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AWARD NUMBER: W81XWH-08-2-0118

TITLE: The STRONG STAR Multidisciplinary PTSD Research Consortium

PRINCIPAL INVESTIGATOR: Randy Strong. Ph.D.

CONTRACTING ORGANIZATION: University of Texas Health Science Center at
San Antonio
San Antonio, TX 78229

REPORT DATE: September 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE September 2012		2. REPORT TYPE Annual		3. DATES COVERED 1 September 2011 – 31 August 2012	
4. TITLE AND SUBTITLE The STRONG STAR Multidisciplinary PTSD Research Consortium				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-08-2-0118	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Randy Strong, Ph.D. Alan Frazer, Ph.D. David Morilak, Ph.D. Email: strong@uthscsa.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Texas Health Science Center at San Antonio San Antonio, TX 78229				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Traumatic stress is a requirement for the development of PTSD. However, the majority of trauma-exposed persons do not develop PTSD. Therefore, examination of the typical effects of a stressor may not identify the critical components of PTSD risk or pathogenesis. One obvious explanation for individual differences in vulnerability to PTSD is that there may be genetic predisposition to susceptibility to precipitating stressors. However, to date, very few genetic polymorphisms for PTSD have been identified. An alternative mechanism that would impart lifelong vulnerability to PTSD is stable alterations in gene expression programmed by exposure to early life stressors. Therefore, the hypothesis to be addressed by this project is that early life exposure to stress or glucocorticoids programs a distinct neurochemical and behavioral phenotype during adulthood characterized by vulnerability to stressors that trigger PTSD. Moreover, we hypothesize that the susceptibility to PTSD can be reversed in adult offspring by anti-depressants which have been reported to reverse the epigenetic changes in expression of selected genes caused by stress. To address this hypothesis, the following specific aims are proposed: 1. To generate and characterize models of early life stress: prenatal stress; prenatal glucocorticoid receptor stimulation; and perinatal stress and perinatal glucocorticoid exposure. 2. To determine adult predictors of vulnerability to stress: as determined by behavioral, physiological, and molecular and neurochemical measures. 3. To determine adult vulnerability to stress: Adult offspring from models developed in Specific Aim 1 are exposed to a model of traumatic stress and then a fear conditioning paradigm. Behavioral, physiological and molecular neurochemical measures are made. 4. To determine the effects of treatments with the SSRI sertraline in adults exposed to early life stress.					
15. SUBJECT TERMS Rats, prenatal stress, PTSD, open field test, social interaction test, fear conditioning, extinction, glucocorticoid receptors, norepinephrine.					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
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A. INTRODUCTION:

Traumatic stress is a requirement for the development of PTSD. However, the majority of trauma-exposed persons do not develop PTSD. Therefore, examination of the typical effects of a stressor may not identify the critical components of PTSD risk or pathogenesis. One obvious explanation for individual differences in vulnerability to PTSD is that there may be genetic predisposition to susceptibility to precipitating stressors. However, to date, very few genetic polymorphisms for PTSD have been identified. An alternative mechanism that would impart lifelong vulnerability to PTSD is stable alterations in gene expression programmed by exposure to early life stressors. Therefore, the hypothesis to be addressed by this project is that early life exposure to stress or glucocorticoids programs a distinct neurochemical and behavioral phenotype during adulthood characterized by vulnerability to stressors that trigger PTSD. Moreover, we hypothesize that the susceptibility to PTSD can be reversed in adult offspring by anti-depressants that have been reported to reverse the epigenetic changes in expression of selected genes caused by stress. To address this hypothesis, we proposed the following specific aims: 1. To generate and characterize models of early life stress: prenatal stress; prenatal glucocorticoid receptor stimulation; and perinatal stress and perinatal glucocorticoid exposure. 2. To determine adult predictors of vulnerability to stress: as determined by behavioral, physiological, and molecular and neurochemical measures. 3. To determine adult vulnerability to stress: Adult offspring from models developed in Specific Aim 1 are exposed to a model of traumatic stress and then a fear conditioning paradigm. Behavioral, physiological and molecular neurochemical measures are made. 4. To determine the effects of treatments with the SSRI sertraline in adults exposed to early life stress.

B. BODY:

We have spent considerable time and effort developing and validating the Chronic plus Acute Prolonged Stress (CAPS) model, which we believe reasonably models the chronic stress of deployment plus the acute stress of a traumatic event, to induce behavioral changes resembling PTSD in rats. At the end of last year we initiated experiments to begin testing a second model of early life trauma, i.e. testing the effects of pharmacological manipulation of corticosteroid function during pregnancy to determine the role of corticosterone during maternal stress on behavioral and neurochemical phenotypes in adult offspring (Model 3). We made a minor modification to the approach in which, instead of testing dexamethasone (a synthetic glucocorticoid) administered *in utero*, we administered the natural glucocorticoid agonist corticosterone. We also began another model in which we will measure the effect of the corticosteroid antagonist metyrapone administered during prenatal stress to determine the role of glucocorticoids in the effects of prenatal stress on subsequent adult changes in neurochemistry and behavior in the offspring. As discussed previously, we decided against using the synthetic glucocorticoid receptor agonist dexamethasone to determine if it would mimic the effects of stress, because it is a pure glucocorticoid receptor agonist whereas the endogenous glucocorticoid (corticosterone) is an agonist at both glucocorticoid and mineralocorticoid receptors. Therefore, the natural glucocorticoid more closely mimics the effects of stress. Moreover, by using the antagonist metyrapone and the agonist corticosterone, we hope to be able to determine unambiguously whether corticosterone is involved in the effects of maternal stress. In order to design an intervention, we need to know whether the naturally occurring

corticosterone is involved in the effects of maternal stress, as opposed to other stress-responsive hormones such as catecholamines.

Results: Some pups in each treatment group were tested on behavioral tests and molecular biology parameters as adults (Model 3, Tasks 1 and 2, Steps 4 and 5).

Behavioral Testing:

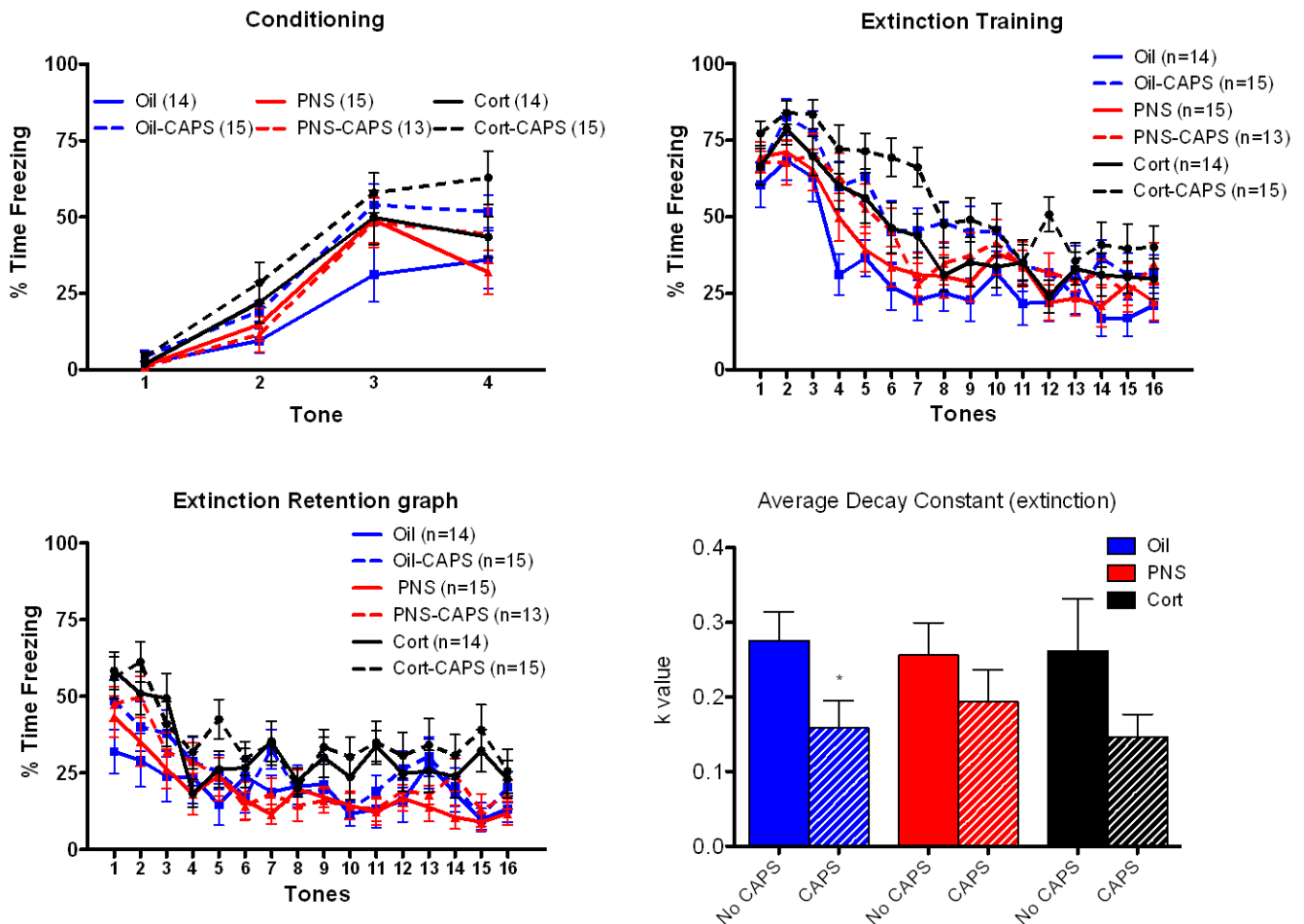


Figure 1. The effects of prenatal stress (PNS) or prenatal cort (CORT) on fear conditioning and extinction.

Fear Conditioning: As shown in Figure 1, there was a main effect of chronic plus acute prolonged stress (CAPS), resulting in an overall increase in freezing in animals stressed as adults, regardless of prenatal treatment.

