *Neurogastroenterol Motil* (2006) **18**:91–103. doi:10.1111/j.1365-2982.2005.00730.x
40. Bush G, Luu P, Posner MI. Cognitive and emotional influences in anterior cingulate cortex. *Trends Cogn Sci* (2000) **4**:215–22. doi:10.1016/S1364-6613(00)01483-2
41. Kravets JL, Reyes BA, Unterwald EM, van Bockstaele EJ. Direct targeting of peptidergic amygdalar neurons by noradrenergic afferents: linking stress-integrative circuitry. *Brain Struct Funct* (2013) **220**:541–58. doi:10.1007/s00429-013-0674-8
42. Jasmin L, Burkey AR, Card JP, Basbaum AI. Transneuronal labeling of a nociceptive pathway, the spino-(trigemino-)parabrachio-amygdaloid, in the rat. *J Neurosci* (1997) **17**:3751–65.
43. Gustafsson JK, Greenwood-van Meerveld B. Amygdala activation by corticosterone alters visceral and somatic pain in cycling female rats. *Am J Physiol Gastrointest Liver Physiol* (2011) **300**:G1080–5. doi:10.1152/ajpgi.00349.2010
44. Johnson AC, Myers B, Lazovic J, Towner R, Greenwood-van Meerveld B. Brain activation in response to visceral stimulation in rats with amygdala implants of corticosterone: an FMRI study. *PLoS One* (2010) **5**:e8573. doi:10.1371/journal.pone.0008573
45. Johnson AC, Tran L, Schulkin J, Greenwood-van Meerveld B. Importance of stress receptor-mediated mechanisms in the amygdala on visceral pain perception in an intrinsically anxious rat. *Neurogastroenterol Motil* (2012) **24**(479–86):e219. doi:10.1111/j.1365-2982.2012.01899.x
46. Myers B, Dittmeyer K, Greenwood-van Meerveld B. Involvement of amygdaloid corticosterone in altered visceral and somatic sensation. *Behav Brain Res* (2007) **181**:163–7. doi:10.1016/j.bbr.2007.03.031

47. Myers B, Greenwood-van Meerveld B. Divergent effects of amygdala glucocorticoid and mineralocorticoid receptors in the regulation of visceral and somatic pain. *Am J Physiol Gastrointest Liver Physiol* (2010) **298**:G295–303. doi:10.1152/ajpgi.00298.2009
48. Myers B, Greenwood-van Meerveld B. Differential involvement of amygdala corticosteroid receptors in visceral hyperalgesia following acute or repeated stress. *Am J Physiol Gastrointest Liver Physiol* (2012) **302**:G260–6. doi:10.1152/ajpgi.00353.2011
49. Myers B, Schulkin J, Greenwood-van Meerveld B. Sex steroids localized to the amygdala increase pain responses to visceral stimulation in rats. *J Pain* (2011) **12**:486–94. doi:10.1016/j.jpain.2010.10.007
50. Tran L, Greenwood-van Meerveld B. Lateralized amygdala activation: importance in the regulation of anxiety and pain behavior. *Physiol Behav* (2012) **105**:371–5. doi:10.1016/j.physbeh.2011.08.038
51. Venkova K, Foreman RD, Greenwood-van Meerveld B. Mineralocorticoid and glucocorticoid receptors in the amygdala regulate distinct responses to colorectal distension. *Neuropharmacology* (2009) **56**:514–21. doi:10.1016/j.neuropharm.2008.10.004
52. Felice VD, Gibney SM, Gosselin RD, Dinan TG, O'Mahony SM, Cryan JF. Differential activation of the prefrontal cortex and amygdala following psychological stress and colorectal distension in the maternally separated rat. *Neuroscience* (2014) **267**:252–62. doi:10.1016/j.neuroscience.2014.01.064
53. Grover M, Drossman DA. Functional abdominal pain. *Curr Gastroenterol Rep* (2010) **12**:391–8. doi:10.1007/s11894-010-0125-0
54. Le Bars D, Villanueva L, Bouhassira D, Willer JC. Diffuse noxious inhibitory controls (DNIC) in animals and in man. *Patol Fiziol Eksp Ter* (1992) **4**: 55–65.
55. King CD, Wong F, Currie T, Mauderli AP, Fillingim RB, Riley JL III. Deficiency in endogenous modulation of prolonged heat pain in patients with irritable bowel syndrome and temporomandibular disorder. *Pain* (2009) **143**:172–8. doi:10.1016/j.pain.2008.12.027
56. Heymen S, Maixner W, Whitehead WE, Klatzkin RR, Mechlin B, Light KC. Central processing of noxious somatic stimuli in patients with irritable bowel syndrome compared with healthy controls. *Clin J Pain* (2010) **26**:104–9. doi:10.1097/AJP.0b013e3181bff800
57. Bouhassira D, Moisset X, Jouet P, Duboc H, Coffin B, Sabate JM. Changes in the modulation of spinal pain processing are related to severity in irritable bowel syndrome. *Neurogastroenterol Motil* (2013) **25**:623–e468. doi:10.1111/nmo.12123
58. Wilder-Smith CH, Schindler D, Lovblad K, Redmond SM, Nirkko A. Brain functional magnetic resonance imaging of rectal pain and activation of endogenous inhibitory mechanisms in irritable bowel syndrome patient subgroups and healthy controls. *Gut* (2004) **53**:1595–601. doi:10.1136/gut.2003.028514
59. Yang M, Roman K, Chen DF, Wang ZG, Lin Y, Stephens RL Jr. GLT-1 overexpression attenuates bladder nociception and local/cross-organ sensitization of bladder nociception. *Am J Physiol Renal Physiol* (2011) **300**:F1353–9. doi:10.1152/ajprenal.00009.2011
60. Bradesi S, Herman J, Mayer EA. Visceral analgesics: drugs with a great potential in functional disorders? *Curr Opin Pharmacol* (2008) **8**:697–703. doi:10.1016/j.coph.2008.08.009
61. Blackshaw LA, Page AJ, Young RL. Metabotropic glutamate receptors as novel therapeutic targets on visceral sensory pathways. *Front Neurosci* (2011) **5**:40. doi:10.3389/fnins.2011.00040
62. Joshi SK, Mikusa JP, Weaver B, Honore P. Morphine and ABT-594 (a nicotinic acetylcholine agonist) exert centrally mediated antinociception in the rat cyclophosphamide cystitis model of visceral pain. *J Pain* (2008) **9**:146–56. doi:10.1016/j.jpain.2007.09.004
63. Fioramonti J, Bueno L. Role of cannabinoid receptors in the control of gastrointestinal motility and perception. *Expert Rev Gastroenterol Hepatol* (2008) **2**:385–97. doi:10.1586/17474124.2.3.385
64. Hughes PA, Castro J, Harrington AM, Isaacs N, Moretta M, Hicks GA, et al. Increased kappa-opioid receptor expression and function during chronic visceral hypersensitivity. *Gut* (2014) **63**:1199–200. doi:10.1136/gutjnl-2013-306240
65. Gonzalez-Cano R, Merlos M, Baeyens JM, Cendan CM. sigma1 receptors are involved in the visceral pain induced by intracolonic administration of capsaicin in mice. *Anesthesiology* (2013) **118**:691–700. doi:10.1097/ALN.0b013e318280a60a
66. Hyland NP, Golubeva AV. GABA receptors in the bladder and bowel: therapeutic potential for positive allosteric modulators? *Br J Pharmacol* (2014). doi:10.1111/bph.12617
67. Lindstrom E, Brusberg M, Ravnefjord A, Kakol-Palm D, Pahlman I, Noven A, et al. Oral baclofen reduces visceral pain-related pseudo-affective responses to colorectal distension in rats: relation between plasma exposure and efficacy. *Scand J Gastroenterol* (2011) **46**:652–62. doi:10.3109/00365521.2011.560677
68. Zhou L, Huang J, Gao J, Zhang G, Jiang J. NMDA and AMPA receptors in the anterior cingulate cortex mediates visceral pain in visceral hypersensitivity rats. *Cell Immunol* (2014) **287**:86–90. doi:10.1016/j.cellimm.2013.12.001
69. Cao DY, Bai G, Ji Y, Traub RJ. Epigenetic upregulation of metabotropic glutamate receptor 2 in the spinal cord attenuates oestrogen-induced visceral hypersensitivity. *Gut* (2014). doi:10.1136/gutjnl-2014-307748
70. Crock LW, Kolber BJ, Morgan CD, Sadler KE, Vogt SK, Bruchas MR, et al. Central amygdala metabotropic glutamate receptor 5 in the modulation of visceral pain. *J Neurosci* (2012) **32**:14217–26. doi:10.1523/JNEUROSCI.1473-12.2012
71. Tran L, Schulkin J, Ligon CO, Greenwood-van Meerveld B. Epigenetic modulation of chronic anxiety and pain by histone deacetylation. *Mol Psychiatry* (2014). doi:10.1038/mp.2014.122
72. Kawabata A, Kawao N, Kitano T, Matsunami M, Satoh R, Ishiki T, et al. Colonic hyperalgesia triggered by proteinase-activated receptor-2 in mice: involvement of endogenous bradykinin. *Neurosci Lett* (2006) **402**:167–72. doi:10.1016/j.neulet.2006.03.074
73. Couture R, Harrisson M, Vianna RM, Cloutier F. Kinin receptors in pain and inflammation. *Eur J Pharmacol* (2001) **429**:161–76. doi:10.1016/S0014-2999(01)01318-8
74. Qin HY, Luo JL, Qi SD, Xu HX, Sung JJ, Bian ZX. Visceral hypersensitivity induced by activation of transient receptor potential vanilloid type 1 is mediated through the serotonin pathway in rat colon. *Eur J Pharmacol* (2010) **647**:75–83. doi:10.1016/j.ejphar.2010.08.019
75. Xu GY, Shenoy M, Winston JH, Mittal S, Pasricha PJ. P2X receptor-mediated visceral hyperalgesia in a rat model of chronic visceral hypersensitivity. *Gut* (2008) **57**:1230–7. doi:10.1136/gut.2007.134221
76. de Carvalho Rocha HA, Dantas BP, Rolim TL, Costa BA, de Medeiros AC. Main ion channels and receptors associated with visceral hypersensitivity in irritable bowel syndrome. *Ann Gastroenterol* (2014) **27**:200–6.
77. Gralnek IM, Hays RD, Kilbourne A, Naliboff B, Mayer EA. The impact of irritable bowel syndrome on health-related quality of life. *Gastroenterology* (2000) **119**:654–60. doi:10.1053/gast.2000.16484
78. Whitehead WE, Burnett CK, Cook EW III, Taub E. Impact of irritable bowel syndrome on quality of life. *Dig Dis Sci* (1996) **41**:2248–53. doi:10.1007/BF02071408
79. Mishra S, Singh A, Pandey BL. Current state of pharmacology and therapeutics in irritable bowel syndrome with special reference to brain-gut axis. *Int J Basic Clin Pharmacol* (2013) **2**:122–9. doi:10.5455/2319-2003.ijbcp20130303
80. Holdgate A, Pollock T. Nonsteroidal anti-inflammatory drugs (NSAIDs) versus opioids for acute renal colic. *Cochrane Database Syst Rev* (2004) **1**:Cd004137. doi:10.1002/14651858.CD004137.pub2
81. Morgan G. Beneficial effects of NSAIDs in the gastrointestinal tract. *Eur J Gastroenterol Hepatol* (1999) **11**:393–400. doi:10.1097/00042737-199904000-00006
82. Leventer SM, Raudibaugh K, Frissora CL, Kassem N, Keogh JC, Phillips J, et al. Clinical trial: dextofisopam in the treatment of patients with diarrhoea-predominant or alternating irritable bowel syndrome. *Aliment Pharmacol Ther* (2008) **27**:197–206. doi:10.1111/j.1365-2036.2007.03566.x
83. Lazaraki G, Chatzimavroudis G, Katsinelos P. Recent advances in pharmacological treatment of irritable bowel syndrome. *World J Gastroenterol* (2014) **20**:8867–85. doi:10.3748/wjg.v20.i27.8867
84. Camilleri M, Andresen V. Current and novel therapeutic options for irritable bowel syndrome management. *Dig Liver Dis* (2009) **41**:854–62. doi:10.1016/j.dld.2009.07.009
85. Jadallah KA, Kullab SM, Sanders DS. Constipation-predominant irritable bowel syndrome: a review of current and emerging drug therapies. *World J Gastroenterol* (2014) **20**:8898–909. doi:10.3748/wjg.v20.i27.8898
86. Vanuytsel T, Tack JF, Boeckxstaens GE. Treatment of abdominal pain in irritable bowel syndrome. *J Gastroenterol* (2014) **49**:1193–205. doi:10.1007/s00535-014-0966-7
87. Benyamin R, Trescot AM, Datta S, Buenaventura R, Adlaka R, Sehgal N, et al. Opioid complications and side effects. *Pain Physician* (2008) **11**:S105–20.

88. Tsang BK, He Z, Wongchanapai W, Ho IK, Eichhorn JH. Visceral analgesic tolerance to intrathecal butorphanol in rats. *Can J Anaesth* (1998) **45**:1019–23. doi:10.1007/BF03012311
89. Saito Y, Kaneko M, Kirihara Y, Sakura S, Kosaka Y. Characteristics of tolerance to somatic and visceral antinociception after continuous epidural infusion of morphine in rats. *Anesth Analg* (1998) **87**:1340–5. doi:10.1097/00000539-199812000-00024
90. Ness TJ, Follett KA. The development of tolerance to intrathecal morphine in rat models of visceral and cutaneous pain. *Neurosci Lett* (1998) **248**:33–6. doi:10.1016/S0304-3940(98)00327-9
91. Lian B, Vera-Portocarrero L, King T, Ossipov MH, Porreca F. Opioid-induced latent sensitization in a model of non-inflammatory viscerosomatic hypersensitivity. *Brain Res* (2010) **1358**:64–70. doi:10.1016/j.brainres.2010.08.032
92. Mao J, Mayer DJ. Spinal cord neuroplasticity following repeated opioid exposure and its relation to pathological pain. *Ann N Y Acad Sci* (2001) **933**:175–84. doi:10.1111/j.1749-6632.2001.tb05823.x
93. Hay JL, White JM, Bochner F, Somogyi AA, Semple TJ, Rounsefell B. Hyperalgesia in opioid-managed chronic pain and opioid-dependent patients. *J Pain* (2009) **10**:316–22. doi:10.1016/j.jpain.2008.10.003
94. Arner S, Rawal N, Gustafsson LL. Clinical experience of long-term treatment with epidural and intrathecal opioids – a nationwide survey. *Acta Anaesthesiol Scand* (1988) **32**:253–9. doi:10.1111/j.1399-6576.1988.tb02725.x
95. Davis MP. Drug management of visceral pain: concepts from basic research. *Pain Res Treat* (2012) **2012**:265605. doi:10.1155/2012/265605
96. Kozlowski C, Green A, Grundy D, Boissonade F, Bountra C. The 5-HT3 receptor antagonist alosetron inhibits the colorectal distention induced depressor response and spinal c-fos expression in the anaesthetised rat. *Gut* (2000) **46**:474–80. doi:10.1136/gut.46.4.474
97. Mayer EA, Berman S, Derbyshire SW, Suyenobu B, Chang L, Fitzgerald L, et al. The effect of the 5-HT3 receptor antagonist, alosetron, on brain responses to visceral stimulation in irritable bowel syndrome patients. *Aliment Pharmacol Ther* (2002) **16**:1357–66. doi:10.1046/j.1365-2036.2002.01287.x
98. Camilleri M, Mayer E, Drossman D, Heath A, Dukes G, Mcsorley D, et al. Improvement in pain and bowel function in female irritable bowel patients with alosetron, a 5-HT. *Aliment Pharmacol Ther* (1999) **13**:1149–59. doi:10.1046/j.1365-2036.1999.00610.x
99. Degen L, Matzinger D, Merz M, Appel-Dingemanse S, Osborne S, Luchinger S, et al. Tegaserod, a 5-HT4 receptor partial agonist, accelerates gastric emptying and gastrointestinal transit in healthy male subjects. *Aliment Pharmacol Ther* (2001) **15**:1745–51. doi:10.1046/j.1365-2036.2001.01103.x
100. Coffin B, Farmachidi JP, Rueegg P, Bastie A, Bouhassira D. Tegaserod, a 5-HT4 receptor partial agonist, decreases sensitivity to rectal distension in healthy subjects. *Aliment Pharmacol Ther* (2003) **17**:577–85. doi:10.1046/j.1365-2036.2003.01449.x
101. Beglinger C. Tegaserod: a novel, selective 5-HT4 receptor partial agonist for irritable bowel syndrome. *Int J Clin Pract* (2002) **56**:47–51.