Fear Extinction: Also shown in Figure 1, similar to our recently published data, we found a significant main effect of adult CAPS exposure, and a CAPS by tone interaction on fear extinction. Likewise prenatal stress had no effect on fear extinction. However, there was a main effect of prenatal treatment that was driven by prenatal CORT (significantly different than Oil on

tones 4,6-7), indicating that both prenatal CORT and CAPS increase freezing and delay extinction. There was no CAPS by CORT interaction, indicating that the CAPS and CORT effects were additive. Likewise, when the rate constant for decay was analyzed for each individual animal, we found a significant main effect of CAPS treatment with a significant post-hoc difference in animals treated with oil vehicle prenatally.

Fear Retention: There was a main effect of prenatal treatment and prenatal treatment by tone interaction. Prenatal CORT exposure increases overall freezing and delays extinction in the adult offspring. PNS had no effect on fear retention in contrast to what we had found earlier. Overall, the data indicate that, in terms of behavior, prenatal corticosterone exposure alone elicits a distinct behavioral profile from prenatal stress, even though the dose of CORT was titrated to mimic the fetal exposure from maternal stress. This may suggest a compensatory mechanism, activated by maternal stress that buffers prenatal corticosterone exposure in some manner, or, as suggested by our neurochemical data (below), that other factors in addition to CORT also play a role in the prenatal stress effect.

Neurochemical Testing:

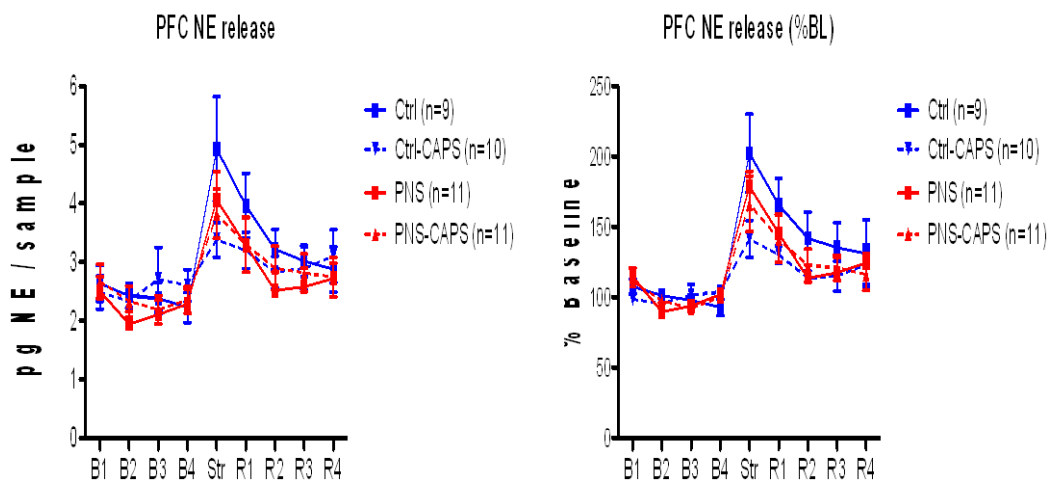


Figure 2. Microdialysis in PNS vs Saline animals with and without CAPS.

We have repeatedly measured reductions in mRNA for TH in the pontine region that contains the locus ceruleus. However, the functional significance of this had not been tested. We hypothesized that the decrease in TH mRNA would be reflected by a reduction in norepinephrine (NE) release in the prefrontal cortex, suggesting a mechanism whereby prenatal stress impaired stress-responsiveness. We therefore measured NE release by microdialysis in the prefrontal cortex of rats exposed to adult stress (CAPS) and/or prenatal stress. We found a CAPS by sample interaction on NE released in the mPFC. CAPS treatment blunted the acute immobilization stress-induced increase in NE in control (ie non-PNS) animals only, whereas CAPS had no effect in the PNS treated animals, which were moderately lower than the unstressed control animals overall. It still remains for us to ensure that this CAPS effect in prenatal control animals was not

due to repeated exposure to immobilization stress, which also comprises part of the CAPS treatment. We plan to do this in the coming year.

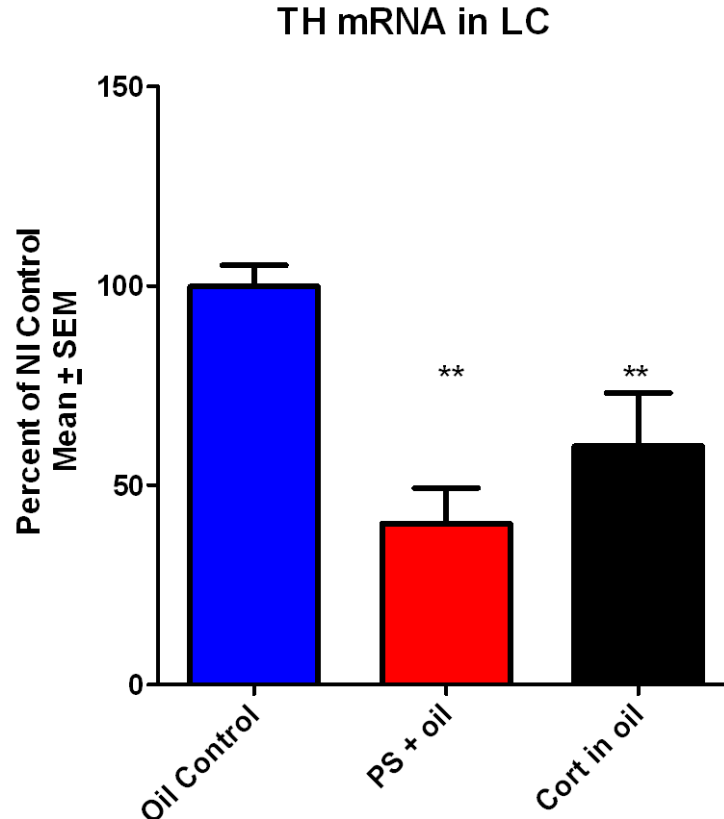


Figure 3. Comparison of the effects of prenatal corticosterone (CORT) and prenatal stress (PS) on TH mRNA in the pontine region of the brain containing the locus ceruleus. Data are expressed as mean $\pm$ SEM of the offspring of non-immobilized (NI) mothers, i.e. non-prenatal stress (NPS) ** $p < 0.01$ Significantly different from oil control.

As discussed above, we have repeatedly measured by RT-PCR a reduction in mRNA for TH in the region of the pons containing the locus ceruleus in adult rats exposed to prenatal stress. Therefore, to determine if this effect could be due to prenatal exposure to corticosterone, we compared the effects of prenatal CORT and prenatal stress on TH mRNA in the pontine region containing the locus ceruleus. As shown in Figure 3, there was a significant reduction in TH mRNA in rats exposed to prenatal stress as we have reported previously. Similarly, prenatal CORT also reduced TH mRNA. These results are consistent with the conclusion that prenatal stress may produce an effect on TH mRNA by increasing CORT. However, the significance of this is not clear since the effect on NE release in the PFC did not seem to be altered by PNS.

We also measured TH mRNA in the locus ceruleus (LC) by *in situ* hybridization to see if the reductions in TH mRNA measured by RT-PCR were localized to LC neurons. The results are shown in Figure 4. We found no effect of PNS or CAPS on the TH signal in the LC. This may be due to one of two factors. First, these data came from animals that had been previously subjected to surgery for microdialysis cannulation, and this could have influenced TH expression, masking

the PNS effects seen by qPCR. Alternatively, it is possible that the major changes in TH mRNA expression occurred in cell groups other than the LC in this same region of pons, including noradrenergic A5 or A7 cell groups in the ventrolateral and dorsolateral pons.

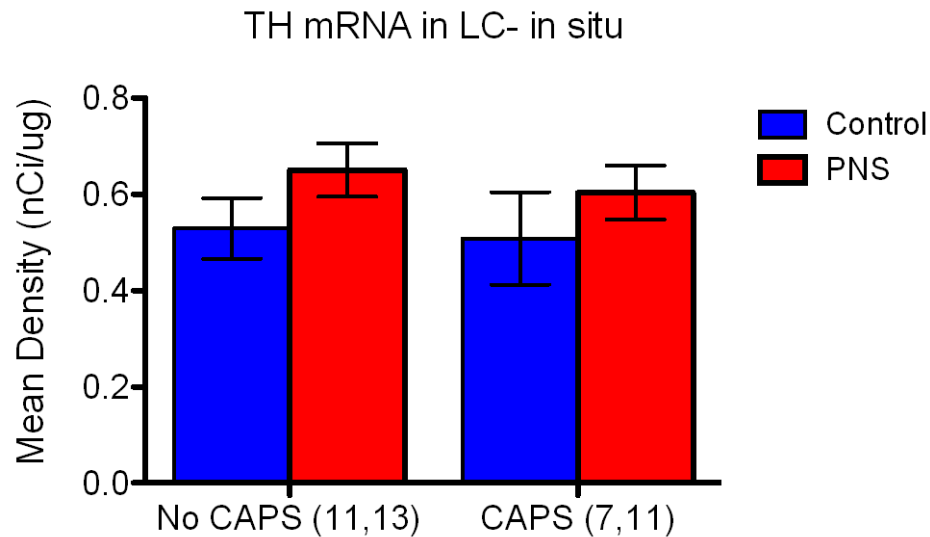


Figure 4. Effect of adult stress and prenatal stress on LC TH mRNA by ub situ hybridization. Data are expressed as the mean $\pm$ SEM of the number of animals in parentheses.

We have also consistently found that prenatal stress (PNS) reduces glucocorticoid receptors in the medial prefrontal cortex and the hippocampus in adulthood. We therefore determined whether this too might be mediated by prenatal increases in CORT (Figure 5).

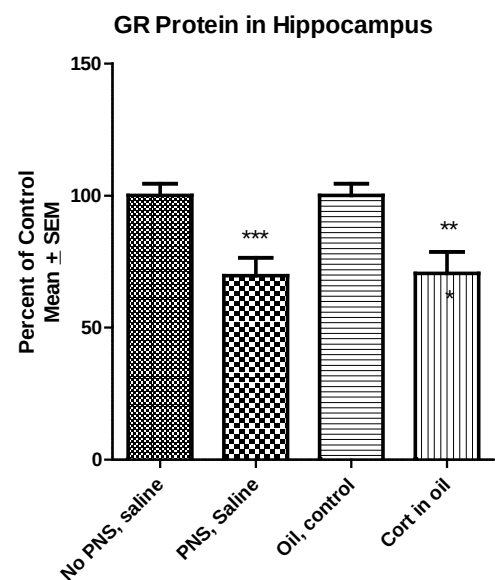
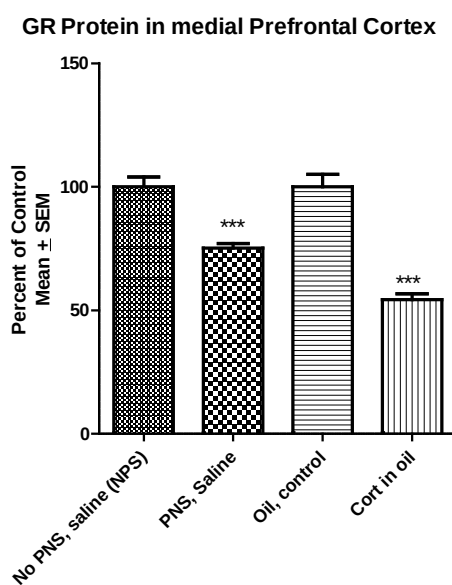


Figure 5. Effect of prenatal stress and prenatal CORT exposure on glucocorticoid receptor protein in the medial prefrontal cortex and hippocampus. Data are expressed as the mean $\pm$ SEM. **, significantly different from no PNS or Oil controls.

As shown in Figure 5, prenatal corticosterone at a level produced by maternal stress reduced glucocorticoid receptor protein in the medial prefrontal cortex and the hippocampus, indicating that elevated corticosterone during prenatal stress is sufficient to explain the alterations in GR protein in adults exposed to prenatal stress.

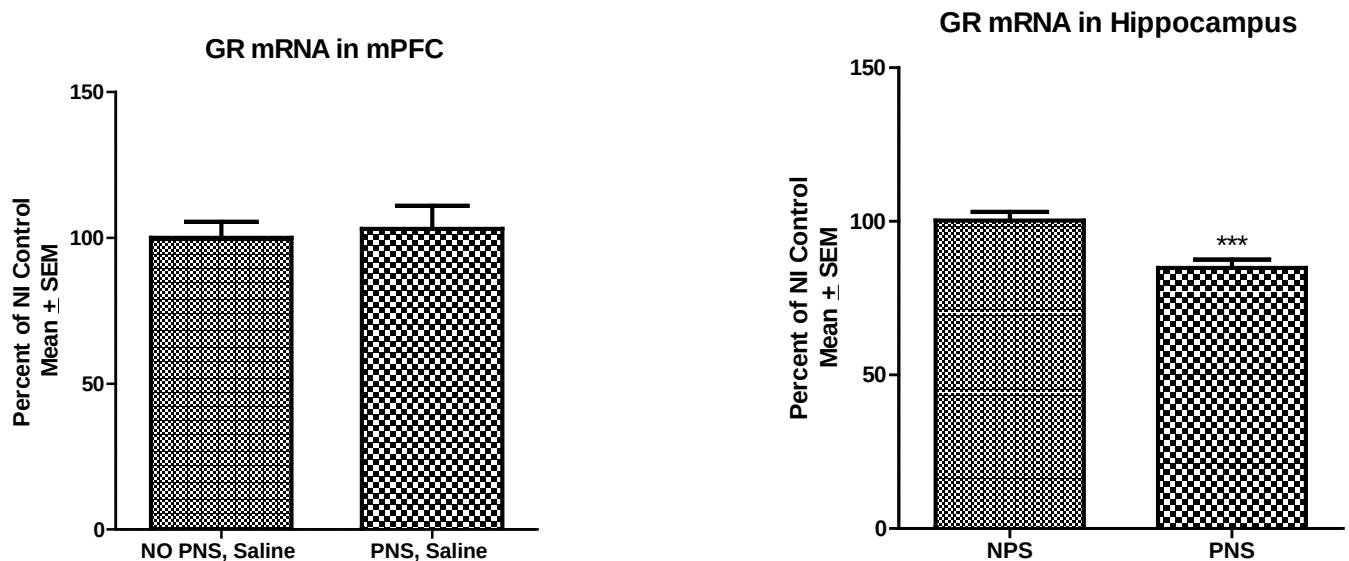


Figure 6. Effect of prenatal stress on glucocorticoid receptor mRNA in the medial prefrontal cortex and hippocampus. Data are expressed as the mean $\pm$ SEM. ***, significantly different from no PNS controls (NPS).

We began to determine whether the prenatal stress-induced decreases in GR protein expression in the adult mPFC and hippocampus resulted from reduced mRNA expression. The results are shown in Figure 6. As can be seen the results were mixed. There was no effect of PNS on GR in the mPFC, but there was a small but significant decrease in mRNA for GR in the hippocampus in adult rats exposed to maternal stress.

Thus, we hypothesize that the reductions in GR protein might be attributable to post-transcriptional mechanisms. One of our goals is to determine if micro-RNA plays a role. We will work with Dr. Doug Williamson in the Genomics Core to investigate that question.

KEY RESEARCH ACCOMPLISHMENTS:

- **We have established a dose of corticosterone that mimics the levels of corticosterone in mothers and pups exposed to prenatal stress.**

- **We found that prenatal corticosterone treatment produces a neurochemical phenotype similar to prenatal stress characterized by reduced GR protein in prefrontal cortex.**
- **We also found that, like prenatal stress, prenatal corticosterone reduced TH mRNA, suggesting that prenatal stress produces its effects on TH mRNA through a mechanism dependent on corticosterone.**
- **However, studies examining release of norepinephrine in the mPFC suggest that compensatory mechanisms maintain release of NE in the adults exposed to PNS.**
- **There was a reduction in NE release capability following adults CAPS stress, which could account for some of the behavioral effects, e.g., the extinction deficit.**

REPORTABLE OUTCOMES:

STRONG STAR reported in FY3 on the acceptance and advance online publication of the first manuscript by Dr. Randy Strong's research team. That manuscript was officially published in the print edition of the journal *Neuroscience* on 29 September 2011. A copy is attached in the appendix of this report.

- Green, M. K., Rani, C. S. S., Joshi, A., Soto-Piña, A. E., Martinez, P. A., Frazer, A., Strong, R., & Morilak, D. A. (2011). Prenatal stress induced long term stress vulnerability, compromising stress response systems in the brain and impairing extinction of conditioned fear after adult stress. *Neuroscience*, 192, 438-451. doi:10.1016/j.neuroscience.2011.06.041

Dr. Randy Strong's research team worked throughout the year on preparing, submitting, and responding to reviewer critiques of a manuscript reporting their latest efforts and successes in developing a rat model for PTSD. In July 2012, their efforts were rewarded with notice of acceptance and online publication by the journal *Neuropharmacology*. The manuscript is officially scheduled for the November 2012 edition, but complete, fully citable portions of that issue – including this manuscript – were published online in July and August. A copy is attached in the appendix of this report.

- Roth, M. K., Bingham, B., Shah, A., Joshi, A., Frazer, A., Strong, R., & Morilak, D. A.; for the STRONG STAR Consortium. (2012). Effects of Chronic Plus Acute Prolonged Stress on measures of coping style, anxiety, and evoked HPA-axis reactivity. *Neuropharmacology*, 63(6), 1118-1126. doi:10.1016/j.neuropharm.2012.07.034

Members of Dr. Strong's research team presented two posters reporting STRONG STAR study findings at the annual meeting of the Society for Neuroscience, held in Washington, D.C., from 12-16 November 2011. Copies of the poster abstracts as published by the conference online are attached in the appendix of this report.

- Bingham, B., Kaddapakam, S., Strong, R., & Morilak, D. A. (2011, November). *Potential role of corticosterone in the long-lasting effects of prenatal stress*. Poster presented at the annual meeting of the Society for Neuroscience, Washington, D.C.
- Green, M. K., Joshi, A., Shah, A., Frazer, A., Strong, R., & Morilak, D. A. (2011, November). *Effects of prenatal stress and combined chronic plus acute adult stress on anxiety-like behavior and evoked HPA axis activity in rats*. Poster presented at the annual meeting of the Society for Neuroscience, Washington, D.C.

CONCLUSION:

During this year we tested the effects of pharmacological manipulation of corticosteroid function during pregnancy to determine the role of corticosterone during maternal stress on behavioral and neurochemical phenotypes in adult offspring (Model 3). We found that prenatal corticosterone treatment programs a neurochemical phenotype similar to prenatal stress characterized by reduced GR protein in prefrontal cortex and hippocampus and reduced TH mRNA in the locus ceruleus. We also found that prenatal corticosterone also influenced responses to fear conditioning and extinction, independent of adult stress. At the same time we continued to develop, refine and validate the CAPS stress procedure as a model of PTSD. This led to the publication of a substantive paper (Roth et al., 2012), in which we further validated the CAPS model, describing additional changes in stress coping behavior, anxiety-like behaviors, and acute stress reactivity of the HPA axis that resemble several key components of PTSD.

Our models have begun to bear fruit regarding their applicability to research performed by other members of STRONG STAR. We recently had a request from Dr. Doug Williamson (Genomics Core) concerning providing him with tissue from our models to help inform his genome-wide studies of molecular markers.