102. Liang LX, Zhang Q, Qian W, Hou XH. Antinociceptive property of tegaserod in a rat model of chronic visceral hypersensitivity. *Chin J Dig Dis* (2005) **6**:21–5. doi:10.1111/j.1443-9573.2005.00182.x
103. Ravnefjord A, Brusberg M, Kang D, Bauer U, Larsson H, Lindstrom E, et al. Involvement of the transient receptor potential vanilloid 1 (TRPV1) in the development of acute visceral hyperalgesia during colorectal distension in rats. *Eur J Pharmacol* (2009) **611**:85–91. doi:10.1016/j.ejphar.2009.03.058
104. Blackshaw LA, Brierley SM, Hughes PA. TRP channels: new targets for visceral pain. *Gut* (2010) **59**:126–35. doi:10.1136/gut.2009.179523
105. Ravnefjord A, Brusberg M, Larsson H, Lindstrom E, Martinez V. Effects of pregabalin on visceral pain responses and colonic compliance in rats. *Br J Pharmacol* (2008) **155**:407–16. doi:10.1038/bjp.2008.259
106. Million M, Wang L, Adelson DW, Roman F, Diop L, Tache Y. Pregabalin decreases visceral pain and prevents spinal neuronal activation in rats. *Gut* (2007) **56**:1482–4. doi:10.1136/gut.2007.129304
107. Olesen SS, Graversen C, Olesen AE, Frokjaer JB, Wilder-Smith O, van Goor H, et al. Randomised clinical trial: pregabalin attenuates experimental visceral pain through sub-cortical mechanisms in patients with painful chronic pancreatitis. *Aliment Pharmacol Ther* (2011) **34**:878–87. doi:10.1111/j.1365-2036.2011.04802.x
108. O'Mahony SM, Coelho AM, Fitzgerald P, Lee K, Winchester W, Dinan TG, et al. The effects of gabapentin in two animal models of co-morbid anxiety and visceral hypersensitivity. *Eur J Pharmacol* (2011) **667**:169–74. doi:10.1016/j.ejphar.2011.05.055
109. Stephens RL Jr. Glutamate transporter activators as anti-nociceptive agents. *Eurasian J Med* (2011) **43**:182–5. doi:10.5152/eajm.2011.39
110. Roman K, Yang M, Stephens RL. Characterization of the visceral antinociceptive effect of glial glutamate transporter GLT-1 upregulation by ceftriaxone. *ISRN Pain* (2013) **2013**:1–10. doi:10.1155/2013/726891
111. Lindstrom E, Brusberg M, Hughes PA, Martin CM, Brierley SM, Phillis BD, et al. Involvement of metabotropic glutamate 5 receptor in visceral pain. *Pain* (2008) **137**:295–305. doi:10.1016/j.pain.2007.09.008
112. Lin Y, Tian G, Roman K, Handy C, Travers JB, Lin CL, et al. Increased glial glutamate transporter EAAT2 expression reduces visceral nociceptive response in mice. *Am J Physiol Gastrointest Liver Physiol* (2009) **296**:G129–34. doi:10.1152/ajpgi.90556.2008
113. Lin Y, Roman K, Foust KD, Kaspar BK, Bailey MT, Stephens RL. Glutamate transporter GLT-1 upregulation attenuates visceral nociception and hyperalgesia via spinal mechanisms not related to anti-inflammatory or probiotic effects. *Pain Res Treat* (2011) **2011**:507029. doi:10.1155/2011/507029
114. Gosselin RD, O'Connor RM, Tramullas M, Julio-Pieper M, Dinan TG, Cryan JF. Riluzole normalizes early-life stress-induced visceral hypersensitivity in rats: role of spinal glutamate reuptake mechanisms. *Gastroenterology* (2010) **138**:2418–25. doi:10.1053/j.gastro.2010.03.003
115. Bradesi S, Golovatscka V, Ennes HS, Mcroberts JA, Karagiannides I, Bakirtzi K, et al. Role of astrocytes and altered regulation of spinal glutamatergic neurotransmission in stress-induced visceral hyperalgesia in rats. *Am J Physiol Gastrointest Liver Physiol* (2011) **301**:G580–9. doi:10.1152/ajpgi.00182.2011
116. Whitehead WE, Palsson OS, Levy RR, Feld AD, Turner M, von Korff M. Comorbidity in irritable bowel syndrome. *Am J Gastroenterol* (2007) **102**:2767–76. doi:10.1111/j.1572-0241.2007.01540.x
117. Whitehead WE, Palsson O, Jones KR. Systematic review of the comorbidity of irritable bowel syndrome with other disorders: what are the causes and implications? *Gastroenterology* (2002) **122**:1140–56. doi:10.1053/gast.2002.32392
118. Vandvik PO, Wilhelmsen I, Ihlebaek C, Farup PG. Comorbidity of irritable bowel syndrome in general practice: a striking feature with clinical implications. *Aliment Pharmacol Ther* (2004) **20**:1195–203. doi:10.1111/j.1365-2036.2004.02250.x
119. Vandvik PO, Lydersen S, Farup PG. Prevalence, comorbidity and impact of irritable bowel syndrome in Norway. *Scand J Gastroenterol* (2006) **41**:650–6. doi:10.1080/00365520500442542
120. Spetalen S, Sandvik L, Blomhoff S, Jacobsen MB. Rectal visceral sensitivity in women with irritable bowel syndrome without psychiatric comorbidity compared with healthy volunteers. *Gastroenterol Res Pract* (2009) **2009**:130684. doi:10.1155/2009/130684
121. Kumano H, Kaiya H, Yoshiuchi K, Yamanaka G, Sasaki T, Kuboki T. Comorbidity of irritable bowel syndrome, panic disorder, and agoraphobia in a Japanese representative sample. *Am J Gastroenterol* (2004) **99**:370–6. doi:10.1111/j.1572-0241.2004.04048.x
122. Irwin C, Falsetti SA, Lydiard RB, Ballenger JC, Brock CD, Brener W. Comorbidity of posttraumatic stress disorder and irritable bowel syndrome. *J Clin Psychiatry* (1996) **57**:576–8. doi:10.4088/JCP.v57n1204
123. Hillila MT, Siivola MT, Farkkila MA. Comorbidity and use of health-care services among irritable bowel syndrome sufferers. *Scand J Gastroenterol* (2007) **42**:799–806. doi:10.1080/00365520601113927
124. Garakani A, Win T, Virk S, Gupta S, Kaplan D, Masand PS. Comorbidity of irritable bowel syndrome in psychiatric patients: a review. *Am J Ther* (2003) **10**:61–7. doi:10.1097/00045391-200301000-00014
125. Frissora CL, Koch KL. Symptom overlap and comorbidity of irritable bowel syndrome with other conditions. *Curr Gastroenterol Rep* (2005) **7**:264–71. doi:10.1007/s11894-005-0018-9
126. Park HJ, Jarrett M, Cain K, Heitkemper M. Psychological distress and GI symptoms are related to severity of bloating in women with irritable bowel syndrome. *Res Nurs Health* (2008) **31**:98–107. doi:10.1002/nur.20237
127. Resendiz-Figueroa FE, Ortiz-Garrido OM, Pulido D, Arcila-Martinez D, Schmulson M. Impact of the anxiety characteristics and depression on clinical aspects and quality of life in patients with irritable bowel syndrome. *Rev Gastroenterol Mex* (2008) **73**:3–10.
128. Mikocka-Walus A, Turnbull D, Moulding N, Wilson I, Andrews JM, Holtmann G. Psychological comorbidity and complexity of gastrointestinal symptoms in

clinically diagnosed irritable bowel syndrome patients. *J Gastroenterol Hepatol* (2008) **23**:1137–43. doi:10.1111/j.1440-1746.2007.05245.x
129. Gralnek IM, Hays RD, Kilbourne AM, Chang L, Mayer EA. Racial differences in the impact of irritable bowel syndrome on health-related quality of life. *J Clin Gastroenterol* (2004) **38**:782–9. doi:10.1097/01.mcg.0000140190.65405.fb
130. Gupta A, Silman AJ. Psychological stress and fibromyalgia: a review of the evidence suggesting a neuroendocrine link. *Arthritis Res Ther* (2004) **6**:98–106. doi:10.1186/ar1140
131. Boudewijn van Houdenhove M, Egle U. The role of life stress in fibromyalgia. *Curr Rheumatol Rep* (2005) **7**:365–70. doi:10.1007/s11926-005-0021-z
132. Gunter WD, Shepard JD, Foreman RD, Myers DA, Greenwood-van Meerveld B. Evidence for visceral hypersensitivity in high-anxiety rats. *Physiol Behav* (2000) **69**:379–82. doi:10.1016/S0031-9384(99)00254-1
133. Bravo JA, Dinan TG, Cryan JF. Alterations in the central CRF system of two different rat models of comorbid depression and functional gastrointestinal disorders. *Int J Neuropsychopharmacol* (2011) **14**:666–83. doi:10.1017/S1461145710000994
134. Tramullas M, Dinan TG, Cryan JF. Chronic psychosocial stress induces visceral hyperalgesia in mice. *Stress* (2012) **15**:281–92. doi:10.3109/10253890.2011.622816
135. O'Mahony SM, Marchesi JR, Scully P, Codling C, Ceolho AM, Quigley EM, et al. Early life stress alters behavior, immunity, and microbiota in rats: implications for irritable bowel syndrome and psychiatric illnesses. *Biol Psychiatry* (2009) **65**:263–7. doi:10.1016/j.biopsych.2008.06.026
136. Nishiyama H, Mizuta Y, Isomoto H, Takeshima F, Omagari K, Miyahara Y, et al. Chronic visceral hypersensitivity renders defecation more susceptible to stress via a serotonergic pathway in rats. *Dig Dis Sci* (2004) **49**:763–9. doi:10.1023/B:DDAS.0000030086.03506.c7
137. Geuze E, Westenberg HG, Jochims A, de Kloet CS, Bohus M, Vermetten E, et al. Altered pain processing in veterans with posttraumatic stress disorder. *Arch Gen Psychiatry* (2007) **64**:76–85. doi:10.1001/archpsyc.64.1.76
138. Staud R, Domingo M. Evidence for abnormal pain processing in fibromyalgia syndrome. *Pain Med* (2001) **2**:208–15. doi:10.1046/j.1526-4637.2001.01030.x
139. Coss-Adame E, Rao SS. Brain and gut interactions in irritable bowel syndrome: new paradigms and new understandings. *Curr Gastroenterol Rep* (2014) **16**:379. doi:10.1007/s11894-014-0379-z
140. Mayer EA, Chang L, Lembo T. Brain-gut interactions: implications for newer therapy. *Eur J Surg Suppl* (1998) **582**:50–5.
141. Fukudo S, Muranaka M, Nomura T, Satake M. Brain-gut interactions in irritable bowel syndrome: physiological and psychological aspect. *Nihon Rinsho* (1992) **50**:2703–11.
142. Mulak A, Bonaz B. Irritable bowel syndrome: a model of the brain-gut interactions. *Med Sci Monit* (2004) **10**:Ra55–62.
143. Cryan JF, O'Mahony SM. The microbiome-gut-brain axis: from bowel to behavior. *Neurogastroenterol Motil* (2011) **23**:187–92. doi:10.1111/j.1365-2982.2010.01664.x
144. Borre YE, Moloney RD, Clarke G, Dinan TG, Cryan JF. The impact of microbiota on brain and behavior: mechanisms & therapeutic potential. *Adv Exp Med Biol* (2014) **817**:373–403. doi:10.1007/978-1-4939-0897-4_17
145. Moloney RD, Desbonnet L, Clarke G, Dinan TG, Cryan JF. The microbiome: stress, health and disease. *Mamm Genome* (2014) **25**:49–74. doi:10.1007/s00335-013-9488-5
146. Bonaz B, Pellissier S, Sinniger V, Clarençon D, Peinnequin A, Canini F. *The Irritable Bowel Syndrome: How Stress can Affect the Amygdala Activity and the Brain-Gut Axis.* InTech (2012).
147. Labus JS, Dinov ID, Jiang Z, Ashe-Mcnalley C, Zamanyan A, Shi Y, et al. Irritable bowel syndrome in female patients is associated with alterations in structural brain networks. *Pain* (2014) **155**:137–49. doi:10.1016/j.pain.2013.09.020
148. Icenhour A, Langhorst J, Benson S, Schlamann M, Hampel S, Engler H, et al. Neural circuitry of abdominal pain-related fear learning and reinstatement in irritable bowel syndrome. *Neurogastroenterol Motil* (2015) **27**:114–27. doi:10.1111/nmo.12489
149. Selye H. A syndrome produced by diverse nocuous agents. 1936. *J Neuropsychiatry Clin Neurosci* (1936) **10**:230–1. doi:10.1176/jnp.10.2.230a
150. Carrasco GA, van de Kar LD. Neuroendocrine pharmacology of stress. *Eur J Pharmacol* (2003) **463**:235–72. doi:10.1016/S0014-2999(03)01285-8
151. Charmandari E, Tsigos C, Chrousos G. Endocrinology of the stress response. *Annu Rev Physiol* (2005) **67**:259–84. doi:10.1146/annurev.physiol.67.040403.120816
152. Habib KE, Gold PW, Chrousos GP. Neuroendocrinology of stress. *Endocrinol Metab Clin North Am* (2001) **30**:695–728. doi:10.1016/S0889-8529(05)70208-5
153. McEwen BS. Physiology and neurobiology of stress and adaptation: central role of the brain. *Physiol Rev* (2007) **87**:873–904. doi:10.1152/physrev.00041.2006
154. Smith SM, Vale WW. The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress. *Dialogues Clin Neurosci* (2006) **8**:383–95.
155. Chrousos GP. Regulation and dysregulation of the hypothalamic-pituitary-adrenal axis. The corticotropin-releasing hormone perspective. *Endocrinol Metab Clin North Am* (1992) **21**:833–58.
156. Whitnall MH. Regulation of the hypothalamic corticotropin-releasing hormone neurosecretory system. *Prog Neurobiol* (1993) **40**:573–629. doi:10.1016/0301-0082(93)90035-Q
157. McEwen BS. The neurobiology of stress: from serendipity to clinical relevance. *Brain Res* (2000) **886**:172–89. doi:10.1016/S0006-8993(00)02950-4
158. McEwen BS. Brain on stress: how the social environment gets under the skin. *Proc Natl Acad Sci U S A* (2012) **109**(Suppl 2):17180–5. doi:10.1073/pnas.1121254109
159. Nutt DJ, Malizia AL. Structural and functional brain changes in posttraumatic stress disorder. *J Clin Psychiatry* (2004) **65**(Suppl 1):11–7.