APPENDICES:

Reprint: Green, M. K., Rani, C. S. S., Joshi, A., Soto-Piña, A. E., Martinez, P. A., Frazer, A., Strong, R., & Morilak, D. A. (2011). Prenatal stress induced long term stress vulnerability, compromising stress response systems in the brain and impairing extinction of conditioned fear after adult stress. *Neuroscience*, 192, 438-451. doi:10.1016/j.neuroscience.2011.06.041

Reprint: Roth, M. K., Bingham, B., Shah, A., Joshi, A., Frazer, A., Strong, R., & Morilak, D. A.; for the STRONG STAR Consortium. (2012). Effects of Chronic Plus Acute Prolonged Stress on measures of coping style, anxiety, and evoked HPA-axis reactivity. *Neuropharmacology*, 63(6), 1118-1126. doi:10.1016/j.neuropharm.2012.07.034

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SUPPORTING DATA:

Shown in the body of the report.

PRENATAL STRESS INDUCES LONG TERM STRESS VULNERABILITY, COMPROMISING STRESS RESPONSE SYSTEMS IN THE BRAIN AND IMPAIRING EXTINCTION OF CONDITIONED FEAR AFTER ADULT STRESS

M. K. GREEN, C. S. S. RANI, A. JOSHI,
A. E. SOTO-PIÑA, P. A. MARTINEZ, A. FRAZER,
R. STRONG AND D. A. MORILAK*

Department of Pharmacology and Center for Biomedical Neuroscience,
University of Texas Health Science Center at San Antonio, 7703
Floyd Curl Drive, San Antonio, TX 78229, USA

Abstract—Stress is a risk factor for the development of affective disorders, including depression, post-traumatic stress disorder, and other anxiety disorders. However, not all individuals who experience either chronic stress or traumatic acute stress develop such disorders. Thus, other factors must confer a vulnerability to stress, and exposure to early-life stress may be one such factor. In this study we examined prenatal stress (PNS) as a potential vulnerability factor that may produce stable changes in central stress response systems and susceptibility to develop fear- and anxiety-like behaviors after adult stress exposure. Pregnant Sprague–Dawley rats were immobilized for 1 h daily during the last week of pregnancy. Controls were unstressed. The male offspring were then studied as adults. As adults, PNS or control rats were first tested for shock-probe defensive burying behavior, then half from each group were exposed to a combined chronic plus acute prolonged stress (CAPS) treatment, consisting of chronic intermittent cold stress (4 °C, 6 h/d, 14 days) followed on day 15 by a single session of sequential acute stressors (social defeat, immobilization, cold swim). After CAPS or control treatment, different groups were tested for open field exploration, social interaction, or cued fear conditioning and extinction. Rats were sacrificed at least 5 days after behavioral testing for measurement of tyrosine hydroxylase (TH) and glucocorticoid receptor (GR) expression in specific brain regions, and plasma adrenocorticotrophic hormone (ACTH) and corticosterone. Shock-probe burying, open field exploration and social interaction were unaffected by any treatment. However, PNS elevated basal corticosterone, decreased GR protein levels in hippocampus and prefrontal cortex, and decreased TH mRNA expression in noradrenergic neurons in the dorsal pons. Further, rats exposed to PNS plus CAPS showed attenuated extinction of cue-conditioned fear. These results suggest that PNS induces vulnerability to subsequent adult stress, resulting in an enhanced fear-like behavioral profile, and dysregulation of brain noradrenergic and hypothalamic–pituitary–adrenal axis (HPA) activity. © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: prenatal stress, traumatic stress, vulnerability, fear extinction, tyrosine hydroxylase, HPA axis.

Affective disorders, such as depression, post-traumatic stress disorder (PTSD) and other anxiety disorders, have long been considered to be stress-related/stress-initiated disorders. Responses to acute stressors are thought to be adaptive in the short term by increasing, for example, access to energy stores, increasing cardiovascular tone, and enhancing behavioral response capabilities. However, when these systems are repeatedly activated, as with chronic stress, dysregulation of a number of hormonal and neurotransmitter systems may occur (McEwen, 2003). Chronic stress is a risk factor, and possibly a causal factor, in the development of depression and anxiety disorders (Kendler et al., 1999; Koenen et al., 2002, 2007; Gilmer et al., 2005; Jordanova et al., 2007). Indeed, a number of physiological and anatomical alterations associated with chronic stress are hallmarks of depression and anxiety disorders (Board et al., 1956; Nemeroff et al., 1984, 1991; Gold et al., 1986; Holsboer et al., 1986; Weisse, 1992; Heuser et al., 1998; Arborelius et al., 1999; Manji et al., 2001; McEwen, 2003). In addition to cumulative or chronic stress, severe acute stress is also associated with mood and anxiety disorders, most prominently with PTSD. (Jordanova et al., 2007; American Psychiatric Association, 2000). However, not all individuals exposed to chronic or acute-traumatic stress in adulthood develop depression or anxiety disorders, suggesting that some other factor or factors, either genetic, epigenetic, or experience-based, contribute to susceptibility to develop affective disorders. Therefore, to fully understand the mechanisms underlying these disorders, it is insufficient to simply examine the response to stressors. Rather, the factors involved in predisposing for failure to recover from the normal response to stress must be identified (Yehuda and LeDoux, 2007).

Early life stress is one potential factor. For example, early life stress is a risk factor for PTSD, specifically, a history of trauma, childhood abuse/neglect, low education and IQ, low socio-economic status, or loss of a parent in childhood (Bremner et al., 1993; Breslau et al., 1999; Widom, 1999; Koenen et al., 2002, 2007). In rodents, prenatal stress (PNS) produces several behavioral and physiological changes that may be indicative of later stress vulnerability (e.g., Weinstock et al., 1992; Valle et al., 1997; Lemaire et al., 2000).

*Corresponding author. Tel: +1-210-567-4174; fax: +1-210-567-4303.

E-mail address: morilak@uthscsa.edu (D. A. Morilak).

Abbreviations: ACTH, adrenocorticotrophic hormone; CAPS, chronic plus acute prolonged stress; CORT, corticosterone; GR, glucocorticoid receptor; HPA axis, hypothalamic–pituitary–adrenal axis; HR, high responder rats; ITI, inter-trial interval; LC, locus coeruleus; NE, nor-epinephrine; PD, postnatal day; PNS, prenatal stress; PTSD, posttraumatic stress disorder; TH, tyrosine hydroxylase; WKY, Wistar-Kyoto rats.

Two of the most prominent systems involved in stress adaptation, the brain noradrenergic system and the hypothalamic–pituitary–adrenal (HPA) axis, have also been implicated in stress-related pathology. Norepinephrine (NE) is released in response to stress (Morilak et al., 2005; Aston-Jones et al., 1999), chronic stress alters noradrenergic signaling (Buffalari and Grace, 2009; Kitayama et al., 2008; Ma and Morilak, 2005), and noradrenergic dysregulation is reported in numerous affective disorders, including depression and PTSD (Ressler and Nemeroff, 1999; Strawn and Geraciotti, 2008). Likewise, the HPA axis is activated in response to acute stress, resulting in release of adrenocorticotrophic hormone (ACTH) and corticosterone (CORT) and this response is altered after chronic stress (Dallman, 1993; Ma and Morilak, 2005). Furthermore, HPA axis dysregulation is a consistent component of several affective disorders, including depression, panic disorders, obsessive-compulsive disorder, and PTSD (Nemeroff et al., 1984; Gold et al., 1986; Souetre et al., 1988; Abelson et al., 2007; Kluge et al., 2007; Mason et al., 1986; Pitman and Orr, 1990).

It is being increasingly recognized that changes in executive function and cognitive capability are also prominent features of mood and anxiety disorders (Beck, 1976; Beck et al., 1987; Mathews and Mackintosh, 1998; Coles and Heimberg, 2002). Moreover, in the context of stress, both the brain noradrenergic system and the HPA axis are involved in regulation and dysregulation of cognitive processes such as learning and memory (de Quervain et al., 2009), including specifically conditioned fear and extinction learning (McIntyre et al., 2002; Mueller et al., 2008; Gourley et al., 2009). Impaired cognition, maladaptive fear responses, and impaired extinction of learned fear are primary symptoms of a number of affective disorders, with these fear-related symptoms being most relevant to anxiety disorders such as panic disorder, phobias, obsessive-compulsive disorder, and PTSD (Sutker et al., 1995; Fossati et al., 1999; Koenen et al., 2001; Moritz et al., 2002; Kangaratnam and Asbjørnsen, 2007; Blechert et al., 2007; Wessa and Flor, 2007). Therefore, it is possible that the mechanisms by which vulnerability factors such as prenatal stress may induce long-lasting susceptibility to develop psychopathology upon adult stress exposure could include dysregulation of the HPA axis and/or brain noradrenergic system, resulting specifically in maladaptive responses to fear-provoking stimuli and an impaired ability to extinguish fear responses in non-stressful conditions.

Thus, the purpose of the present study was to examine neurobiological correlates of adult stress vulnerability induced by PNS exposure. We measured the effects of PNS followed by a combined chronic plus acute prolonged stress (CAPS) treatment as adults, on tyrosine hydroxylase (TH) expression in the locus coeruleus (LC) and adrenal medulla, HPA status, and glucocorticoid receptor (GR) protein levels in the prefrontal cortex (PFC) and hippocampus. In the same rats, we also tested the vulnerability of PNS-exposed adult rats to develop fear or anxiety-like behaviors following exposure to CAPS treatment, on measures of acute stress reactivity, social interaction,

fear conditioning, and extinction. We hypothesized that PNS exposure would produce stable, long-term changes in central and peripheral stress response systems, and a vulnerability to subsequent adult stress exposure such that the behavioral impact of adult stress would be greater. Portions of this work have been presented in abstract form (Green et al., 2010).

EXPERIMENTAL PROCEDURES

Animals

Timed-pregnant (6 days pregnant upon arrival) female Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) were singly housed throughout pregnancy. On postnatal day (PD) 5, litters were culled to eight pups each, maximizing the number of males retained (typically, three to six per litter), and weaned on PD 21. Upon weaning, male pups were pair-housed with a littermate until PD 41–45, depending on the experiment, at which time they were singly housed prior to starting the adult stress or unstressed control treatments. The rats were housed in Plexiglas cages (25×45×15 cm³) on a 12/12 h light-dark cycle (lights on at 7:00 h) with food and water available *ad libitum*. In total, 141 adult male offspring (from 63 litters—33 stressed and 30 unstressed) were used in these experiments. In addition, for the social defeat procedure, six adult male Long–Evans rats (Harlan), weighing at least 400 g, were used as defeators. They were housed, together with an ovariectomized female, in large resident cages (80×55×40 cm³) in a separate room on the same 12/12 h light cycle. All experiments were conducted during the light phase. All procedures were conducted according to NIH guidelines for the care and use of laboratory animals and were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio. All efforts were made to minimize animal pain, suffering or discomfort, and to minimize the number of rats used.