160. Lupien SJ, Mcewen BS, Gunnar MR, Heim C. Effects of stress throughout the lifespan on the brain, behaviour and cognition. *Nat Rev Neurosci* (2009) **10**:434–45. doi:10.1038/nrn2639
161. O'Malley D, Quigley EM, Dinan TG, Cryan JF. Do interactions between stress and immune responses lead to symptom exacerbations in irritable bowel syndrome? *Brain Behav Immun* (2011) **25**:1333–41. doi:10.1016/j.bbi.2011.04.009
162. Munck A, Guyre PM, Holbrook NJ. Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. *Endocr Rev* (1984) **5**:25–44. doi:10.1210/edrv-5-1-25
163. Bamberger CM, Schulte HM, Chrousos GP. Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. *Endocr Rev* (1996) **17**:245–61. doi:10.1210/edrv-17-3-245
164. McEwen BS, Stellar E. Stress and the individual. Mechanisms leading to disease. *Arch Intern Med* (1993) **153**:2093–101. doi:10.1001/archinte.153.18.2093
165. Buckley MM, O'Halloran KD, Rae MG, Dinan TG, O'Malley D. Modulation of enteric neurons by interleukin-6 and corticotropin-releasing factor contributes to visceral hypersensitivity and altered colonic motility in a rat model of irritable bowel syndrome. *J Physiol* (2014) **592**:5235–50. doi:10.1113/jphysiol.2014.279968
166. Greenwood-van Meerveld B, Johnson AC, Cochrane S, Schulkin J, Myers DA. Corticotropin-releasing factor 1 receptor-mediated mechanisms inhibit colonic hypersensitivity in rats. *Neurogastroenterol Motil* (2005) **17**:415–22. doi:10.1111/j.1365-2982.2005.00648.x
167. Trimble N, Johnson AC, Foster A, Greenwood-van Meerveld B. Corticotropin-releasing factor receptor 1-deficient mice show decreased anxiety and colonic sensitivity. *Neurogastroenterol Motil* (2007) **19**:754–60. doi:10.1111/j.1365-2982.2007.00951.x
168. Tran L, Schulkin J, Greenwood-van Meerveld B. Importance of CRF receptor-mediated mechanisms of the bed nucleus of the stria terminalis in the processing of anxiety and pain. *Neuropsychopharmacology* (2014) **39**:2633–45. doi:10.1038/npp.2014.117
169. Tache Y, Martinez V, Wang L, Million M. CRF1 receptor signaling pathways are involved in stress-related alterations of colonic function and viscerosensitivity: implications for irritable bowel syndrome. *Br J Pharmacol* (2004) **141**:1321–30. doi:10.1038/sj.bjp.0705760
170. Tache Y, Martinez V, Million M, Maillot C. Role of corticotropin releasing factor receptor subtype 1 in stress-related functional colonic alterations: implications in irritable bowel syndrome. *Eur J Surg* (2002) (Suppl) **587**:16–22.
171. Schwetz I, Mcroberts JA, Coutinho SV, Bradesi S, Gale G, Fanselow M, et al. Corticotropin-releasing factor receptor 1 mediates acute and delayed stress-induced visceral hyperalgesia in maternally separated Long-Evans rats. *Am J Physiol Gastrointest Liver Physiol* (2005) **289**:G704–12. doi:10.1152/ajpgi.00498.2004
172. Million M, Wang L, Wang Y, Adelson DW, Yuan PQ, Maillot C, et al. CRF2 receptor activation prevents colorectal distension induced visceral pain and

spinal ERK1/2 phosphorylation in rats. *Gut* (2006) **55**:172–81. doi:10.1136/gut.2004.051391
173. Tache Y, Million M, Nelson AG, Lamy C, Wang L. Role of corticotropin-releasing factor pathways in stress-related alterations of colonic motor function and viscerosensibility in female rodents. *Gend Med* (2005) **2**:146–54. doi:10.1016/S1550-8579(05)80043-9
174. Martinez V, Tache Y. CRF1 receptors as a therapeutic target for irritable bowel syndrome. *Curr Pharm Des* (2006) **12**:4071–88. doi:10.2174/138161206778743637
175. Larauche M, Gourcerol G, Wang L, Pambukchian K, Brunnhuber S, Adelson DW, et al. Cortagine, a CRF1 agonist, induces stresslike alterations of colonic function and visceral hypersensitivity in rodents primarily through peripheral pathways. *Am J Physiol Gastrointest Liver Physiol* (2009) **297**:G215–27. doi:10.1152/ajpgi.00072.2009
176. Tache Y. Corticotrophin-releasing factor 1 activation in the central amygdale and visceral hyperalgesia. *Neurogastroenterol Motil* (2015) **27**:1–6. doi:10.1111/nmo.12495
177. Myers DA, Gibson M, Schulkin J, Greenwood van-Meerveld B. Corticosterone implants to the amygdala and type 1 CRH receptor regulation: effects on behavior and colonic sensitivity. *Behav Brain Res* (2005) **161**:39–44. doi:10.1016/j.bbr.2005.03.001
178. Hornig M. The role of microbes and autoimmunity in the pathogenesis of neuropsychiatric illness. *Curr Opin Rheumatol* (2013) **25**:488–795. doi:10.1097/BOR.0b013e32836208de
179. Longstreth GF, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC. Functional bowel disorders. *Gastroenterology* (2006) **130**:1480–91. doi:10.1053/j.gastro.2005.11.061
180. Dinan TG, Cryan J, Shanahan F, Keeling PW, Quigley EM. IBS: an epigenetic perspective. *Nat Rev Gastroenterol Hepatol* (2010) **7**:465–71. doi:10.1038/nrgastro.2010.99
181. Elsenbruch S. Abdominal pain in irritable Bowel syndrome: a review of putative psychological, neural and neuro-immune mechanisms. *Brain Behav Immun* (2011) **25**:386–94. doi:10.1016/j.bbi.2010.11.010
182. Mayer EA. The neurobiology of stress and gastrointestinal disease. *Gut* (2000) **47**:861–9. doi:10.1136/gut.47.6.861
183. Mayer EA, Naliboff BD, Chang L, Coutinho SV. Stress and irritable bowel syndrome. *Am J Physiol Gastrointest Liver Physiol* (2001) **280**:G519–24.
184. Mayer EA, Craske M, Naliboff BD. Depression, anxiety, and the gastrointestinal system. *J Clin Psychiatry* (2001) **62**(Suppl 8):28–36.
185. McEwen BS. Protective and damaging effects of stress mediators. *N Engl J Med* (1998) **338**:171–9. doi:10.1056/NEJM199801153380307
186. Fukudo S. Stress and visceral pain: focusing on irritable bowel syndrome. *Pain* (2013) **154**(Suppl 1):S63–70. doi:10.1016/j.pain.2013.09.008
187. Tache Y, Martinez V, Million M, Rivier J. Corticotropin-releasing factor and the brain-gut motor response to stress. *Can J Gastroenterol* (1999) **13**(Suppl A):18A–25A.
188. Barreau F, Ferrier L, Fioramonti J, Bueno L. New insights in the etiology and pathophysiology of irritable bowel syndrome: contribution of neonatal stress models. *Pediatr Res* (2007) **62**:240–5. doi:10.1203/PDR.0b013e3180db2949
189. Chitkara DK, van Tilburg MA, Blois-Martin N, Whitehead WE. Early life risk factors that contribute to irritable bowel syndrome in adults: a systematic review. *Am J Gastroenterol* (2008) **103**:765–74. doi:10.1111/j.1572-0241.2007.01722.x
190. Haglund ME, Nestadt PS, Cooper NS, Southwick SM, Charney DS. Psychobiological mechanisms of resilience: relevance to prevention and treatment of stress-related psychopathology. *Dev Psychopathol* (2007) **19**:889–920. doi:10.1017/S0954579407000430
191. Charney DS. Psychobiological mechanisms of resilience and vulnerability: implications for successful adaptation to extreme stress. *Am J Psychiatry* (2004) **161**:195–216. doi:10.1176/appi.ajp.161.2.195
192. Drossman DA, Whitehead WE, Toner BB, Diamant N, Hu YJB, Bangdiwala SI, et al. What determines severity among patients with painful functional bowel disorders[quest]. *Am J Gastroenterol* (2000) **95**:974–80. doi:10.1111/j.1572-0241.2000.01936.x
193. Dinan TG, Quigley EM, Ahmed SM, Scully P, O'Brien S, O'Mahony L, et al. Hypothalamic-pituitary-gut axis dysregulation in irritable bowel syndrome: plasma cytokines as a potential biomarker? *Gastroenterology* (2006) **130**:304–11. doi:10.1053/j.gastro.2005.11.033
194. Hertig VL, Cain KC, Jarrett ME, Burr RL, Heitkemper MM. Daily stress and gastrointestinal symptoms in women with irritable bowel syndrome. *Nurs Res* (2007) **56**:399–406. doi:10.1097/01.NNR.0000299855.60053.88
195. Lee OY. [Psychosocial factors and visceral hypersensitivity in irritable bowel syndrome]. *Korean J Gastroenterol* (2006) **47**:111–9.
196. Bonaz B, Rivest S. Effect of a chronic stress on CRF neuronal activity and expression of its type 1 receptor in the rat brain. *Am J Physiol* (1998) **275**:R1438–49.
197. Seres G, Kovacs Z, Kovacs A, Kerekgyarto O, Sardi K, Demeter P, et al. Different associations of health related quality of life with pain, psychological distress and coping strategies in patients with irritable bowel syndrome and inflammatory bowel disorder. *J Clin Psychol Med Settings* (2008) **15**:287–95. doi:10.1007/s10880-008-9132-9
198. Minderhoud IM, Oldenburg B, Wismeijer JA, van Berge Henegouwen GP, Smout AJ. IBS-like symptoms in patients with inflammatory bowel disease in remission; relationships with quality of life and coping behavior. *Dig Dis Sci* (2004) **49**:469–74. doi:10.1023/B:DDAS.0000020506.84248.f9
199. Jones MP, Wessinger S, Crowell MD. Coping strategies and interpersonal support in patients with irritable bowel syndrome and inflammatory bowel disease. *Clin Gastroenterol Hepatol* (2006) **4**:474–81. doi:10.1016/j.cgh.2005.12.012
200. Deechakawan W, Cain KC, Jarrett ME, Burr RL, Heitkemper MM. Effect of self-management intervention on cortisol and daily stress levels in irritable bowel syndrome. *Biol Res Nurs* (2013) **15**:26–36. doi:10.1177/1099800411414047
201. Warren JW, Langenberg P, Clauw DJ. The number of existing functional somatic syndromes (FSSs) is an important risk factor for new, different FSSs. *J Psychosom Res* (2013) **74**:12–7. doi:10.1016/j.jpsychores.2012.09.002
202. Tremolaterra F, Gallotta S, Morra Y, Lubrano E, Ciacci C, Iovino P. The severity of irritable bowel syndrome or the presence of fibromyalgia influencing the perception of visceral and somatic stimuli. *BMC Gastroenterol* (2014) **14**:182. doi:10.1186/1471-230X-14-182
203. Thabane M, Kottachchi DT, Marshall JK. Systematic review and meta-analysis: the incidence and prognosis of post-infectious irritable bowel syndrome. *Aliment Pharmacol Ther* (2007) **26**:535–44. doi:10.1111/j.1365-2036.2007.03399.x
204. Thabane M, Marshall JK. Post-infectious irritable bowel syndrome. *World J Gastroenterol* (2009) **15**:3591–6. doi:10.3748/wjg.15.3591
205. Fendt M, Fanselow MS. The neuroanatomical and neurochemical basis of conditioned fear. *Neurosci Biobehav Rev* (1999) **23**:743–60. doi:10.1016/S0149-7634(99)00016-0
206. Mayer EA, Naliboff BD, Chang L. Basic pathophysiologic mechanisms in irritable bowel syndrome. *Dig Dis* (2001) **19**:212–8. doi:10.1159/000050682
207. Rhee SH, Pothoulakis C, Mayer EA. Principles and clinical implications of the brain-gut-enteric microbiota axis. *Nat Rev Gastroenterol Hepatol* (2009) **6**:306–14. doi:10.1038/nrgastro.2009.35
208. Camara RJ, Ziegler R, Begre S, Schoepfer AM, von Kanel R, Swiss Inflammatory Bowel Disease Cohort Study, G. The role of psychological stress in inflammatory bowel disease: quality assessment of methods of 18 prospective studies and suggestions for future research. *Digestion* (2009) **80**:129–39. doi:10.1159/000226087
209. Mawdsley JE, Rampton DS. The role of psychological stress in inflammatory bowel disease. *Neuroimmunomodulation* (2006) **13**:327–36. doi:10.1159/000104861
210. Bonaz BL, Bernstein CN. Brain-gut interactions in inflammatory bowel disease. *Gastroenterology* (2013) **144**:36–49. doi:10.1053/j.gastro.2012.10.003
211. Grenham S, Clarke G, Cryan JF, Dinan TG. Brain-gut-microbe communication in health and disease. *Front Physiol* (2011) **2**:94. doi:10.3389/fphys.2011.00094
212. Aziz Q, Thompson DG. Brain-gut axis in health and disease. *Gastroenterology* (1998) **114**:559–78. doi:10.1016/S0016-5085(98)70540-2
213. Mayer EA. Gut feelings: the emerging biology of gut-brain communication. *Nat Rev Neurosci* (2011) **12**:453–66. doi:10.1038/nrn3071
214. Davari S, Talaei SA, Alaei H, Salami M. Probiotics treatment improves diabetes-induced impairment of synaptic activity and cognitive function: behavioral and electrophysiological proofs for microbiome-gut-brain axis. *Neuroscience* (2013) **240**:287–96. doi:10.1016/j.neuroscience.2013.02.055
215. Borre YE, O'Keeffe GW, Clarke G, Stanton C, Dinan TG, Cryan JF. Microbiota and neurodevelopmental windows: implications for brain disorders. *Trends Mol Med* (2014) **20**:509–18. doi:10.1016/j.molmed.2014.05.002

216. Cryan JF, Dinan TG. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nat Rev Neurosci* (2012) **13**:701–12. doi:10.1038/nrn3346
217. Nicholson JK, Holmes E, Kinross J, Burcelin R, Gibson G, Jia W, et al. Host-gut microbiota metabolic interactions. *Science* (2012) **336**:1262–7. doi:10.1126/science.1223813
218. Sam AH, Troke RC, Tan TM, Bewick GA. The role of the gut/brain axis in modulating food intake. *Neuropharmacology* (2012) **63**:46–56. doi:10.1016/j.neuropharm.2011.10.008
219. Schellekens H, Finger BC, Dinan TG, Cryan JF. Ghrelin signalling and obesity: at the interface of stress, mood and food reward. *Pharmacol Ther* (2012) **135**:316–26. doi:10.1016/j.pharmthera.2012.06.004
220. Ellekilde M, Selfjord E, Larsen CS, Jakesevic M, Rune I, Tranberg B, et al. Transfer of gut microbiota from lean and obese mice to antibiotic-treated mice. *Sci Rep* (2014) **4**:5922. doi:10.1038/srep05922
221. Cox LM, Yamanishi S, Sohn J, Alekseyenko AV, Leung JM, Cho I, et al. Altering the intestinal microbiota during a critical developmental window has lasting metabolic consequences. *Cell* (2014) **158**:705–21. doi:10.1016/j.cell.2014.05.052
222. Clarke G, Cryan JF, Dinan TG, Quigley EM. Review article: probiotics for the treatment of irritable bowel syndrome – focus on lactic acid bacteria. *Aliment Pharmacol Ther* (2012) **35**:403–13. doi:10.1111/j.1365-2036.2011.04965.x
223. Galley JD, Nelson MC, Yu Z, Dowd SE, Walter J, Kumar PS, et al. Exposure to a social stressor disrupts the community structure of the colonic mucosa-associated microbiota. *BMC Microbiol* (2014) **14**:189. doi:10.1186/1471-2180-14-189
224. de Palma G, Collins SM, Bercik P, Verdu EF. The microbiota-gut-brain axis in gastrointestinal disorders: stressed bugs, stressed brain or both? *J Physiol* (2014) **592**:2989–97. doi:10.1113/jphysiol.2014.273995
225. Crumeyrolle-Arias M, Jaglin M, Bruneau A, Vancassel S, Cardona A, Dauge V, et al. Absence of the gut microbiota enhances anxiety-like behavior and neuroendocrine response to acute stress in rats. *Psychoneuroendocrinology* (2014) **42**:207–17. doi:10.1016/j.psyneuen.2014.01.014
226. Ait-Belgnaoui A, Colom A, Braniste V, Ramalho L, Marrot A, Cartier C, et al. Probiotic gut effect prevents the chronic psychological stress-induced brain activity abnormality in mice. *Neurogastroenterol Motil* (2014) **26**:510–20. doi:10.1111/nmo.12295
227. Xu D, Gao J, Gillilland M III, Wu X, Song I, Kao JY, et al. Rifaximin alters intestinal bacteria and prevents stress-induced gut inflammation and visceral hyperalgesia in rats. *Gastroenterology* (2014) **146**:484.e–96.e. doi:10.1053/j.gastro.2013.10.026
228. Scott LV, Clarke G, Dinan TG. The brain-gut axis: a target for treating stress-related disorders. *Mod Trends Pharmacopsychiatri* (2013) **28**:90–9. doi:10.1159/000343971
229. Collins SM, Surette M, Bercik P. The interplay between the intestinal microbiota and the brain. *Nat Rev Microbiol* (2012) **10**:735–42. doi:10.1038/nrmicro2876
230. Bercik P. The microbiota-gut-brain axis: learning from intestinal bacteria? *Gut* (2011) **60**:288–9. doi:10.1136/gut.2010.226779
231. Jeffery IB, O'Toole PW, Ohman L, Claesson MJ, Deane J, Quigley EM, et al. An irritable bowel syndrome subtype defined by species-specific alterations in faecal microbiota. *Gut* (2012) **61**:997–1006. doi:10.1136/gutjnl-2011-301501
232. Matto J, Maunuksela L, Kajander K, Palva A, Korpela R, Kassinen A, et al. Composition and temporal stability of gastrointestinal microbiota in irritable bowel syndrome – a longitudinal study in IBS and control subjects. *FEMS Immunol Med Microbiol* (2005) **43**:213–22. doi:10.1016/j.femsim.2004.08.009
233. Malinen E, Rinttila T, Kajander K, Matto J, Kassinen A, Krogius L, et al. Analysis of the fecal microbiota of irritable bowel syndrome patients and healthy controls with real-time PCR. *Am J Gastroenterol* (2005) **100**:373–82. doi:10.1111/j.1572-0241.2005.40312.x
234. Kassinen A, Krogius-Kurikka L, Makivuokko H, Rinttila T, Paulin L, Corander J, et al. The fecal microbiota of irritable bowel syndrome patients differs significantly from that of healthy subjects. *Gastroenterology* (2007) **133**:24–33. doi:10.1053/j.gastro.2007.04.005
235. Lyra A, Rinttila T, Nikkila J, Krogius-Kurikka L, Kajander K, Malinen E, et al. Diarrhoea-predominant irritable bowel syndrome distinguishable by 16S rRNA gene phylotype quantification. *World J Gastroenterol* (2009) **15**:5936–45. doi:10.3748/wjg.15.5936
236. Tana C, Umesaki Y, Imaoka A, Handa T, Kanazawa M, Fukudo S. Altered profiles of intestinal microbiota and organic acids may be the origin of symptoms in irritable bowel syndrome. *Neurogastroenterol Motil* (2010) **22**(512–9):e114–5. doi:10.1111/j.1365-2982.2009.01427.x
237. Mayer EA, Savidge T, Shulman RJ. Brain-gut microbiome interactions and functional bowel disorders. *Gastroenterology* (2014) **146**:1500–12. doi:10.1053/j.gastro.2014.02.037
238. Theodorou V, Ait Belgnaoui A, Agostini S, Eutamene H. Effect of commensals and probiotics on visceral sensitivity and pain in irritable bowel syndrome. *Gut Microb* (2014) **5**:430–6. doi:10.4161/gmic.29796
239. Distrutti E, Cipriani S, Mencarelli A, Renga B, Fiorucci S. Probiotics VSL#3 protect against development of visceral pain in murine model of irritable bowel syndrome. *PLoS One* (2013) **8**:e63893. doi:10.1371/journal.pone.0063893
240. Dai C, Guandalini S, Zhao DH, Jiang M. Antinociceptive effect of VSL#3 on visceral hypersensitivity in a rat model of irritable bowel syndrome: a possible action through nitric oxide pathway and enhance barrier function. *Mol Cell Biochem* (2012) **362**:43–53. doi:10.1007/s11010-011-1126-5
241. McKernan DP, Fitzgerald P, Dinan TG, Cryan JF. The probiotic *Bifidobacterium infantis* 35624 displays visceral antinociceptive effects in the rat. *Neurogastroenterol Motil* (2010) **22**(1029–35):e268. doi:10.1111/j.1365-2982.2010.01520.x
242. Johnson AC, Greenwood-van Meerveld B, Mcrorie J. Effects of *Bifidobacterium infantis* 35624 on post-inflammatory visceral hypersensitivity in the rat. *Dig Dis Sci* (2011) **56**:3179–86. doi:10.1007/s10620-011-1730-y
243. Agostini S, Goubern M, Tondereau V, Salvador-Cartier C, Bezirard V, Leveque M, et al. A marketed fermented dairy product containing *Bifidobacterium lactis* CNCM I-2494 suppresses gut hypersensitivity and colonic barrier disruption induced by acute stress in rats. *Neurogastroenterol Motil* (2012) **24**:376–e172. doi:10.1111/j.1365-2982.2011.01865.x
244. Verdu EF, Bercik P, Verma-Gandhu M, Huang XX, Blennerhassett P, Jackson W, et al. Specific probiotic therapy attenuates antibiotic induced visceral hypersensitivity in mice. *Gut* (2006) **55**:182–90. doi:10.1136/gut.2005.066100
245. Kamiya T, Wang L, Forsythe P, Goettsche G, Mao Y, Wang Y, et al. Inhibitory effects of *Lactobacillus reuteri* on visceral pain induced by colorectal distension in Sprague-Dawley rats. *Gut* (2006) **55**:191–6. doi:10.1136/gut.2005.070987
246. Rousseaux C, Thuru X, Gelot A, Barnich N, Neut C, Dubuquoy L, et al. *Lactobacillus acidophilus* modulates intestinal pain and induces opioid and cannabinoid receptors. *Nat Med* (2007) **13**:35–7. doi:10.1038/nm1521
247. Eutamene H, Lamine F, Chabo C, Theodorou V, Rochat F, Bergonzelli GE, et al. Synergy between *Lactobacillus paracasei* and its bacterial products to counteract stress-induced gut permeability and sensitivity increase in rats. *J Nutr* (2007) **137**:1901–7.
248. O'Mahony SM, Felice VD, Nally K, Savignac HM, Claesson MJ, Scully P, et al. Disturbance of the gut microbiota in early-life selectively affects visceral pain in adulthood without impacting cognitive or anxiety-related behaviors in male rats. *Neuroscience* (2014) **277**:885–901. doi:10.1016/j.neuroscience.2014.07.054
249. Saadi M, McCallum RW. Rifaximin in irritable bowel syndrome: rationale, evidence and clinical use. *Ther Adv Chronic Dis* (2013) **4**:71–5. doi:10.1177/2040622312472008
250. Schoenfeld P, Pimentel M, Chang L, Lembo A, Chey WD, Yu J, et al. Safety and tolerability of rifaximin for the treatment of irritable bowel syndrome without constipation: a pooled analysis of randomised, double-blind, placebo-controlled trials. *Aliment Pharmacol Ther* (2014) **39**:1161–8. doi:10.1111/apt.12735
251. Schmulson M, Bielsa MV, Carmona-Sanchez R, Hernandez A, Lopez-Colombo A, LOPEZ Vidal Y, et al. Microbiota, gastrointestinal infections, low-grade inflammation, and antibiotic therapy in irritable bowel syndrome: an evidence-based review. *Rev Gastroenterol Mex* (2014) **79**:96–134. doi:10.1016/j.rgmx.2014.01.004
252. Pimentel M, Morales W, Chua K, Barlow G, Weitsman S, Kim G, et al. Effects of rifaximin treatment and retreatment in nonconstipated IBS subjects. *Dig Dis Sci* (2011) **56**:2067–72. doi:10.1007/s10620-011-1728-5
253. Pimentel M, Lembo A, Chey WD, Zakko S, Ringel Y, Yu J, et al. Rifaximin therapy for patients with irritable bowel syndrome without constipation. *N Engl J Med* (2011) **364**:22–32. doi:10.1056/NEJMoa1004409
254. Moraru IG, Portincasa P, Moraru AG, Diculescu M, Dumitrascu DL. Small intestinal bacterial overgrowth produces symptoms in irritable bowel syndrome which are improved by rifaximin. A pilot study. *Rom J Intern Med* (2013) **51**:143–7.

255. Menees SB, Maneerattannaporn M, Kim HM, Chey WD. The efficacy and safety of rifaximin for the irritable bowel syndrome: a systematic review and meta-analysis. *Am J Gastroenterol* (2012) **107**:28–35. doi:10.1038/ajg.2011.355
256. Jolley J. High-dose rifaximin treatment alleviates global symptoms of irritable bowel syndrome. *Clin Exp Gastroenterol* (2011) **4**:43–8. doi:10.2147/CEG.S18051
257. Boltin D, Perets TT, Shporn E, Aizic S, Levy S, Niv Y, et al. Rifaximin for small intestinal bacterial overgrowth in patients without irritable bowel syndrome. *Ann Clin Microbiol Antimicrob* (2014) **13**:49. doi:10.1186/s12941-014-0049-x
258. Leonard BE. Impact of inflammation on neurotransmitter changes in major depression: an insight into the action of antidepressants. *Prog Neuropsychopharmacol Biol Psychiatry* (2014) **48**:261–7. doi:10.1016/j.pnpbp.2013.10.018
259. Skaper SD, Facci L, Giusti P. Neuroinflammation, microglia and mast cells in the pathophysiology of neurocognitive disorders: a review. *CNS Neurol Disord Drug Targets* (2014) **13**:1654–66.
260. Leonard BE. The concept of depression as a dysfunction of the immune system. *Curr Immunol Rev* (2010) **6**:205–12. doi:10.2174/157339510791823835
261. Marchand F, Perretti M, Mcmahon SB. Role of the immune system in chronic pain. *Nat Rev Neurosci* (2005) **6**:521–32. doi:10.1038/nrn1700
262. Bengmark S. Gut microbiota, immune development and function. *Pharmacol Res* (2013) **69**:87–113. doi:10.1016/j.phrs.2012.09.002
263. Leonard BE. The HPA and immune axes in stress: the involvement of the serotonergic system. *Eur Psychiatry* (2005) **20**(Suppl 3):S302–6. doi:10.1016/S0924-9338(05)80180-4
264. Bateman A, Singh A, Kral T, Solomon S. The immune-hypothalamic-pituitary-adrenal axis. *Endocr Rev* (1989) **10**:92–112. doi:10.1210/edrv-10-1-92
265. Genton L, Kudsk KA. Interactions between the enteric nervous system and the immune system: role of neuropeptides and nutrition. *Am J Surg* (2003) **186**:253–8. doi:10.1016/S0002-9610(03)00210-1
266. Hori T, Katafuchi T, Take S, Shimizu N, Niijima A. The autonomic nervous system as a communication channel between the brain and the immune system. *Neuroimmunomodulation* (1995) **2**:203–15. doi:10.1159/000097198
267. Nance DM, Sanders VM. Autonomic innervation and regulation of the immune system (1987-2007). *Brain Behav Immun* (2007) **21**:736–45. doi:10.1016/j.bbi.2007.03.008
268. Graeber MB. Changing face of microglia. *Science* (2010) **330**:783–8. doi:10.1126/science.1190929
269. Lu CL. Spinal microglia: a potential target in the treatment of chronic visceral pain. *J Chin Med Assoc* (2014) **77**:3–9. doi:10.1016/j.jcma.2013.08.008
270. Cervero F. Visceral pain: mechanisms of peripheral and central sensitization. *Ann Med* (1995) **27**:235–9. doi:10.3109/07853899509031965
271. Bradesi S. Role of spinal cord glia in the central processing of peripheral pain perception. *Neurogastroenterol Motil* (2010) **22**:499–511. doi:10.1111/j.1365-2982.2010.01491.x
272. Gosselin RD, Suter MR, Ji RR, Decosterd I. Glial cells and chronic pain. *Neuroscientist* (2010) **16**:519–31. doi:10.1177/1073858409360822
273. Inoue K, Tsuda M. Microglia and neuropathic pain. *Glia* (2009) **57**:1469–79. doi:10.1002/glia.20871
274. Graeber MB, Christie MJ. Multiple mechanisms of microglia: a gatekeeper's contribution to pain states. *Exp Neurol* (2012) **234**:255–61. doi:10.1016/j.expneurol.2012.01.007
275. Scholz J, Woolf CJ. The neuropathic pain triad: neurons, immune cells and glia. *Nat Neurosci* (2007) **10**:1361–8. doi:10.1038/nn1992
276. Tsuda M, Beggs S, Salter MW, Inoue K. Microglia and intractable chronic pain. *Glia* (2013) **61**:55–61. doi:10.1002/glia.22379
277. Tsuda M, Inoue K, Salter MW. Neuropathic pain and spinal microglia: a big problem from molecules in "small" glia. *Trends Neurosci* (2005) **28**:101–7. doi:10.1016/j.tins.2004.12.002
278. Zhang Y, Li H, Li Y, Sun X, Zhu M, Hanley G, et al. Essential role of toll-like receptor 2 in morphine-induced microglia activation in mice. *Neurosci Lett* (2011) **489**:43–7. doi:10.1016/j.neulet.2010.11.063
279. Mika J, Zychowska M, Popiolek-Barczyk K, Rojewska E, Przewlocka B. Importance of glial activation in neuropathic pain. *Eur J Pharmacol* (2013) **716**:106–19. doi:10.1016/j.ejphar.2013.01.072
280. Saab CY, Wang J, Gu C, Garner KN, Al-Chaer ED. Microglia: a newly discovered role in visceral hypersensitivity? *Neuron Glia Biol* (2006) **2**:271–7. doi:10.1017/S1740925X07000439