Prenatal stress treatment

After 1 week in the housing facility, half of the pregnant females were immobilized daily for 1 h, from day 14 of pregnancy until birth (8–9 days). Immobilization involved taping the rat's torso and limbs gently but snugly in a prone position on a flat platform, allowing no movement. Unstressed control pregnant females were left undisturbed during this same time period.

Shock-probe defensive burying test

At PD 41–43, a subset of the offspring ($n=60$) from both groups were tested in the shock probe defensive burying test. This was to evaluate potential differences in active and passive behavioral stress-reactivity as a consequence of the prenatal stress treatment prior to any exposure to adult stress. The rats were placed into a modified cage containing 5 cm of bedding, with a shock probe protruding 6 cm into one end of the cage. The probe was set to deliver 2 mA of current when the probe was touched. After the rat made contact with the probe and received a shock, the current was shut off and the 15 min test began. Behavior was recorded using a CCD camera mounted above the cage and stored to video files for offline scoring and analysis. The dependent measures analyzed were the amount of time spent immobile and the amount of time spent engaged in actively burying the probe. After the shock-probe defensive burying test, these animals were individually housed. Likewise, rats not tested in the shock-probe defensive burying test were also individually housed at this same time point.

Adult stress treatment: chronic plus acute prolonged stress

Beginning between PD 46 and 54, half of the rats that received prenatal stress ($n=35$) and half that did not ($n=34$) received CAPS treatment, which consisted of 2 weeks of chronic intermittent cold stress followed by a single 1-h session of acute prolonged stress on day 15. For cold stress, rats were transported in their cage with food, water and bedding into a cold room at 4 °C for 6 h per day for 14 days. The acute prolonged stress on day 15 consisted of 20 min social defeat, followed immediately by 30 min immobilization, and then 10 min cold swim. For social defeat, the ovariectomized Long–Evans female was removed from the resident cage, and the test rat was placed into the resident cage. Typically within 10–30 s, the resident Long–Evans male rat will attack and defeat the smaller “intruder” Sprague–Dawley test rat. Once defeat occurred, with “defeat” defined as the test rat assuming a supine posture and the resident expressing a dominant posture for at least 4 s, the test rat was placed under a wire mesh cage for 20 min, thus protecting the test rat from further physical contact but allowing continued sensory exposure to the dominant rat. Immobilization was then conducted, as described above, for 30 min. Finally, cold swim was accomplished by placing the rat in a cylindrical tank (30 cm diam×60 cm) filled to a depth of 30 cm with water at 18 °C. The combination of PNS and adult CAPS treatment resulted in four treatment groups: No PNS/No CAPS (i.e. unstressed controls), PNS/No CAPS, No PNS/CAPS, and PNS/CAPS.

Neurochemical and hormonal analyses

All of the rats were sacrificed by rapid decapitation 5–10 days after the last stress day, or at the equivalent time for controls. Trunk blood was collected into tubes containing 10 mM EDTA and was centrifuged at 4000×g for 15 min at 4 °C for the separation and collection of plasma. Plasma was stored at –80 °C until use. Brains were removed, placed in a brain matrix on ice, and the structures of interest dissected. For PFC, a 2 mm coronal section was cut, extending from the frontal pole to approximately plate 8 in the atlas of Paxinos and Watson (1986). The hippocampus was then separated from the lateral margins of the remaining cortex. For the pontine region containing the LC, the cerebellum was removed and the obex located. A slab was cut 3–5 mm anterior to the obex (plates 53–60), and the dorsal half was collected. Brain samples were rapidly frozen in 2-methylbutane on dry ice, and stored at –80 °C until assay.

TH mRNA. TH mRNA was measured in the dorsal pons containing the locus coeruleus and in the adrenal medulla by qPCR. Total RNA was isolated from tissues using the RNeasy Plus Mini kit (Qiagen Inc., Valencia, CA, USA) as described by the manufacturer. The RNA concentration was measured by spectroscopy at 260 nm using the Nanodrop ND-1000 instrument (NanoDrop Technologies, Inc., Wilmington, DE, USA). To check the integrity of RNA, RNA was denatured with 50% formamide loading buffer and run on an E-Gel EX 1% agarose gel using the iBase Power System (Invitrogen Corp., Carlsbad, CA, USA) and the 18S and 28S bands visualized. In all, 250–500 ng RNA was converted to cDNA using random hexamers and TaqMan Multiscribe reverse transcriptase enzyme included in the High Capacity RNA Reverse Transcription kit (Applied Biosystems Inc., Foster City, CA, USA). Reactions included controls without the reverse transcriptase enzyme but with only RNA template, and negative control with the enzyme, but no template in 20 μ l volume. After 2-h incubation at 37 °C, the RNA in the reactions was considered completely converted to cDNA. In order to check the quality of cDNA, cDNA (1 μ l=5 ng) from all reactions was used as template for PCR with rat GAPDH primers and the Go-Taq Green PCR master mix (Promega Corp., Madison, WI, USA). No bands

were detected either in negative control (no template) or in the samples without the reverse transcriptase enzyme, but a 200 bp band was seen in all cDNA-containing samples upon agarose gel electrophoresis.

TH gene expression was then quantified by qPCR using the TaqMan gene expression master mix and TaqMan Gene Expression assay ID Rn00562500_m1, consisting of a set of intron-spanning primers and FAM-labeled probe set for rat TH (Applied Biosystems Inc.) along with the cDNA, equivalent to 0.125–0.25 ng RNA. Assays were performed in triplicate and first validated using the Applied Biosystems Inc. PRISM 7900 Sequence Detection System in a 96-well format. Results were normalized to 18S rRNA, which was amplified simultaneously in the same samples using the primer-limited TaqMan VIC-MGB labeled 18S rRNA probe (Applied Biosystems Inc.). Real time PCR data were analyzed by the $2^{-\Delta\Delta C_T}$ method. The average difference in quantification cycle threshold (Cq) of the target gene and the 18S control for each sample was calculated, and the relative expression of TH in other groups was calculated with respect to the value obtained for the no-stress control samples.

TH protein. TH protein was measured in the adrenal medulla using Western blot. Samples were thawed on ice and homogenized in RIPA buffer (radioimmunoprecipitation assay buffer: 50 mM Tris–HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS with Sigma protease inhibitor cocktail (P8340, 1:100 dilution) and phenylmethylsulfonylfluoride (PMSF, 1 mM) added just prior to use). Homogenates were centrifuged at 16,000×g for 15 min at 4 °C and the supernatants transferred to fresh tubes. Protein concentration in the lysates was assessed by MicroBCA method (Pierce Inc., ThermoFisher Scientific, Rockford, IL, USA). Equal amounts of sample protein in 1× NuPage LDS sample buffer (Invitrogen) under denaturing condition were loaded on 4–12% NuPage Bis–Tris SDS gels (Invitrogen) and electrophoresed at 175 V for ~1 h. Proteins separated on the gel were transferred to polyvinylidene fluoride membranes using the iBlot transblot apparatus (Invitrogen). Membranes were probed by simultaneous addition of specific monoclonal antibodies to TH (Sigma, St. Louis, MO, USA) and β -actin (AbCam, Cambridge, MA, USA), followed by secondary antibody consisting of IRDye 800CW conjugated goat polyclonal anti-mouse IgG (LI-COR Biosciences, Lincoln, NE, USA), and the fluorescent signal was scanned and quantified using the Odyssey infrared imaging system (LI-COR). The ratio of TH to actin of each sample was calculated, and the relative expression of TH was computed as percentage of the no-stress control group.

GR protein levels in the hippocampus and PFC. GR protein levels were assayed in the hippocampus and the PFC using an ELISA-based TransAM kit (Active Motif, Carlsbad, CA, USA). Assays were performed according to manufacturer's instructions. Briefly, the brain tissue was homogenized in complete lysis buffer AM2, containing 1 mM dithiothreitol (DTT) and a protease inhibitor cocktail using motorized pestles in 1.5 ml microtubes (RPI, Mount prospect, IL, USA). All steps were conducted at 4 °C. After 30 min incubation on ice, the homogenates were centrifuged at 10,000×g for 10 min and the supernatants were transferred to fresh chilled tubes. Aliquots were frozen or used for protein assay using the BioRad Bradford protein assay. For the ELISA, 20 μ g protein was used in a 96-well format. The homogenates, in complete binding buffer containing 1 mM DTT and herring sperm DNA, were incubated in wells coated with immobilized oligonucleotide containing a consensus GR binding site (5'-GGTACAnnnTGTCT-3'). The bound GR was then detected using a specific GR primary antibody and an HRP-conjugated secondary antibody followed by a colorimetric step for quantification by spectrophotometry in a plate reader. The absorbance at 450 nm after suitable blank correction was used to determine GR levels. The A450 values for each

stress group were calculated as percentage of the no-stress control group.

Plasma ACTH and CORT levels. Levels of circulating ACTH and CORT were analyzed by radioimmunoassay. ACTH was determined from duplicate 100 μ l plasma aliquots according to the manufacturer instructions (ImmuChem double antibody hACTH assay, MP Biomedicals, Orangeburg, NY, USA). The detection limit of the assay is 6 pg/ml, and the inter-assay variability was 10%. CORT was measured in diluted plasma samples according to manufacturer instructions (ImmuChem Double antibody, corticosterone assay, MP Biomedicals). The assay detection limit was 8 ng/ml, and inter-assay variability was 8%.

Behavioral measures

Fear conditioning and extinction. One day after the termination of CAPS treatment (or the comparable control period), a subset of rats in each group ($n=98$; 23–25/grp) was habituated to the two fear conditioning and extinction contexts for 15 min each. Context A is a 30.5×25.4×30.5 cm³ square conditioning chamber (Coulbourn Instruments, Whitehall, PA, USA; model # H10-11R-TC) with metal walls and a grid shock floor attached to a shock generator (Coulbourn, # H13-15). Context B is a modified chamber with black and white vinyl walls forming a circular enclosure and a smooth green vinyl floor placed over the shock grid. Both contexts are enclosed in a 58.4×61×50.8 cm³ sound-attenuating chamber (Coulbourn, # H10-24T). Twenty-four hours after habituation, the rats received cued fear conditioning in Context A. Each rat was placed into the chamber and, after a 5 min acclimation period, experienced four pairings of a tone (10 kHz, 75 dB, 20 s) co-terminating with a shock (0.7 mA, 0.5 s). The average inter-trial interval (ITI) was 120 s.

Seventy-two hours after fear conditioning, the rats experienced fear extinction training consisting of exposure to 10 trials of the tone alone (average ITI 120 s, for a total 27 min extinction session). Then, 24 h later, the rats were tested for retention of fear extinction during exposure to 10 trials of the tone alone. To avoid any contextual effects, all extinction and retention testing was conducted in Context B.

Freezing behavior was recorded and analyzed using the FreezeFrame and FreezeView software (ActiMetrics Software; Coulbourn Instruments # ACT-100). Freezing was defined as all behavior which fell below the motion index threshold of 10 and lasted at least 1 s. Freezing was measured during each 20-s tone presentation on the conditioning, extinction training and extinction retention days. The time spent freezing was then expressed as a percentage of each 20-s sampling period.

Open field exploration. One day after the termination of CAPS treatment (or comparable control period), the remaining rats ($n=43$; 10–12/group) were tested for anxiety-like and exploratory behavior in an open field (60×60×40 cm³) under normal ambient laboratory lighting. The floor of the test arena was marked in a grid pattern of 36 squares, 10×10 cm² each. The test rat was placed into the center of the open field and behavior was recorded for 5 min. The number of line crossings and time spent in the center zone (i.e. the inner 16 squares) were measured.

Social interaction. Twenty-four hours after open field testing, the rats were tested in the same arena for social interaction with a novel male con-specific, weight-matched to within ± 5 g of the test rat. The con-specific “stimulus rats” had all been previously habituated to interacting in the arena with other stimulus rats so that their behavior would be constant during testing. The amount of time that the test rat spent engaged in social behaviors (sniffing, chasing, climbing, etc.) was measured during the 5-min test.

Statistical analyses

For the shock-probe defensive burying data, differences in immobility time and in active burying time between rats in the two prenatal stress conditions (prenatally stressed and controls) were analyzed by *t*-tests. All neurochemical and plasma hormone measures were analyzed by two-way ANOVA (prenatal stress×adult stress). Similarly, for the open field and social interaction tests, group differences in the number of line crossings, time spent in the center zone of the open field, and social interaction time were each analyzed by a two-way ANOVA (prenatal stress×adult stress). For the fear-conditioning and extinction tests, group differences in percent freezing were analyzed for each session by a three-way ANOVA (prenatal stress×adult stress×tone) with repeated measures over tone. To determine if there were differences in the retention of conditioned fear, freezing levels in response to the first tone presentation on the extinction training day were analyzed by two-way ANOVA (prenatal stress×adult stress). Likewise, tone 1 was analyzed in the same way on the extinction retention day as a measure of retention of extinction that occurred the day before (see Milad et al., 2004; Vidal-Gonzalez et al., 2006; Muigg et al., 2008). Following the primary ANOVA, in order to better assess and compare the rate of extinction across groups, a non-linear regression analysis was performed. An exponential decay function was best-fit to the freezing data for each rat, from which the rate constants (*k*) were derived. The rate constants for each treatment group were then compared by two-way ANOVA (prenatal stress×adult stress). On the extinction training day, an increase in freezing was always observed from tone 1 to tone 2. Thus, in order to capture the true rate of extinction from the peak level of freezing, and also to obviate any potential group differences in extinction attributable solely to different starting points on tone 1, tone 1 was not included in the regression analysis. Further, cases for which a line could not be fit to the data were excluded from the regression analysis, resulting in the exclusion of one to two animals per group. Upon examination of these cases, no consistent pattern could be discerned, and the data from these cases were included in the primary ANOVA for freezing data. In all analyses, significance was determined at $P<0.05$. Sources of significant main effects or interactions were then determined by analysis with the Newman–Keuls post hoc test.

RESULTS

Shock-probe defensive burying test

Prior to administering the adult stress treatment, there were no significant differences between rats that received prenatal stress and rats that did not in either time spent immobile (Table 1; $t_{58}=0.381$, $P>0.05$), nor in time spent burying the shock probe ($t_{58}=0.599$, $P>0.05$).

TH expression in the locus coeruleus and adrenal medulla

Prenatal stress significantly reduced TH mRNA expression in homogenates of rostral pons containing the LC, irrespective of exposure to CAPS (Fig. 1; $F_{(1,66)}=9.745$,

Table 1. Shock probe defensive burying behavior before adult stress

	Controls	PNS
Immobilization time (s)	150.9±31.95	166.2±24.99
Burying time (s)	97.77±20.11	81.56±18.24

Data expressed as mean±SEM, $n=28$ –32/group.

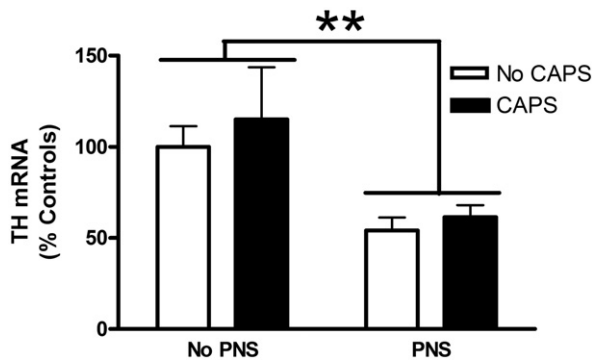


Fig. 1. Tyrosine hydroxylase mRNA expression in the rostral pons containing the locus coeruleus. Prenatal stress produced a significant decrease in TH mRNA expression in the LC region. ** main effect of PNS, $P=0.003$. Data expressed as mean percent of controls $\pm$ SEM, $n=17$ –19/group.

$P<0.01$). There was no main effect of CAPS ($F_{(1,66)}=0.488$, $P>0.05$) nor an interaction between PNS and CAPS ($F_{(1,66)}=0.061$, $P>0.05$). However, effects in the adrenal medulla were different (Fig. 2). For TH mRNA, there was no main effect of PNS (Fig. 2A; $F_{(1,33)}=2.606$, $P>0.05$) nor was there an effect of CAPS ($F_{(1,33)}=0.343$, $P>0.05$), but there was a significant PNS $\times$ CAPS interaction ($F_{(1,33)}=6.06$, $P<0.05$) such that PNS alone induced

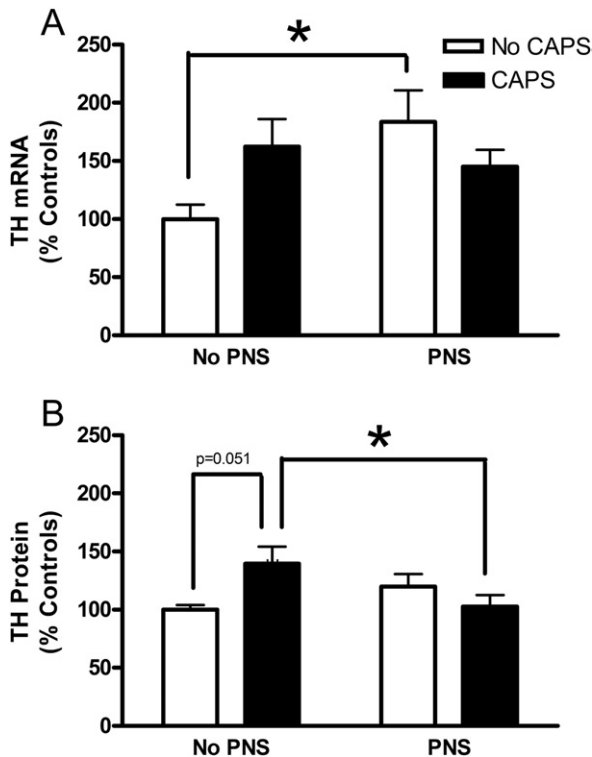


Fig. 2. Tyrosine hydroxylase mRNA and protein expression in the adrenal medulla. (A) PNS alone significantly increased TH mRNA expression in the adrenal medulla compared to unstressed controls, * $P=0.03$ by Newman–Keuls post hoc comparisons, $n=8$ –10/group. (B) CAPS alone significantly increased TH protein levels compared to both PNS/CAPS (* $P=0.04$) and unstressed controls ($P=0.051$). Data expressed as mean percent of controls $\pm$ SEM, $n=13$ /group.