281. Riazi K, Galic MA, Kuzmiski JB, Ho W, Sharkey KA, Pittman QJ. Microglial activation and TNFalpha production mediate altered CNS excitability following peripheral inflammation. *Proc Natl Acad Sci U S A* (2008) **105**:17151–6. doi:10.1073/pnas.0806682105
282. Delafoy L, Raymond F, Doherty AM, Eschalier A, Diop L. Role of nerve growth factor in the trinitrobenzene sulfonic acid-induced colonic hypersensitivity. *Pain* (2003) **105**:489–97. doi:10.1016/S0304-3959(03)00266-5
283. Liu PY, Lu CL, Wang CC, Lee IH, Hsieh JC, Chen CC, et al. Spinal microglia initiate and maintain hyperalgesia in a rat model of chronic pancreatitis. *Gastroenterology* (2012) **142**(165–173):e2. doi:10.1053/j.gastro.2011.09.041
284. Bradesi S, Svensson CI, Steinauer J, Pothoulakis C, Yaksh TL, Mayer EA. Role of spinal microglia in visceral hyperalgesia and NK1R up-regulation in a rat model of chronic stress. *Gastroenterology* (2009) **136**(1339–48):e1–2. doi:10.1053/j.gastro.2008.12.044
285. Dunlop SP, Jenkins D, Neal KR, Naesdal J, Borgaonker M, Collins SM, et al. Randomized, double-blind, placebo-controlled trial of prednisolone in post-infectious irritable bowel syndrome. *Aliment Pharmacol Ther* (2003) **18**:77–84. doi:10.1046/j.1365-2036.2003.01640.x
286. Casanova JL, Abel L, Quintana-Murci L. Human TLRs and Il-1Rs in host defense: natural insights from evolutionary, epidemiological, and clinical genetics. *Annu Rev Immunol* (2011) **29**:447–91. doi:10.1146/annurev-immunol-030409-101335
287. Takeda K, Akira S. TLR signaling pathways. *Semin Immunol* (2004) **16**:3–9. doi:10.1016/j.smim.2003.10.003
288. Cario E. Toll-like receptors in inflammatory bowel diseases: a decade later. *Inflamm Bowel Dis* (2010) **16**:1583–97. doi:10.1002/ibd.21282
289. Tramullas M, Finger BC, Moloney RD, Golubeva AV, Moloney G, Dinan TG, et al. Toll-like receptor 4 regulates chronic stress-induced visceral pain in mice. *Biol Psychiatry* (2014) **76**:340–8. doi:10.1016/j.biopsych.2013.11.004
290. Brint EK, Macsharry J, Fanning A, Shanahan F, Quigley EM. Differential expression of toll-like receptors in patients with irritable bowel syndrome. *Am J Gastroenterol* (2011) **106**:329–36. doi:10.1038/ajg.2010.438
291. McKernan DP, Gaszner G, Quigley EM, Cryan JF, Dinan TG. Altered peripheral toll-like receptor responses in the irritable bowel syndrome. *Aliment Pharmacol Ther* (2011) **33**:1045–52. doi:10.1111/j.1365-2036.2011.04624.x
292. McKernan DP, Nolan A, Brint EK, O'Mahony SM, Hyland NP, Cryan JF, et al. Toll-like receptor mRNA expression is selectively increased in the colonic mucosa of two animal models relevant to irritable bowel syndrome. *PLoS One* (2009) **4**:e8226. doi:10.1371/journal.pone.0008226
293. Mbodji K, Torre S, Haas V, Dechelotte P, Marion-Letellier R. Alanyl-glutamine restores maternal deprivation-induced TLR4 levels in a rat neonatal model. *Clin Nutr* (2011) **30**:672–7. doi:10.1016/j.clnu.2011.04.002
294. Barajon I, Serrao G, Arnaboldi F, Opizzi E, Ripamonti G, Balsari A, et al. Toll-like receptors 3, 4, and 7 are expressed in the enteric nervous system and dorsal root ganglia. *J Histochem Cytochem* (2009) **57**:1013–23. doi:10.1369/jhc.2009.953539
295. Ochoa-Cortes F, Ramos-Lomas T, Miranda-Morales M, Spreadbury I, Ibeakanma C, Barajas-Lopez C, et al. Bacterial cell products signal to mouse colonic nociceptive dorsal root ganglia neurons. *Am J Physiol Gastrointest Liver Physiol* (2010) **299**:G723–32. doi:10.1152/ajpgi.00494.2009
296. Lehnardt S, Massillon L, Follett P, Jensen FE, Ratan R, Rosenberg PA, et al. Activation of innate immunity in the CNS triggers neurodegeneration through a toll-like receptor 4-dependent pathway. *Proc Natl Acad Sci U S A* (2003) **100**:8514–9. doi:10.1073/pnas.1432609100
297. Bettoni I, Comelli F, Rossini C, Granucci F, Giagnoni G, Peri F, et al. Glial TLR4 receptor as new target to treat neuropathic pain: efficacy of a new receptor antagonist in a model of peripheral nerve injury in mice. *Glia* (2008) **56**:1312–9. doi:10.1002/glia.20699
298. Hutchinson MR, Bland ST, Johnson KW, Rice KC, Maier SF, Watkins LR. Opioid-induced glial activation: mechanisms of activation and implications for opioid analgesia, dependence, and reward. *ScientificWorldJournal* (2007) **7**:98–111. doi:10.1100/tsw.2007.230
299. Hutchinson MR, Zhang Y, Shridhar M, Evans JH, Buchanan MM, Zhao TX, et al. Evidence that opioids may have toll-like receptor 4 and MD-2 effects. *Brain Behav Immun* (2010) **24**:83–95. doi:10.1016/j.bbi.2009.08.004
300. Lewis SS, Hutchinson MR, Rezvani N, Loram LC, Zhang Y, Maier SF, et al. Evidence that intrathecal morphine-3-glucuronide may cause pain enhancement via toll-like receptor 4/MD-2 and interleukin-1β. *Neuroscience* (2010) **165**:569–83. doi:10.1016/j.neuroscience.2009.10.011

301. Watkins LR, Hutchinson MR, Rice KC, Maier SF. The "toll" of opioid-induced glial activation: improving the clinical efficacy of opioids by targeting glia. *Trends Pharmacol Sci* (2009) **30**:581–91. doi:10.1016/j.tips.2009.08.002
302. Mogil JS. Sex differences in pain and pain inhibition: multiple explanations of a controversial phenomenon. *Nat Rev Neurosci* (2012) **13**:859–66. doi:10.1038/nrn3360
303. Meleine M, Matricon J. Gender-related differences in irritable bowel syndrome: potential mechanisms of sex hormones. *World J Gastroenterol* (2014) **20**:6725–43. doi:10.3748/wjg.v20.i22.6725
304. Mulak A, Tache Y, Larauche M. Sex hormones in the modulation of irritable bowel syndrome. *World J Gastroenterol* (2014) **20**:2433–48. doi:10.3748/wjg.v20.i10.2433
305. Mogil JS, Bailey AL. Sex and gender differences in pain and analgesia. *Prog Brain Res* (2010) **186**:141–57. doi:10.1016/B978-0-444-53630-3.00009-9
306. Adeyemo MA, Spiegel BM, Chang L. Meta-analysis: do irritable bowel syndrome symptoms vary between men and women? *Aliment Pharmacol Ther* (2010) **32**:738–55. doi:10.1111/j.1365-2036.2010.04409.x
307. Heitkemper MM, Chang L. Do fluctuations in ovarian hormones affect gastrointestinal symptoms in women with irritable bowel syndrome? *Gend Med* (2009) **6**(Suppl 2):152–67. doi:10.1016/j.genm.2009.03.004
308. Fillingim RB, King CD, Ribeiro-Dasilva MC, Rahim-Williams B, Riley JL III. Sex, gender, and pain: a review of recent clinical and experimental findings. *J Pain* (2009) **10**:447–85. doi:10.1016/j.jpain.2008.12.001
309. Gui X, Carraway RE, Dobner PR. Endogenous neurotensin facilitates visceral nociception and is required for stress-induced antinociception in mice and rats. *Neuroscience* (2004) **126**:1023–32. doi:10.1016/j.neuroscience.2004.04.034
310. Moloney RD, O'Leary OF, Felice D, Bettler B, Dinan TG, Cryan JF. Early-life stress induces visceral hypersensitivity in mice. *Neurosci Lett* (2012) **512**:99–102. doi:10.1016/j.neulet.2012.01.066
311. Larauche M, Gourcerol G, Million M, Adelson DW, Tache Y. Repeated psychological stress-induced alterations of visceral sensitivity and colonic motor functions in mice: influence of surgery and postoperative single housing on visceromotor responses. *Stress* (2010) **13**:343–54. doi:10.3109/10253891003664166
312. Ouyang A, Wrzos HF. Contribution of gender to pathophysiology and clinical presentation of IBS: should management be different in women? *Am J Gastroenterol* (2006) **101**:S602–9. doi:10.1111/j.1572-0241.2006.00975.x
313. Heitkemper M, Jarrett M. Irritable bowel syndrome: does gender matter? *J Psychosom Res* (2008) **64**:583–7. doi:10.1016/j.jpsychores.2008.02.020
314. Lee OY, Mayer EA, Schmulson M, Chang L, Naliboff B. Gender-related differences in IBS symptoms. *Am J Gastroenterol* (2001) **96**:2184–93. doi:10.1111/j.1572-0241.2001.03961.x
315. North CS, Downs D, Clouse RE, Alrakawi A, Dokucu ME, Cox J, et al. The presentation of irritable bowel syndrome in the context of somatization disorder. *Clin Gastroenterol Hepatol* (2004) **2**:787–95. doi:10.1016/S1542-3565(04)00350-7
316. Heitkemper MM, Jarrett ME, Levy RL, Cain KC, Burr RL, Feld A, et al. Self-management for women with irritable bowel syndrome. *Clin Gastroenterol Hepatol* (2004) **2**:585–96. doi:10.1016/S1542-3565(04)00242-3
317. Drossman DA, Toner BB, Whitehead WE, Diamant NE, Dalton CB, Duncan S, et al. Cognitive-behavioral therapy versus education and desipramine versus placebo for moderate to severe functional bowel disorders. *Gastroenterology* (2003) **125**:19–31. doi:10.1016/S0016-5085(03)00669-3
318. Blanchard EB, Lackner JM, Sanders K, Krasner S, Keefer L, Payne A, et al. A controlled evaluation of group cognitive therapy in the treatment of irritable bowel syndrome. *Behav Res Ther* (2007) **45**:633–48. doi:10.1016/j.brat.2006.07.003
319. Anbardan SJ, Daryani NE, Fereshtehnejad SM, Taba Taba, Vakili S, Keramati MR, et al. Gender role in irritable bowel syndrome: a comparison of irritable bowel syndrome module (ROME III) between male and female patients. *J Neurogastroenterol Motil* (2012) **18**:70–7. doi:10.5056/jnm.2012.18.1.70
320. Heitkemper MM, Cain KC, Jarrett ME, Burr RL, Hertig V, Bond EF. Symptoms across the menstrual cycle in women with irritable bowel syndrome. *Am J Gastroenterol* (2003) **98**:420–30. doi:10.1111/j.1572-0241.2003.07233.x
321. Altman G, Cain KC, Motzer S, Jarrett M, Burr R, Heitkemper M. Increased symptoms in female IBS patients with dysmenorrhea and PMS. *Gastroenterol Nurs* (2006) **29**:4–11. doi:10.1097/00001610-200601000-00002
322. Bjornsson B, Orvar KB, Theodors A, Kjeld M. [The relationship of gastrointestinal symptoms and menstrual cycle phase in young healthy women]. *Laeknabladid* (2006) **92**:677–82.
323. Whitehead WE, Cheskin LJ, Heller BR, Robinson JC, Crowell MD, Benjamin C, et al. Evidence for exacerbation of irritable bowel syndrome during menses. *Gastroenterology* (1990) **98**:1485–9.
324. Laessle RG, Tuschl RJ, Schweiger U, Pirke KM. Mood changes and physical complaints during the normal menstrual cycle in healthy young women. *Psychoneuroendocrinology* (1990) **15**:131–8. doi:10.1016/0306-4530(90)90021-Z
325. Kane SV, Sable K, Hanauer SB. The menstrual cycle and its effect on inflammatory bowel disease and irritable bowel syndrome: a prevalence study. *Am J Gastroenterol* (1998) **93**:1867–72. doi:10.1111/j.1572-0241.1998.540_i.x
326. Lesniewska B, Nowak M, Malendowicz LK. Sex differences in adrenocortical structure and function. XXVIII. ACTH and corticosterone in intact, gonadectomised and gonadal hormone replaced rats. *Horm Metab Res* (1990) **22**:378–81.
327. Kitay JI. Sex differences in adrenal cortical secretion in the rat. *Endocrinology* (1961) **68**:818–24. doi:10.1210/endo-68-5-818
328. Kant GJ, Lenox RH, Bunnell BN, Mougey EH, Pennington LL, Meyerhoff JL. Comparison of stress response in male and female rats: pituitary cyclic AMP and plasma prolactin, growth hormone and corticosterone. *Psychoneuroendocrinology* (1983) **8**:421–8. doi:10.1016/0306-4530(83)90021-5
329. Critchlow V, Liebelt RA, Bar-Sela M, Mountcastle W, Lipscomb HS. Sex difference in resting pituitary-adrenal function in the rat. *Am J Physiol* (1963) **205**:807–15.
330. Allen-Rowlands CF, Allen JP, Greer MA, Wilson M. Circadian rhythmicity of ACTH and corticosterone in the rat. *J Endocrinol Invest* (1980) **3**:371–7. doi:10.1007/BF03349373
331. Chen X-N, Zhu H, Meng Q-Y, Zhou J-N. Estrogen receptor-α and-β regulate the human corticotropin-releasing hormone gene through similar pathways. *Brain Res* (2008) **1223**:1–10. doi:10.1016/j.brainres.2008.05.043
332. Mulak A, Taché Y. Sex difference in irritable bowel syndrome: do gonadal hormones play a role? *Gastroenterol Pol* (2010) **17**:89.
333. McCormick CM, Linkroum W, Sallinen BJ, Miller NW. Peripheral and central sex steroids have differential effects on the HPA axis of male and female rats. *Stress* (2002) **5**:235–47. doi:10.1080/1025389021000061165
334. Atkinson HC, Waddell BJ. Circadian variation in basal plasma corticosterone and adrenocorticotropin in the rat: sexual dimorphism and changes across the estrous cycle. *Endocrinology* (1997) **138**:3842–8. doi:10.1210/en.138.9.3842
335. Bohler HC Jr, Zoeller RT, King JC, Rubin BS, Weber R, Merriam GR. Corticotropin releasing hormone mRNA is elevated on the afternoon of proestrus in the parvocellular paraventricular nuclei of the female rat. *Brain Res Mol Brain Res* (1990) **8**:259–62. doi:10.1016/0169-328X(90)90025-9
336. Buckingham JC, Dohler KD, Wilson CA. Activity of the pituitary-adrenocortical system and thyroid gland during the oestrous cycle of the rat. *J Endocrinol* (1978) **78**:359–66. doi:10.1677/joe.0.0780359
337. Linthorst A. *Interactions Between Corticotropin-Releasing Hormone and Serotonin: Implications for the Aetiology and Treatment of Anxiety Disorders. Anxiety and Anxiolytic Drugs.* Berlin: Springer (2005).