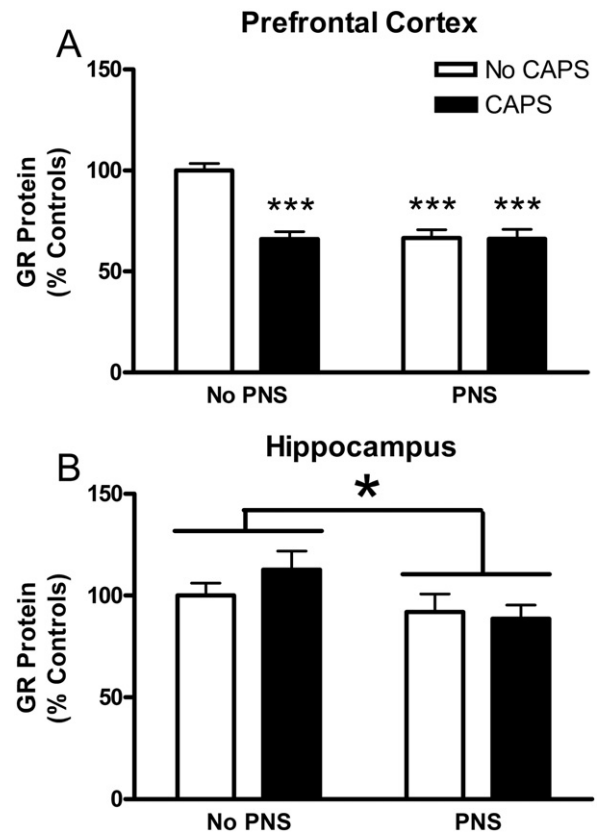


Fig. 3. Effects of PNS and CAPS on glucocorticoid receptor (GR) expression. (A) There was significantly reduced GR protein expression in the prefrontal cortex after either PNS or CAPS alone, as well as the combined PNS/CAPS treatment. *** $P=0.0001$ compared to unstressed controls, $n=18$ –20/group. (B) PNS modestly but significantly reduced GR protein expression in the hippocampus. * Main effect of PNS, $P=0.04$. Data expressed as mean percent of unstressed controls $\pm$ SEM, $n=14$ –16/group.

significantly higher TH mRNA expression compared to controls receiving no PNS. There was also a non-significant elevation of TH mRNA in the group that received CAPS alone ($P=0.09$). Likewise, for TH protein, there was no significant main effect of PNS (Fig. 2B; $F_{(1,48)}=0.664$, $P>0.05$) nor of CAPS ($F_{(1,48)}=1.107$, $P>0.05$), but a significant PNS $\times$ CAPS interaction ($F_{(1,48)}=7.272$, $P<0.01$). Post hoc analyses reveal that rats exposed to CAPS alone had more adrenal TH protein than did control rats (P approaching significance at 0.051) or rats exposed to PNS plus CAPS.

GR levels in the mPFC and hippocampus

In the prefrontal cortex, both PNS and CAPS significantly decreased GR protein (Fig. 3A; $F_{(1,70)}=16.906$, $P<0.0001$ for PNS; $F_{(1,70)}=18.177$, $P<0.0001$ for CAPS) with no apparent additive effect of the two treatments, resulting also in a significant interaction of PNS and CAPS ($F_{(1,70)}=17.171$, $P<0.0001$). Post hoc analyses revealed that the three stress conditions (PNS, CAPS, PNS/CAPS) did not differ from each other. By contrast, in the hippocampus, only a main effect of prenatal stress was evi-

dent (Fig. 3B; $F_{(1,55)}=4.368$, $P<0.05$), causing a significant decrease in GR protein, but no main effect of CAPS ($F_{(1,55)}=0.37$, $P>0.05$) nor an interaction of PNS and CAPS ($F_{(1,55)}=1.06$, $P>0.05$).

Plasma ACTH and CORT

There were no effects of PNS or CAPS on basal plasma ACTH ($F_{(1,100)}=0.091$ for PNS, $F_{(1,100)}=0.003$ for CAPS, $P>0.05$), nor was there a significant interaction ($F_{(1,100)}=1.692$, $P>0.05$). However, prenatal stress induced a significant, long-term elevation in basal CORT (Fig. 4; $F_{(1,99)}=5.068$, $P<0.05$). Adult stress had no effect on basal CORT ($F_{(1,99)}=0.702$, $P>0.05$) nor was there a significant interaction between PNS and CAPS ($F_{(1,99)}=0.123$, $P>0.05$), probably because sacrifice and trunk blood collection for plasma measures took place 5–10 days after the termination of the adult stress treatment.

Fear conditioning and extinction

On all 3 days, there was the expected main effect of Tone ($F_{(3,276)}=107.712$, $P<0.001$ for conditioning; $F_{(9,828)}=38.875$, $P<0.001$; $F_{(9,828)}=21.271$, $P<0.001$ for extinction training and retention, respectively), confirming that the conditioning and extinction protocols were effective. Rats exposed specifically to PNS plus CAPS displayed enhanced fear conditioning and impaired extinction. For fear conditioning, there was a significant interaction between CAPS and Tone (Fig. 5; $F_{(3,276)}=2.813$, $P<0.05$). Post hoc analyses revealed that the PNS/CAPS group expressed significantly higher levels of freezing than did the PNS group on tone 4. There were no other main effects or interactions during conditioning.

On the test day, freezing in response to tone 1 alone was first analyzed to determine if there were any differences in the retention of conditioning from the previous day, and there were not. There were no main effects of PNS ($F_{(1,92)}=1.317$, $P>0.05$), or CAPS ($F_{(1,92)}=0.4607$, $P>0.05$), nor an interaction ($F_{(1,92)}=0.1656$, $P>0.05$). Next, the analysis of the subsequent course of extinction

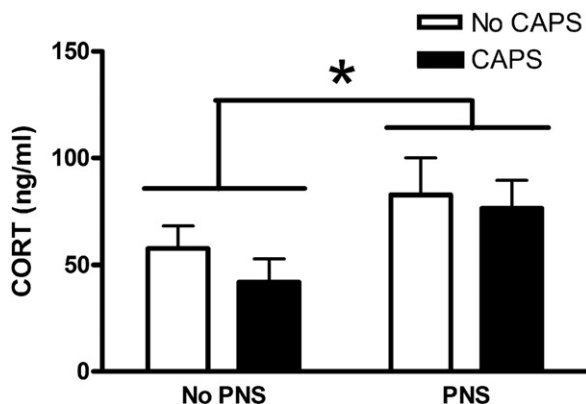


Fig. 4. Effects of PNS and CAPS on basal corticosterone. PNS-treated rats had significantly higher basal levels of circulating corticosterone. * Main effect of PNS, $P=0.03$. Data expressed as mean $\pm$ SEM, $n=25$ –27/group.

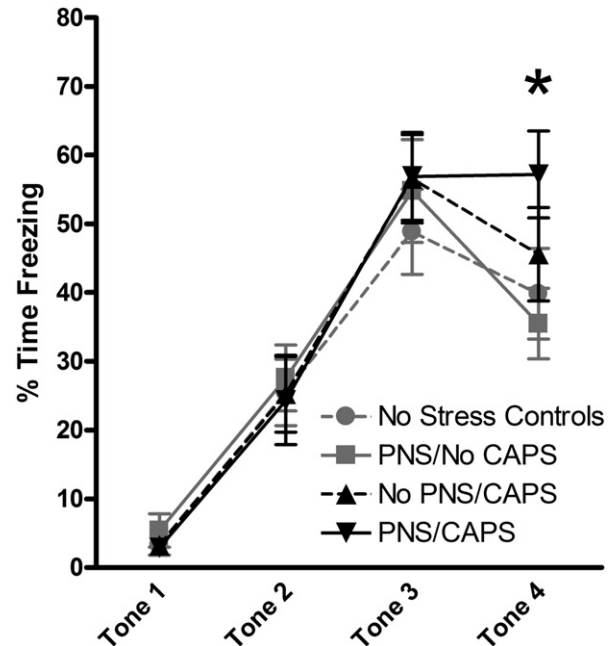


Fig. 5. Combined PNS/CAPS treatment enhanced the expression of learned fear. Rats that received PNS/CAPS displayed enhanced freezing on tone 4 of fear conditioning. * PNS/CAPS vs. PNS/No CAPS, $P=0.009$. Data expressed as mean $\pm$ SEM, $n=23$ –25/group.

training showed that there was a significant effect of CAPS (Fig. 6A; $F_{(1,92)}=3.97$, $P<0.05$) and a significant interaction between CAPS and Tone ($F_{(9,828)}=1.9$, $P<0.05$). Additionally, the main effect of PNS approached significance ($F_{(1,92)}=3.18$, $P=0.078$). Post hoc analyses revealed that the effect of CAPS was manifest as a delay in extinction (i.e. more tones required for extinction), as CAPS-treated rats had significantly higher freezing on tones 3–5 compared to unstressed controls (Fig. 6A). Subsequent post hoc comparisons between groups indicated that this was driven largely by a delayed extinction profile specifically in the combined PNS/CAPS group, in which freezing behavior remained elevated longer than in the other groups. The PNS/CAPS group had significantly more freezing than unstressed controls on tones 3–5, and more than both the PNS-only and CAPS-only groups on tone 5 (Fig. 6A, B). To further assess differences specifically in the rate of extinction, an exponential decay function was fit to each rat's freezing data, and the resulting rate constants (k) were compared by two-way ANOVA. Confirming the results of the primary ANOVA, there was a significant effect of CAPS ($F_{(1,89)}=7.522$, $P<0.01$), reflecting a slower rate of extinction, that was especially evident in the PNS/CAPS group. Post hoc analyses showed that the PNS/CAPS group had significantly slower rates of extinction than both the unstressed control group and the PNS only group (Fig. 7A, B). These analyses suggest that the extinction deficit induced by CAPS, and most prominently in the PNS/CAPS group, was driven primarily by a decrease in the rate of extinction. By contrast, also as seen in Fig. 7, the effect of PNS was primarily to elevate freezing (i.e. increased fear) without affecting the rate of extinction.