338. Lu CL, Hsieh JC, Dun NJ, Oprea TI, Wang PS, Luo JC, et al. Estrogen rapidly modulates 5-hydroxytrytophan-induced visceral hypersensitivity via GPR30 in rats. *Gastroenterology* (2009) **137**:1040–50. doi:10.1053/j.gastro.2009.03.047
339. Warnock JK, Clayton AH. Chronic episodic disorders in women. *Psychiatr Clin North Am* (2003) **26**:725–40. doi:10.1016/S0193-953X(03)00042-X
340. Mermelstein PG. Membrane-localised oestrogen receptor alpha and beta influence neuronal activity through activation of metabotropic glutamate receptors. *J Neuroendocrinol* (2009) **21**:257–62. doi:10.1111/j.1365-2826.2009.01838.x
341. Aloisi AM, Affaitati G, Ceccarelli I, Fiorenzani P, Lerza R, Rossi C, et al. Estradiol and testosterone differently affect visceral pain-related behavioural responses in male and female rats. *Eur J Pain* (2010) **14**:602–7. doi:10.1016/j.ejpain.2009.10.016
342. Sapsed-Byrne S, Ma D, Ridout D, Holdcroft A. Estrous cycle phase variations in visceromotor and cardiovascular responses to colonic distension in the anesthetized rat. *Brain Res* (1996) **742**:10–6. doi:10.1016/S0006-8993(96)00989-4
343. Holdcroft A, Sapsed-Byrne S, Ma D, Hammal D, Forsling ML. Sex and oestrous cycle differences in visceromotor responses and vasopressin release in response to colonic distension in male and female rats anaesthetized with halothane. *Br J Anaesth* (2000) **85**:907–10. doi:10.1093/bja/85.6.907
344. Ji Y, Tang B, Traub RJ. The visceromotor response to colorectal distention fluctuates with the estrous cycle in rats. *Neuroscience* (2008) **154**:1562–7. doi:10.1016/j.neuroscience.2008.04.070

345. Sorge RE, Martin LJ, Isbester KA, Sotocinal SG, Rosen S, Tuttle AH, et al. Olfactory exposure to males, including men, causes stress and related analgesia in rodents. *Nat Methods* (2014) **11**:629–32. doi:10.1038/nmeth.2935
346. Petrenko AB, Yamakura T, Baba H, Shimoji K. The role of N-methyl-D-aspartate (NMDA) receptors in pain: a review. *Anesth Analg* (2003) **97**:1108–16. doi:10.1213/01.ANE.0000081061.12235.55
347. Holzer P, Holzer-Petsche U. Tachykinin receptors in the gut: physiological and pathological implications. *Curr Opin Pharmacol* (2001) **1**:583–90. doi:10.1016/S1471-4892(01)00100-X
348. Blackshaw LA, Gebhart GF. The pharmacology of gastrointestinal nociceptive pathways. *Curr Opin Pharmacol* (2002) **2**:642–9. doi:10.1016/S1471-4892(02)00211-4
349. Coutinho SV, Urban MO, Gebhart GF. Role of glutamate receptors and nitric oxide in the rostral ventromedial medulla in visceral hyperalgesia. *Pain* (1998) **78**:59–69. doi:10.1016/S0304-3959(98)00137-7
350. McRoberts JA, Coutinho SV, Marvizon JC, Grady EF, Tognetto M, Sengupta JN, et al. Role of peripheral N-methyl-D-aspartate (NMDA) receptors in visceral nociception in rats. *Gastroenterology* (2001) **120**:1737–48. doi:10.1053/gast.2001.24848
351. Goudet C, Magnaghi V, Landry M, Nagy F, Gereau RWT, Pin JP. Metabotropic receptors for glutamate and GABA in pain. *Brain Res Rev* (2009) **60**:43–56. doi:10.1016/j.brainresrev.2008.12.007
352. Chen Y, Bacon G, Sher E, Clark BP, Kallman MJ, Wright RA, et al. Evaluation of the activity of a novel metabotropic glutamate receptor antagonist (±)-2-amino-2-(3-cis and trans-carboxycyclobutyl-3-(9-thioxanthyl)propionic acid) in the in vitro neonatal spinal cord and in an in vivo pain model. *Neuroscience* (2000) **95**:787–93. doi:10.1016/S0306-4522(99)00496-0
353. Ji G, Neugebauer V. Reactive oxygen species are involved in group I mGluR-mediated facilitation of nociceptive processing in amygdala neurons. *J Neurophysiol* (2010) **104**:218–29. doi:10.1152/jn.00223.2010
354. Bianchi R, Rezzani R, Borsani E, Rodella L. mGlu5 receptor antagonist decreases Fos expression in spinal neurons after noxious visceral stimulation. *Brain Res* (2003) **960**:263–6. doi:10.1016/S0006-8993(02)03697-1
355. Liaw W, Stephens R, Binns B, Chu Y, Sepkuty J, Johns R, et al. Spinal glutamate uptake is critical for maintaining normal sensory transmission in rat spinal cord. *Pain* (2005) **115**:60–70. doi:10.1016/j.pain.2005.02.006
356. Tao YX, Gu J, Stephens RL Jr. Role of spinal cord glutamate transporter during normal sensory transmission and pathological pain states. *Mol Pain* (2005) **1**:30. doi:10.1186/1744-8069-1-30
357. Latremoliere A, Woolf CJ. Central sensitization: a generator of pain hypersensitivity by central neural plasticity. *J Pain* (2009) **10**:895–926. doi:10.1016/j.jpain.2009.06.012
358. Hwang JH, Yaksh TL. The effect of spinal GABA receptor agonists on tactile allodynia in a surgically-induced neuropathic pain model in the rat. *Pain* (1997) **70**:15–22. doi:10.1016/S0304-3959(96)03249-6
359. Rode F, Jensen DG, Blackburn-Munro G, Bjerrum OJ. Centrally-mediated antinociceptive actions of GABA(A) receptor agonists in the rat spared nerve injury model of neuropathic pain. *Eur J Pharmacol* (2005) **516**:131–8. doi:10.1016/j.ejphar.2005.04.034
360. Reichl S, Augustin M, Zahn PK, Pogatzki-Zahn EM. Peripheral and spinal GABAergic regulation of incisional pain in rats. *Pain* (2012) **153**:129–41. doi:10.1016/j.pain.2011.09.028
361. Hyland NP, Cryan JF. A gut feeling about GABA: focus on GABA(B) receptors. *Front Pharmacol* (2010) **1**:124. doi:10.3389/fphar.2010.00124
362. Abelli L, Conte B, Somma V, Maggi CA, Giuliani S, Meli A. A method for studying pain arising from the urinary bladder in conscious, freely-moving rats. *J Urol* (1989) **141**:148–51.
363. Hara K, Saito Y, Kirihara Y, Yamada Y, Sakura S, Kosaka Y. The interaction of antinociceptive effects of morphine and GABA receptor agonists within the rat spinal cord. *Anesth Analg* (1999) **89**:422–7. doi:10.1213/00000539-199908000-00032
364. Brusberg M, Ravnefjord A, Martinsson R, Larsson H, Martinez V, Lindstrom E. The GABA(B) receptor agonist, baclofen, and the positive allosteric modulator, CGP7930, inhibit visceral pain-related responses to colorectal distension in rats. *Neuropharmacology* (2009) **56**:362–7. doi:10.1016/j.neuropharm.2008.09.006
365. Liu LS, Shenoy M, Pasricha PJ. The analgesic effects of the GABAB receptor agonist, baclofen, in a rodent model of functional dyspepsia. *Neurogastroenterol Motil* (2011) **23**(356–61):e160–1. doi:10.1111/j.1365-2982.2010.01649.x
366. Lu Y, Westlund KN. Effects of baclofen on colon inflammation-induced Fos, CGRP and SP expression in spinal cord and brainstem. *Brain Res* (2001) **889**:118–30. doi:10.1016/S0006-8993(00)03124-3
367. Sengupta JN. Visceral pain: the neurophysiological mechanism. *Handb Exp Pharmacol* (2009) **194**:31–74. doi:10.1007/978-3-540-79090-7_2
368. Smid SD, Young RL, Cooper NJ, Blackshaw LA. GABA(B)R expressed on vagal afferent neurones inhibit gastric mechanosensitivity in ferret proximal stomach. *Am J Physiol Gastrointest Liver Physiol* (2001) **281**:G1494–501.
369. Partosoedarso ER, Young RL, Blackshaw LA. GABA(B) receptors on vagal afferent pathways: peripheral and central inhibition. *Am J Physiol Gastrointest Liver Physiol* (2001) **280**:G658–68.
370. Page AJ, Blackshaw LA. GABA(B) receptors inhibit mechanosensitivity of primary afferent endings. *J Neurosci* (1999) **19**:8597–602.
371. Sengupta JN, Medda BK, Shaker R. Effect of GABA(B) receptor agonist on distension-sensitive pelvic nerve afferent fibers innervating rat colon. *Am J Physiol Gastrointest Liver Physiol* (2002) **283**:G1343–51. doi:10.1152/ajpgi.00124.2002
372. Isomoto S, Kaibara M, Sakurai-Yamashita Y, Nagayama Y, Uezono Y, Yano K, et al. Cloning and tissue distribution of novel splice variants of the rat GABAB receptor. *Biochem Biophys Res Commun* (1998) **253**:10–5. doi:10.1006/bbrc.1998.9706
373. Cryan JF, Kaupmann K. Don't worry 'B' happy!: a role for GABA(B) receptors in anxiety and depression. *Trends Pharmacol Sci* (2005) **26**:36–43. doi:10.1016/j.tips.2004.11.004
374. Houghton LA, Fell C, Whorwell PJ, Jones I, Sudworth DP, Gale JD. Effect of a second-generation alpha2delta ligand (pregabalin) on visceral sensation in hypersensitive patients with irritable bowel syndrome. *Gut* (2007) **56**:1218–25. doi:10.1136/gut.2006.110858
375. Gale JD, Houghton LA. Alpha 2 delta (alpha(2)delta) ligands, gabapentin and pregabalin: what is the evidence for potential use of these ligands in irritable bowel syndrome. *Front Pharmacol* (2011) **2**:28. doi:10.3389/fphar.2011.00028
376. Mozaffari S, Nikfar S, Abdollahi M. Metabolic and toxicological considerations for the latest drugs used to treat irritable bowel syndrome. *Expert Opin Drug Metab Toxicol* (2013) **9**:403–21. doi:10.1517/17425255.2013.759558
377. Zhang MM, Liu SB, Chen T, Koga K, Zhang T, Li YQ, et al. Effects of NB001 and gabapentin on irritable bowel syndrome-induced behavioral anxiety and spontaneous pain. *Mol Brain* (2014) **7**:47. doi:10.1186/1756-6606-7-47
378. Camilleri M. Genetics and irritable bowel syndrome: from genomics to intermediate phenotype and pharmacogenetics. *Dig Dis Sci* (2009) **54**:2318–24. doi:10.1007/s10620-009-0903-4
379. Camilleri M. Genetics of human gastrointestinal sensation. *Neurogastroenterol Motil* (2013) **25**:458–66. doi:10.1111/nmo.12132
380. Camilleri M, Busciglio I, Carlson P, Mckinzie S, Burton D, Baxter K, et al. Candidate genes and sensory functions in health and irritable bowel syndrome. *Am J Physiol Gastrointest Liver Physiol* (2008) **295**:G219–25. doi:10.1152/ajpgi.90202.2008
381. Ek WE, Reznichenko A, Ripke S, Niesler B, Zucchelli M, Rivera NV, et al. Exploring the genetics of irritable bowel syndrome: a GWA study in the general population and replication in multinational case-control cohorts. *Gut* (2014). doi:10.1136/gutjnl-2014-307997
382. Camilleri M, Carlson P, Acosta A, Busciglio I, Nair AA, Gibbons SJ, et al. RNA sequencing shows transcriptomic changes in rectosigmoid mucosa in patients with irritable bowel syndrome-diarrhea: a pilot case-control study. *Am J Physiol Gastrointest Liver Physiol* (2014) **306**:G1089–98. doi:10.1152/ajpgi.00068.2014
383. Swan C, Duroudier NP, Campbell E, Zaitoun A, Hastings M, Dukes GE, et al. Identifying and testing candidate genetic polymorphisms in the irritable bowel syndrome (IBS): association with TNFSF15 and TNFalpha. *Gut* (2013) **62**:985–94. doi:10.1136/gutjnl-2011-301213
384. Saito YA. The role of genetics in IBS. *Gastroenterol Clin North Am* (2011) **40**:45–67. doi:10.1016/j.gtc.2010.12.011
385. Beyder A, Mazzone A, Strege PR, Tester DJ, Saito YA, Bernard CE, et al. Loss-of-function of the voltage-gated sodium channel NaV1.5 (channelopathies) in patients with irritable bowel syndrome. *Gastroenterology* (2014) **146**:1659–68. doi:10.1053/j.gastro.2014.02.054
386. Camilleri M, Shin A, Busciglio I, Carlson P, Acosta A, Bharucha AE, et al. Genetic variation in GPBAR1 predisposes to quantitative changes in colonic transit and bile acid excretion. *Am J Physiol Gastrointest Liver Physiol* (2014) **307**:G508–16. doi:10.1152/ajpgi.00178.2014

387. Wouters MM, Lambrechts D, Knapp M, Cleynen I, Whorwell P, Agreus L, et al. Genetic variants in CDC42 and NXPH1 as susceptibility factors for constipation and diarrhoea predominant irritable bowel syndrome. *Gut* (2014) **63**:1103–11. doi:10.1136/gutjnl-2013-304570
388. Jacobson LH, Cryan JF. Genetic approaches to modeling anxiety in animals. *Curr Top Behav Neurosci* (2010) **2**:161–201. doi:10.1007/7854_2009_31
389. Jacobson LH, Cryan JF. Feeling strained? Influence of genetic background on depression-related behavior in mice: a review. *Behav Genet* (2007) **37**:171–213. doi:10.1007/s10519-006-9106-3
390. Lariviere WR, Wilson SG, Laughlin TM, Kokayeff A, West EE, Adhikari SM, et al. Heritability of nociception. III. Genetic relationships among commonly used assays of nociception and hypersensitivity. *Pain* (2002) **97**:75–86. doi:10.1016/S0304-3959(01)00492-4
391. Mogil JS, Wilson SG, Bon K, Lee SE, Chung K, Raber P, et al. Heritability of nociception II. 'Types' of nociception revealed by genetic correlation analysis. *Pain* (1999) **80**:83–93. doi:10.1016/S0304-3959(98)00196-1
392. Mogil JS, Wilson SG, Bon K, Lee SE, Chung K, Raber P, et al. Heritability of nociception I: responses of 11 inbred mouse strains on 12 measures of nociception. *Pain* (1999) **80**:67–82. doi:10.1016/S0304-3959(98)00197-3
393. Elsenbruch S, Wang L, Hollerbach S, Schedlowski M, Tougas G. Pseudoaffective visceromotor responses and HPA axis activation following colorectal distension in rats with increased cholinergic sensitivity. *Neurogastroenterol Motil* (2004) **16**:801–9. doi:10.1111/j.1365-2982.2004.00563.x
394. O'Malley D, Julio-Pieper M, O'Mahony SM, Dinan TG, Cryan JF. Differential visceral pain sensitivity and colonic morphology in four common laboratory rat strains. *Exp Physiol* (2014) **99**:359–67. doi:10.1113/expphysiol.2013.076109
395. Tang WY, Ho SM. Epigenetic reprogramming and imprinting in origins of disease. *Rev Endocr Metab Disord* (2007) **8**:173–82. doi:10.1007/s11154-007-9042-4
396. Bai G, Wei D, Zou S, Ren K, Dubner R. Inhibition of class II histone deacetylases in the spinal cord attenuates inflammatory hyperalgesia. *Mol Pain* (2010) **6**:51. doi:10.1186/1744-8069-6-51
397. Chiechio S, Copani A, Zammataro M, Battaglia G, Gereau RWT, Nicoletti F. Transcriptional regulation of type-2 metabotropic glutamate receptors: an epigenetic path to novel treatments for chronic pain. *Trends Pharmacol Sci* (2010) **31**:153–60. doi:10.1016/j.tips.2009.12.003
398. Denk F, McMahon SB. Chronic pain: emerging evidence for the involvement of epigenetics. *Neuron* (2012) **73**:435–44. doi:10.1016/j.neuron.2012.01.012
399. Chiechio S, Zammataro M, Morales ME, Busceti CL, Drago F, Gereau RWT, et al. Epigenetic modulation of mGlu2 receptors by histone deacetylase inhibitors in the treatment of inflammatory pain. *Mol Pharmacol* (2009) **75**:1014–20. doi:10.1124/mol.108.054346
400. Tran L, Chaloner A, Sawalha AH, Greenwood van-Meerveld B. Importance of epigenetic mechanisms in visceral pain induced by chronic water avoidance stress. *Psychoneuroendocrinology* (2013) **38**:898–906. doi:10.1016/j.psyneuen.2012.09.016
401. Hong S, Zheng G, Wiley JW. Epigenetic regulation of genes that modulate chronic stress-induced visceral pain in the peripheral nervous system. *Gastroenterology* (2015) **148**:148–57. doi:10.1053/j.gastro.2014.09.032
402. Winston JH, Li Q, Sarna SK. Chronic prenatal stress epigenetically modifies spinal cord BDNF expression to induce sex-specific visceral hypersensitivity in offspring. *Neurogastroenterol Motil* (2014) **26**:715–30. doi:10.1111/nmo.12326
403. van den Wijngaard RM, Stanisor OI, van Diest SA, Welting O, Wouters MM, Cailotto C, et al. Susceptibility to stress induced visceral hypersensitivity in maternally separated rats is transferred across generations. *Neurogastroenterol Motil* (2013) **25**:e780–90. doi:10.1111/nmo.12202
404. Theodorou V. Susceptibility to stress-induced visceral sensitivity: a bad legacy for next generations. *Neurogastroenterol Motil* (2013) **25**:927–30. doi:10.1111/nmo.12260
405. Geranton SM, Morenilla-Palao C, Hunt SP. A role for transcriptional repressor methyl-CpG-binding protein 2 and plasticity-related gene serum- and glucocorticoid-inducible kinase 1 in the induction of inflammatory pain states. *J Neurosci* (2007) **27**:6163–73. doi:10.1523/JNEUROSCI.1306-07.2007
406. Geranton SM, Fratto V, Tochiki KK, Hunt SP. Descending serotonergic controls regulate inflammation-induced mechanical sensitivity and methyl-CpG-binding protein 2 phosphorylation in the rat superficial dorsal horn. *Mol Pain* (2008) **4**:35. doi:10.1186/1744-8069-4-35
407. Stilling RM, Dinan TG, Cryan JF. Microbial genes, brain & behaviour – epigenetic regulation of the gut-brain axis. *Genes Brain Behav* (2013) **13**:69–86. doi:10.1111/gbb.12109
408. O'Mahony SM, Tramullas M, Fitzgerald P, Cryan JF. Rodent models of colorectal distension. *Curr Protoc Neurosci* (2012) **61**:9.40.1–13. doi:10.1002/0471142301.ns0940s61
409. Ness TJ, Gebhart GF. Colorectal distension as a noxious visceral stimulus: physiologic and pharmacologic characterization of pseudaffective reflexes in the rat. *Brain Res* (1988) **450**:153–69. doi:10.1016/0006-8993(88)91555-7
410. Bradesi S, Schwetz I, Ennes HS, Lamy CM, Ohning G, Fanselow M, et al. Repeated exposure to water avoidance stress in rats: a new model for sustained visceral hyperalgesia. *Am J Physiol Gastrointest Liver Physiol* (2005) **289**:G42–53. doi:10.1152/ajpgi.00500.2004
411. Christianson JA, Gebhart GF. Assessment of colon sensitivity by luminal distension in mice. *Nat Protoc* (2007) **2**:2624–31. doi:10.1038/nprot.2007.392
412. Larsson M, Arvidsson S, Ekman C, Bayati A. A model for chronic quantitative studies of colorectal sensitivity using balloon distension in conscious mice – effects of opioid receptor agonists. *Neurogastroenterol Motil* (2003) **15**:371–81. doi:10.1046/j.1365-2982.2003.00418.x
413. Nijsen MJ, Ongenae NG, Coulie B, Meulemans AL. Telemetric animal model to evaluate visceral pain in the freely moving rat. *Pain* (2003) **105**:115–23. doi:10.1016/S0304-3959(03)00170-2
414. Welting O, van den Wijngaard RM, de Jonge WJ, Holman R, Boeckxstaens GE. Assessment of visceral sensitivity using radio telemetry in a rat model of maternal separation. *Neurogastroenterol Motil* (2005) **17**:838–45. doi:10.1111/j.1365-2982.2005.00677.x
415. Arvidsson S, Larsson M, Larsson H, Lindstrom E, Martinez V. Assessment of visceral pain-related pseudo-affective responses to colorectal distension in mice by intracolonic manometric recordings. *J Pain* (2006) **7**:108–18. doi:10.1016/j.jpain.2005.09.003
416. Tammpere A, Brusberg M, Axenborg J, Hirsch I, Larsson H, Lindstrom E. Evaluation of pseudo-affective responses to noxious colorectal distension in rats by manometric recordings. *Pain* (2005) **116**:220–6. doi:10.1016/j.pain.2005.04.012
417. Al-Chaer ED, Kawasaki M, Pasricha PJ. A new model of chronic visceral hypersensitivity in adult rats induced by colon irritation during postnatal development. *Gastroenterology* (2000) **119**:1276–85. doi:10.1053/gast.2000.19576
418. Wang Z, Bradesi S, Maarek JM, Lee K, Winchester WJ, Mayer EA, et al. Regional brain activation in conscious, nonrestrained rats in response to noxious visceral stimulation. *Pain* (2008) **138**:233–43. doi:10.1016/j.pain.2008.04.018
419. Marques AH, O'Connor TG, Roth C, Susser E, Bjorke-Monsen AL. The influence of maternal prenatal and early childhood nutrition and maternal prenatal stress on offspring immune system development and neurodevelopmental disorders. *Front Neurosci* (2013) **7**:120. doi:10.3389/fnins.2013.00120
420. Bale TL, Baram TZ, Brown AS, Goldstein JM, Insel TR, McCarthy MM, et al. Early life programming and neurodevelopmental disorders. *Biol Psychiatry* (2010) **68**:314–9. doi:10.1016/j.biopsych.2010.05.028
421. Morgane PJ, Mokler DJ, Galler JR. Effects of prenatal protein malnutrition on the hippocampal formation. *Neurosci Biobehav Rev* (2002) **26**:471–83. doi:10.1016/S0149-7634(02)00012-X
422. Sampath D, Sabitha KR, Hegde P, Jayakrishnan HR, Kutty BM, Chattarji S, et al. A study on fear memory retrieval and REM sleep in maternal separation and isolation stressed rats. *Behav Brain Res* (2014) **273C**:144–54. doi:10.1016/j.bbr.2014.07.034
423. Takase K, Yamamoto Y, Yagami T. Maternal deprivation in the middle of a stress hyporesponsive period decreases hippocampal calcineurin expression and causes abnormal social and cognitive behaviours in adult male Wistar rats: relevance to negative symptoms of schizophrenia. *Behav Brain Res* (2012) **232**:306–15. doi:10.1016/j.bbr.2012.04.016
424. Paus T, Keshavan M, Giedd JN. Why do many psychiatric disorders emerge during adolescence? *Nat Rev Neurosci* (2008) **9**:947–57. doi:10.1038/nrn2513
425. Larauche M, Mulak A, Tache Y. Stress-related alterations of visceral sensation: animal models for irritable bowel syndrome study. *J Neurogastroenterol Motil* (2011) **17**:213–34. doi:10.5056/jnm.2011.17.3.213
426. Bradesi S, Mayer EA. Experimental models of stress and pain: do they help to develop new therapies? *Dig Dis* (2009) **27**(Suppl 1):55–67. doi:10.1159/000268122

427. Chaloner A, Greenwood-van Meerveld B. Early life adversity as a risk factor for visceral pain in later life: importance of sex differences. *Front Neurosci* (2013) **7**:13. doi:10.3389/fnins.2013.00013
428. Mayer EA, Bradesi S, Chang L, Spiegel BM, Bueller JA, Naliboff BD. Functional GI disorders: from animal models to drug development. *Gut* (2008) **57**:384–404. doi:10.1136/gut.2006.101675
429. Holschneider DP, Bradesi S, Mayer EA. The role of experimental models in developing new treatments for irritable bowel syndrome. *Expert Rev Gastroenterol Hepatol* (2011) **5**:43–57. doi:10.1586/egh.10.88
430. Qin HY, Wu JC, Tong XD, Sung JJ, Xu HX, Bian ZX. Systematic review of animal models of post-infectious/post-inflammatory irritable bowel syndrome. *J Gastroenterol* (2011) **46**:164–74. doi:10.1007/s00535-010-0321-6
431. Bulmer DC, Grundy D. Achieving translation in models of visceral pain. *Curr Opin Pharmacol* (2011) **11**:575–81. doi:10.1016/j.coph.2011.09.008
432. Folks DG. The interface of psychiatry and irritable bowel syndrome. *Curr Psychiatry Rep* (2004) **6**:210–5. doi:10.1007/s11920-004-0066-0
433. Lee S, Wu J, Ma YL, Tsang A, Guo WJ, Sung J. Irritable bowel syndrome is strongly associated with generalized anxiety disorder: a community study. *Aliment Pharmacol Ther* (2009) **30**:643–51. doi:10.1111/j.1365-2036.2009.04074.x
434. Thijssen AY, Jonkers DM, Leue C, van der Veek PP, Vidakovic-Vukic M, van Rood YR, et al. Dysfunctional cognitions, anxiety and depression in irritable bowel syndrome. *J Clin Gastroenterol* (2010) **44**:e236–41. doi:10.1097/MCG.0b013e3181eed5d8
435. Bakshi VP, Kalin NH. Corticotropin-releasing hormone and animal models of anxiety: gene-environment interactions. *Biol Psychiatry* (2000) **48**:1175–98. doi:10.1016/S0006-3223(00)01082-9
436. Delic S, Streif S, Deussing JM, Weber P, Ueffing M, Holter SM, et al. Genetic mouse models for behavioral analysis through transgenic RNAi technology. *Genes Brain Behav* (2008) **7**:821–30. doi:10.1111/j.1601-183X.2008.00412.x
437. Deussing JM, Wurst W. Dissecting the genetic effect of the CRH system on anxiety and stress-related behaviour. *C R Biol* (2005) **328**:199–212. doi:10.1016/j.crvi.2005.01.001
438. Kimura M, Muller-Preuss P, Lu A, Wiesner E, Flachskamm C, Wurst W, et al. Conditional corticotropin-releasing hormone overexpression in the mouse forebrain enhances rapid eye movement sleep. *Mol Psychiatry* (2010) **15**:154–65. doi:10.1038/mp.2009.46
439. Kolber BJ, Boyle MP, Wieczorek L, Kelley CL, Onwuzurike CC, Nettles SA, et al. Transient early-life forebrain corticotropin-releasing hormone elevation causes long-lasting anxiogenic and despair-like changes in mice. *J Neurosci* (2010) **30**:2571–81. doi:10.1523/JNEUROSCI.4470-09.2010
440. Lu A, Steiner MA, Whittle N, Vogl AM, Walser SM, Ableitner M, et al. Conditional mouse mutants highlight mechanisms of corticotropin-releasing hormone effects on stress-coping behavior. *Mol Psychiatry* (2008) **13**:1028–42. doi:10.1038/mp.2008.51
441. Homberg JR, Lesch KP. Looking on the bright side of serotonin transporter gene variation. *Biol Psychiatry* (2011) **69**:513–9. doi:10.1016/j.biopsych.2010.09.024
442. Ferguson SM, Neumaier JF. Grateful DREADDs: engineered receptors reveal how neural circuits regulate behavior. *Neuropsychopharmacology* (2012) **37**:296–7. doi:10.1038/npp.2011.179
443. Videlock EJ, Adeyemo M, Licudine A, Hirano M, Ohning G, Mayer M, et al. Childhood trauma is associated with hypothalamic-pituitary-adrenal axis responsiveness in irritable bowel syndrome. *Gastroenterology* (2009) **137**:1954–62. doi:10.1053/j.gastro.2009.08.058
444. O'Mahony SM, Hyland NP, Dinan TG, Cryan JF. Maternal separation as a model of brain-gut axis dysfunction. *Psychopharmacology (Berl)* (2011) **214**:71–88. doi:10.1007/s00213-010-2010-9
445. Plotsky PM, Meaney MJ. Early, postnatal experience alters hypothalamic corticotropin-releasing factor (CRF) mRNA, median eminence CRF content and stress-induced release in adult rats. *Brain Res Mol Brain Res* (1993) **18**:195–200. doi:10.1016/0169-328X(93)90189-V
446. Rosztoczy A, Fioramonti J, Jarmay K, Barreau F, Wittmann T, Bueno L. Influence of sex and experimental protocol on the effect of maternal deprivation on rectal sensitivity to distension in the adult rat. *Neurogastroenterol Motil* (2003) **15**:679–86. doi:10.1046/j.1350-1925.2003.00451.x
447. Fish EW, Shahrokh D, Bagot R, Caldji C, Bredy T, Szyf M, et al. Epigenetic programming of stress responses through variations in maternal care. *Ann N Y Acad Sci* (2004) **1036**:167–80. doi:10.1196/annals.1330.011
448. Francis DD, Meaney MJ. Maternal care and the development of stress responses. *Curr Opin Neurobiol* (1999) **9**:128–34. doi:10.1016/S0959-4388(99)80016-6
449. Francis D, Diorio J, Liu D, Meaney MJ. Nongenomic transmission across generations of maternal behavior and stress responses in the rat. *Science* (1999) **286**:1155–8. doi:10.1126/science.286.5442.1155
450. Szyf M, Weaver I, Meaney M. Maternal care, the epigenome and phenotypic differences in behavior. *Reprod Toxicol* (2007) **24**:9–19. doi:10.1016/j.reprotox.2007.05.001
451. Barreau F, Cartier C, Ferrier L, Fioramonti J, Bueno L. Nerve growth factor mediates alterations of colonic sensitivity and mucosal barrier induced by neonatal stress in rats. *Gastroenterology* (2004) **127**:524–34. doi:10.1053/j.gastro.2004.05.019
452. Coutinho SV, Plotsky PM, Sablad M, Miller JC, Zhou H, Bayati AI, et al. Neonatal maternal separation alters stress-induced responses to viscerosomatic nociceptive stimuli in rat. *Am J Physiol Gastrointest Liver Physiol* (2002) **282**:G307–16. doi:10.1152/ajpgi.00240.2001
453. Millstein RA, Holmes A. Effects of repeated maternal separation on anxiety- and depression-related phenotypes in different mouse strains. *Neurosci Biobehav Rev* (2007) **31**:3–17. doi:10.1016/j.neubiorev.2006.05.003
454. Savignac HM, Dinan TG, Cryan JF. Resistance to early-life stress in mice: effects of genetic background and stress duration. *Front Behav Neurosci* (2011) **5**:13. doi:10.3389/fnbeh.2011.00013
455. Weiss IC, Franklin TB, Vizi S, Mansuy IM. Inheritable effect of unpredictable maternal separation on behavioral responses in mice. *Front Behav Neurosci* (2011) **5**:3. doi:10.3389/fnbeh.2011.00003
456. Lin C, Al-Chaer ED. Long-term sensitization of primary afferents in adult rats exposed to neonatal colon pain. *Brain Res* (2003) **971**:73–82. doi:10.1016/S0006-8993(03)02358-8
457. Bonaz B, Tache Y. Water-avoidance stress-induced c-fos expression in the rat brain and stimulation of fecal output: role of corticotropin-releasing factor. *Brain Res* (1994) **641**:21–8. doi:10.1016/0006-8993(94)91810-4
458. Enck P, Merlin V, Erckenbrecht JF, Wienbeck M. Stress effects on gastrointestinal transit in the rat. *Gut* (1989) **30**:455–9. doi:10.1136/gut.30.4.455
459. Schwetz I, Bradesi S, Mcroberts JA, Sablad M, Miller JC, Zhou H, et al. Delayed stress-induced colonic hypersensitivity in male Wistar rats: role of neurokinin-1 and corticotropin-releasing factor-1 receptors. *Am J Physiol Gastrointest Liver Physiol* (2004) **286**:G683–91. doi:10.1152/ajpgi.00358.2003
460. Gue M, del Rio-Lacheze C, Eutamene H, Theodorou V, Fioramonti J, Bueno L. Stress-induced visceral hypersensitivity to rectal distension in rats: role of CRF and mast cells. *Neurogastroenterol Motil* (1997) **9**:271–9. doi:10.1046/j.1365-2982.1997.d01-63.x
461. Bradesi S, Eutamene H, Garcia-Villar R, Fioramonti J, Bueno L. Acute and chronic stress differently affect visceral sensitivity to rectal distension in female rats. *Neurogastroenterol Motil* (2002) **14**:75–82. doi:10.1046/j.1365-2982.2002.00305.x
462. Larauche M, Mulak A, Kim YS, Labus J, Million M, Tache Y. Visceral analgesia induced by acute and repeated water avoidance stress in rats: sex difference in opioid involvement. *Neurogastroenterol Motil* (2012) **24**:1031–e547. doi:10.1111/j.1365-2982.2012.01980.x
463. Bennett EJ, Tennant CC, Piesse C, Badcock CA, Kellow JE. Level of chronic life stress predicts clinical outcome in irritable bowel syndrome. *Gut* (1998) **43**:256–61. doi:10.1136/gut.43.2.256
464. Chang L. Review article: epidemiology and quality of life in functional gastrointestinal disorders. *Aliment Pharmacol Ther* (2004) **20**(Suppl 7):31–9. doi:10.1111/j.1365-2036.2004.02183.x
465. Choung RS, Locke GR III, Zinsmeister AR, Schleck CD, Talley NJ. Psychosocial distress and somatic symptoms in community subjects with irritable bowel syndrome: a psychological component is the rule. *Am J Gastroenterol* (2009) **104**:1772–9. doi:10.1038/ajg.2009.239
466. Lackner JM, Brasel AM, Quigley BM, Keefer L, Krasner SS, Powell C, et al. The ties that bind: perceived social support, stress, and IBS in severely affected patients. *Neurogastroenterol Motil* (2010) **22**:893–900. doi:10.1111/j.1365-2982.2010.01516.x
467. Yang PC, Jury J, Soderholm JD, Sherman PM, Mckay DM, Perdue MH. Chronic psychological stress in rats induces intestinal sensitization to luminal antigens. *Am J Pathol* (2006) **168**:104–14. doi:10.2353/ajpath.2006.050575
468. Hong S, Fan J, Kemmerer ES, Evans S, Li Y, Wiley JW. Reciprocal changes in vanilloid (TRPV1) and endocannabinoid (CB1) receptors contribute to

visceral hyperalgesia in the water avoidance stressed rat. *Gut* (2009) **58**:202–10. doi:10.1136/gut.2008.157594
469. Larsson MH, Miketa A, Martinez V. Lack of interaction between psychological stress and DSS-induced colitis affecting colonic sensitivity during colorectal distension in mice. *Stress* (2009) **12**:434–44. doi:10.1080/10253890802626603
470. Zheng J, Babygirija R, Bulbul M, Cerjak D, Ludwig K, Takahashi T. Hypothalamic oxytocin mediates adaptation mechanism against chronic stress in rats. *Am J Physiol Gastrointest Liver Physiol* (2010) **299**:G946–53. doi:10.1152/ajpgi.00483.2009
471. Patel S, Hillard CJ. Adaptations in endocannabinoid signaling in response to repeated homotypic stress: a novel mechanism for stress habituation. *Eur J Neurosci* (2008) **27**:2821–9. doi:10.1111/j.1460-9568.2008.06266.x
472. Black LV, Ness TJ, Robbins MT. Effects of oxytocin and prolactin on stress-induced bladder hypersensitivity in female rats. *J Pain* (2009) **10**:1065–72. doi:10.1016/j.jpain.2009.04.007
473. Sanson M, Bueno L, Fioramonti J. Involvement of cannabinoid receptors in inflammatory hypersensitivity to colonic distension in rats. *Neurogastroenterol Motil* (2006) **18**:949–56. doi:10.1111/j.1365-2982.2006.00819.x
474. Winston JH, Xu GY, Sarna SK. Adrenergic stimulation mediates visceral hypersensitivity to colorectal distension following heterotypic chronic stress. *Gastroenterology* (2010) **138**(294–304):e3. doi:10.1053/j.gastro.2009.09.054
475. Seo HJ, Ham HD, Jin HY, Lee WH, Hwang HS, Park SA, et al. Chronic administration of monosodium glutamate under chronic variable stress impaired hypothalamic-pituitary-adrenal axis function in rats. *Korean J Physiol Pharmacol* (2010) **14**:213–21. doi:10.4196/kjpp.2010.14.4.213
476. Tagliari B, Dos Santos TM, Cunha AA, Lima DD, Delwing D, Sitta A, et al. Chronic variable stress induces oxidative stress and decreases butyrylcholinesterase activity in blood of rats. *J Neural Transm* (2010) **117**:1067–76. doi:10.1007/s00702-010-0445-0
477. Willner P. Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation. *Psychopharmacology (Berl)* (1997) **134**:319–29. doi:10.1007/s002130050473
478. Babygirija R, Zheng J, Ludwig K, Takahashi T. Central oxytocin is involved in restoring impaired gastric motility following chronic repeated stress in mice. *Am J Physiol Regul Integr Comp Physiol* (2010) **298**:R157–65. doi:10.1152/ajpregu.00328.2009
479. Griffiths J, Shanks N, Anisman H. Strain-specific alterations in consumption of a palatable diet following repeated stressor exposure. *Pharmacol Biochem Behav* (1992) **42**:219–27. doi:10.1016/0091-3057(92)90519-L
480. Muscat R, Willner P. Suppression of sucrose drinking by chronic mild unpredictable stress: a methodological analysis. *Neurosci Biobehav Rev* (1992) **16**:507–17. doi:10.1016/S0149-7634(05)80192-7
481. Finger BC, Dinan TG, Cryan JF. High-fat diet selectively protects against the effects of chronic social stress in the mouse. *Neuroscience* (2011) **192**:351–60. doi:10.1016/j.neuroscience.2011.06.072
482. Reber SO, Obermeier F, Straub RH, Falk W, Neumann ID. Chronic intermittent psychosocial stress (social defeat/overcrowding) in mice increases the severity of an acute DSS-induced colitis and impairs regeneration. *Endocrinology* (2006) **147**:4968–76. doi:10.1210/en.2006-0347
483. Vicario M, Alonso C, Guilarte M, Serra J, Martinez C, Gonzalez-Castro AM, et al. Chronic psychosocial stress induces reversible mitochondrial damage and corticotropin-releasing factor receptor type-1 upregulation in the rat intestine and IBS-like gut dysfunction. *Psychoneuroendocrinology* (2012) **37**:65–77. doi:10.1016/j.psyneuen.2011.05.005
484. Cohen H, Jotkowitz A, Buskila D, Pelles-Avraham S, Kaplan Z, Neumann L, et al. Post-traumatic stress disorder and other co-morbidities in a sample population of patients with irritable bowel syndrome. *Eur J Intern Med* (2006) **17**:567–71. doi:10.1016/j.ejim.2006.07.011
485. Drossman DA, Leserman J, Nachman G, Li ZM, Gluck H, Toomey TC, et al. Sexual and physical abuse in women with functional or organic gastrointestinal disorders. *Ann Intern Med* (1990) **113**:828–33. doi:10.7326/0003-4819-113-11-828
486. Savas LS, White DL, Wieman M, Daci K, Fitzgerald S, Laday Smith S, et al. Irritable bowel syndrome and dyspepsia among women veterans: prevalence and association with psychological distress. *Aliment Pharmacol Ther* (2009) **29**:115–25. doi:10.1111/j.1365-2036.2008.03847.x
487. White DL, Savas LS, Daci K, Elserag R, Graham DP, Fitzgerald SJ, et al. Trauma history and risk of the irritable bowel syndrome in women veterans. *Aliment Pharmacol Ther* (2010) **32**:551–61. doi:10.1111/j.1365-2036.2010.04387.x
488. Rau V, Decola JP, Fanselow MS. Stress-induced enhancement of fear learning: an animal model of posttraumatic stress disorder. *Neurosci Biobehav Rev* (2005) **29**:1207–23. doi:10.1016/j.neubiorev.2005.04.010
489. Stam R. PTSD and stress sensitisation: a tale of brain and body Part 2: animal models. *Neurosci Biobehav Rev* (2007) **31**:558–84. doi:10.1016/j.neubiorev.2007.01.001
490. Stam R, Akkermans LM, Wiegant VM. Trauma and the gut: interactions between stressful experience and intestinal function. *Gut* (1997) **40**:704–9. doi:10.1136/gut.40.6.704
491. Wang W, Liu Y, Zheng H, Wang HN, Jin X, Chen YC, et al. A modified single-prolonged stress model for post-traumatic stress disorder. *Neurosci Lett* (2008) **441**:237–41. doi:10.1016/j.neulet.2008.06.031
492. Stam R, van Laar TJ, Akkermans LM, Wiegant VM. Variability factors in the expression of stress-induced behavioural sensitisation. *Behav Brain Res* (2002) **132**:69–76. doi:10.1016/S0166-4328(01)00387-4
493. Collins SM, Vallance B, Barbara G, Borgaonkar M. Putative inflammatory and immunological mechanisms in functional bowel disorders. *Baillieres Best Pract Res Clin Gastroenterol* (1999) **13**:429–36. doi:10.1053/bega.1999.0037
494. Spiller R, Garsed K. Postinfectious irritable bowel syndrome. *Gastroenterology* (2009) **136**:1979–88. doi:10.1053/j.gastro.2009.02.074
495. Long Y, Liu Y, Tong J, Qian W, Hou X. Effectiveness of trimebutine maleate on modulating intestinal hypercontractility in a mouse model of postinfectious irritable bowel syndrome. *Eur J Pharmacol* (2010) **636**:159–65. doi:10.1016/j.ejphar.2010.03.037
496. McLean PG, Picard C, Garcia-Villar R, More J, Fioramonti J, Bueno L. Effects of nematode infection on sensitivity to intestinal distension: role of tachykinin NK2 receptors. *Eur J Pharmacol* (1997) **337**:279–82. doi:10.1016/S0014-2999(97)01275-2
497. Coelho AM, Fioramonti J, Bueno L. Systemic lipopolysaccharide influences rectal sensitivity in rats: role of mast cells, cytokines, and vagus nerve. *Am J Physiol Gastrointest Liver Physiol* (2000) **279**:G781–90.
498. O'Mahony SM, Clarke G, Mckernan DP, Bravo JA, Dinan TG, Cryan JF. Differential visceral nociceptive, behavioural and neurochemical responses to an immune challenge in the stress-sensitive Wistar Kyoto rat strain. *Behav Brain Res* (2013) **253**:310–7. doi:10.1016/j.bbr.2013.07.023
499. Ohman L, Simren M. Pathogenesis of IBS: role of inflammation, immunity and neuroimmune interactions. *Nat Rev Gastroenterol Hepatol* (2010) **7**:163–73. doi:10.1038/nrgastro.2010.4
500. Akiho H, Ihara E, Nakamura K. Low-grade inflammation plays a pivotal role in gastrointestinal dysfunction in irritable bowel syndrome. *World J Gastrointest Pathophysiol* (2010) **1**:97–105. doi:10.4291/wjgp.v1.i3.97
501. Barbara G, de Giorgio R, Stanghellini V, Cremon C, Corinaldesi R. A role for inflammation in irritable bowel syndrome? *Gut* (2002) **51**(Suppl 1):i41–4. doi:10.1136/gut.51.suppl_1.i41
502. Collins SM, Piche T, Rampal P. The putative role of inflammation in the irritable bowel syndrome. *Gut* (2001) **49**:743–5. doi:10.1136/gut.49.6.743
503. Keohane J, O'Mahony C, O'Mahony L, O'Mahony S, Quigley EM, Shanahan F. Irritable bowel syndrome-type symptoms in patients with inflammatory bowel disease: a real association or reflection of occult inflammation? *Am J Gastroenterol* (2010) **105**(1788):1789–94. doi:10.1038/ajg.2010.156
504. Long MD, Drossman DA. Inflammatory bowel disease, irritable bowel syndrome, or what?: a challenge to the functional-organic dichotomy. *Am J Gastroenterol* (2010) **105**:1796–8. doi:10.1038/ajg.2010.162
505. Spiller R, Garsed K. Infection, inflammation, and the irritable bowel syndrome. *Dig Liver Dis* (2009) **41**:844–9. doi:10.1016/j.dld.2009.07.007
506. Barbara G, Cremon C, Stanghellini V. Inflammatory bowel disease and irritable bowel syndrome: similarities and differences. *Curr Opin Gastroenterol* (2014) **30**:352–8. doi:10.1097/MOG.0000000000000070
507. Ji Y, Tang B, Traub RJ. Modulatory effects of estrogen and progesterone on colorectal hyperalgesia in the rat. *Pain* (2005) **117**:433–42. doi:10.1016/j.pain.2005.07.011
508. Palecek J, Willis WD. The dorsal column pathway facilitates visceromotor responses to colorectal distention after colon inflammation in rats. *Pain* (2003) **104**:501–7. doi:10.1016/S0304-3959(03)00075-7
509. Burton MB, Gebhart GF. Effects of intracolonic acetic acid on responses to colorectal distension in the rat. *Brain Res* (1995) **672**:77–82. doi:10.1016/0006-8993(94)01382-R
510. Coutinho SV, Meller ST, Gebhart GF. Intracolonic zymosan produces visceral hyperalgesia in the rat that is mediated by spinal NMDA and

non-NMDA receptors. *Brain Res* (1996) **736**:7–15. doi:10.1016/0006-8993(96)00661-0

511. Traub RJ, Murphy A. Colonic inflammation induces fos expression in the thoracolumbar spinal cord increasing activity in the spinoparabrachial pathway. *Pain* (2002) **95**:93–102. doi:10.1016/S0304-3959(01)00381-5
512. Adam B, Liebregts T, Gschossmann JM, Krippner C, Scholl F, Ruwe M, et al. Severity of mucosal inflammation as a predictor for alterations of visceral sensory function in a rat model. *Pain* (2006) **123**:179–86. doi:10.1016/j.pain.2006.02.029
513. Gschossmann JM, Liebregts T, Adam B, Buenger L, Ruwe M, Gerken G, et al. Long-term effects of transient chemically induced colitis on the visceromotor response to mechanical colorectal distension. *Dig Dis Sci* (2004) **49**:96–101. doi:10.1023/B:DDAS.0000011609.68882.3a
514. Zhou Q, Price DD, Caudle RM, Verne GN. Visceral and somatic hypersensitivity in a subset of rats following Tnbs-induced colitis. *Pain* (2008) **134**:9–15. doi:10.1016/j.pain.2007.03.029
515. Verma-Gandhu M, Verdu EF, Bercik P, Blennerhassett PA, Al-Mutawaly N, Ghia JE, et al. Visceral pain perception is determined by the duration of colitis and associated neuropeptide expression in the mouse. *Gut* (2007) **56**:358–64. doi:10.1136/gut.2006.100016
516. Larsson MH, Rapp L, Lindstrom E. Effect of DSS-induced colitis on visceral sensitivity to colorectal distension in mice. *Neurogastroenterol Motil* (2006) **18**:144–52. doi:10.1111/j.1365-2982.2005.00736.x
517. Deiteren A, Vermeulen W, Moreels TG, Pelckmans PA, de Man JG, de Winter BY. The effect of chemically induced colitis, psychological stress and their combination on visceral pain in female Wistar rats. *Stress* (2014) **17**:431–44. doi:10.3109/10253890.2014.951034
518. Reber SO, Obermeier F, Straub RH, Veenema AH, Neumann ID. Aggravation of DSS-induced colitis after chronic subordinate colony (CSC) housing is partially mediated by adrenal mechanisms. *Stress* (2008) **11**:225–34. doi:10.1080/10253890701733351
519. Veenema AH, Reber SO, Selch S, Obermeier F, Neumann ID. Early life stress enhances the vulnerability to chronic psychosocial stress and experimental colitis in adult mice. *Endocrinology* (2008) **149**:2727–36. doi:10.1210/en.2007-1469
520. Melgar S, Engstrom K, Jagervall A, Martinez V. Psychological stress reactivates dextran sulfate sodium-induced chronic colitis in mice. *Stress* (2008) **11**:348–62. doi:10.1080/10253890701820166
521. Saunders PR, Miceli P, Vallance BA, Wang L, Pinto S, Tougas G, et al. Noradrenergic and cholinergic neural pathways mediate stress-induced reactivation of colitis in the rat. *Auton Neurosci* (2006) **124**:56–68. doi:10.1016/j.autneu.2005.12.002
522. Collins SM, Mchugh K, Jacobson K, Khan I, Riddell R, Murase K, et al. Previous inflammation alters the response of the rat colon to stress. *Gastroenterology* (1996) **111**:1509–15. doi:10.1016/S0016-5085(96)70012-4
523. Liebregts T, Adam B, Bertel A, Lackner C, Neumann J, Talley NJ, et al. Psychological stress and the severity of post-inflammatory visceral hyperalgesia. *Eur J Pain* (2007) **11**:216–22. doi:10.1016/j.ejpain.2006.02.007
524. Armuzzi A, Cremonini F, Ojetti V, Bartolozzi F, Canducci F, Candelli M, et al. Effect of *Lactobacillus* GG supplementation on antibiotic-associated gastrointestinal side effects during *Helicobacter pylori* eradication therapy: a pilot study. *Digestion* (2001) **63**:1–7. doi:10.1159/000051865
525. Bercik P, Denou E, Collins J, Jackson W, Lu J, Jury J, et al. The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. *Gastroenterology* (2011) **141**:e1–3. doi:10.1053/j.gastro.2011.04.052

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