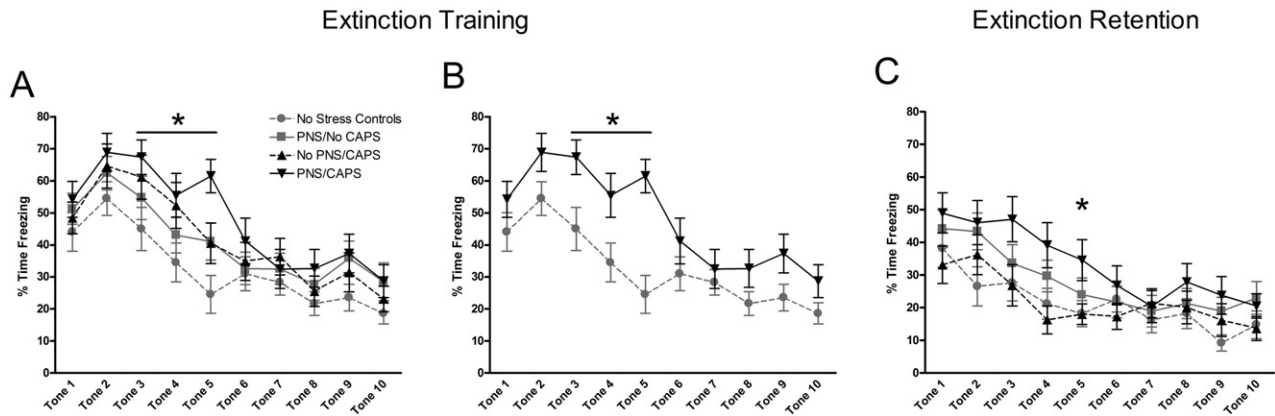


Fig. 6. Combined PNS/CAPS treatment impaired extinction. (A) CAPS caused a delay in extinction, as freezing in the CAPS-treated rats remained significantly elevated during tones 3–5 compared to non-CAPS-treated rats (* $P < 0.05$, post hoc comparisons by Newman–Keuls). (B) Specific comparison of extinction in the PNS/CAPS and control groups showed that freezing in the PNS/CAPS group also remained significantly elevated during tones 3–5 compared to unstressed controls (* $P < 0.05$, post hoc comparisons by Newman–Keuls), and during tone 5 compared to both the No PNS/CAPS and PNS/No CAPS groups (for clarity, only the PNS-CAPS and control groups are shown). (C) During extinction retention testing on the following day, overall freezing was elevated by PNS alone (* main effect of PNS, $P < 0.05$). Although the PNS/CAPS group again appeared to be the most affected, that specific comparison was not significant ($P < 0.10$). Data in all panels expressed as mean $\pm$ SEM, $n = 23$ –25/group.

PNS treatment also elevated freezing during extinction retention, tested on the following day (Fig. 6C). Freezing in response to tone 1 was first analyzed, as above. There was no significant effect of CAPS ($F_{(1,92)} = 0.0002$, $P > 0.05$) nor an interaction between PNS and CAPS ($F_{(1,92)} = 0.6936$, $P > 0.05$). However, the main effect of PNS approached significance ($F_{(1,92)} = 3.505$, $P = 0.064$), suggesting that rats exposed to PNS had modestly elevated freezing even at the outset of the extinction retention day. Subsequent analysis of the full course of extinction retention by ANOVA revealed only a significant main effect of PNS ($F_{(1,92)} = 5.798$, $P < 0.05$). The PNS/CAPS group once again appeared to be most affected (Fig. 6C), although this specific comparison was not significant ($P < 0.10$). That PNS induced an overall elevation in freezing without affecting the trajectory of extinction on retention day was confirmed by analyzing the rate constants derived from the exponential decay curves fit to the extinction retention data, for which there were no significant differences (Fig. 7C, D). Thus, unlike the effect of CAPS, and especially of PNS/CAPS, on the rate of extinction during training, the effect of PNS on extinction retention was an overall elevation in freezing, evident from trial 1 on.

Open field exploration and social interaction

In the open field test, there were no significant main effects of PNS or CAPS on number of line crossings (Table 2; $F_{(1,39)} = 0.057$ for PNS, $F_{(1,39)} = 0.028$ for CAPS, $P > 0.05$) or time spent in the center zone ($F_{(1,39)} = 2.058$ for PNS, $F_{(1,39)} = 0.119$ for CAPS, $P > 0.05$). Likewise, there was no significant interaction between PNS and CAPS on either measure ($F_{(1,39)} = 0.195$, $P > 0.05$ for line crossings; $F_{(1,39)} = 3.303$, $P > 0.05$ for center time). Similarly, in the social interaction test, there were no significant main effects or interactions of PNS and CAPS on time spent interacting with a novel conspecific (Table 2; $F_{(1,39)} = 0.261$

for PNS, $F_{(1,39)} = 0.578$ for CAPS, $F_{(1,39)} = 0.041$ for PNS $\times$ CAPS, all $P > 0.05$).

DISCUSSION

The hypothesis tested in this experiment was that prenatal stress produces a vulnerability to severe stressors in adulthood, such that those rats that experienced prenatal stress would exhibit a greater detrimental behavioral effect following adult stress, which might be accounted for by specific neurochemical changes in the brain and/or periphery. We found that PNS induced stable baseline alterations on several neurochemical parameters independent of adult stress exposure, and also induced a greater sensitivity to adult stress in some, but not all of the neurochemical and behavioral measures. We found that adult stress alone impaired extinction, and that effect was exacerbated by PNS. Thus, we conclude that prenatal stress exposure induces long term and stable changes in brain and peripheral stress response systems that represent a potential vulnerability to subsequent adult stress.

The rats were stressed during the last week of pregnancy (from E14 to birth), a critical period in development of the fetal HPA system, and also of potential sensitivity to maternal glucocorticoids. GR and mineralocorticoid receptor (MR) receptors are expressed in the developing rat brain at E13–E16 (Kitraki et al., 1996; Diaz et al., 1998), and fetal production of CORT, as well as maternal CORT concentrations, increase from E16 to E19 (Dupouy et al., 1975). Further, placental expression and activity of the enzyme 11 β -hydroxysteroid dehydrogenase 2, which protects the fetus from maternal CORT by metabolizing it to inactive 11-dehydrocorticosterone, decreases from E16 to birth (Waddell et al., 1998). Functionally, a comparison of stress exposure during the second and third weeks of gestation found a lasting change in HPA regulation only in

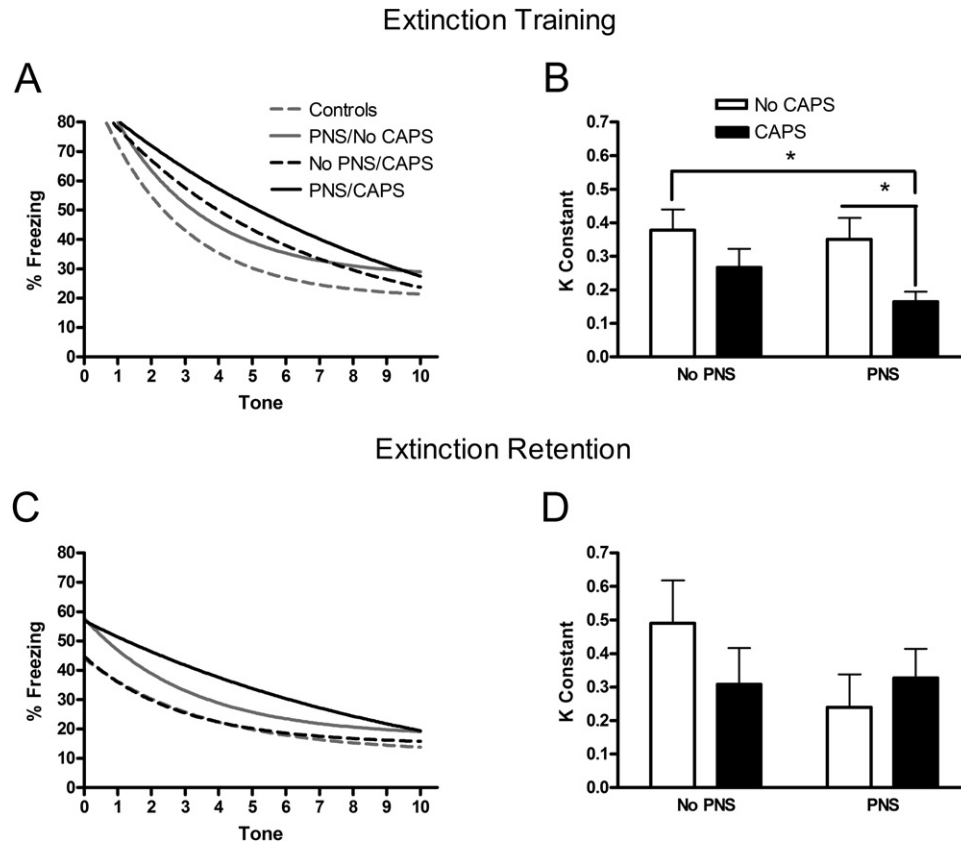


Fig. 7. Effects of PNS and CAPS on rates of extinction. Exponential decay curves were fit to each rat's freezing data, providing a rate of decay (k constant) for each animal. The k -values were then compared by two-way ANOVA to determine group differences in the rate of extinction. (A) For the purposes of illustration, exponential decay curves were fit to each group's mean data, representing the average rate of extinction for each group on training day. (B) In the analysis of k , CAPS produced a slower rate of extinction during training, with the PNS/CAPS rats displaying a significantly slower rate compared to the PNS alone and unstressed control groups (* $P < 0.05$ by Newman–Keuls post hoc comparison). (C) Exponential decay curves were fit to each group's mean data to illustrate the average rate of extinction on retention day. (D) There were no significant effects of PNS or CAPS on k , the rate constant for extinction on the retention day. Data expressed as mean $\pm$ SEM, $n = 23$ –25/group.

the offspring of mothers stressed during the third week (Koenig et al., 2005).

In the present study, PNS induced a 50% reduction in TH mRNA in the dorsal pons, including noradrenergic neurons in the LC and subcoeruleus. This effect was specific to the brain, as there was, if anything, a modest elevation of TH mRNA and/or protein in the adrenal medulla after either PNS or CAPS. PNS also produced stable, long-term changes in the HPA axis. Adult offspring exposed to PNS had significantly elevated basal circulating CORT and reduced GR expression in the hippocampus and PFC, suggesting potential impairment of HPA negative feedback mechanisms. These neurochemical changes are consistent with effects of PNS reported previously in the

literature. For example, PNS has been shown to affect regulation of the HPA axis, including alterations in circadian rhythm (Koehl et al., 1999), elevated CORT, and delayed recovery after mild stress (Weinstock et al., 1992; Barbazanges et al., 1996; Valle et al., 1997; Koenig et al., 2005; Fan et al., 2009). PNS has also been shown to decrease neurogenesis (Lemaire et al., 2000), and the expression of both the GR and the MR in the hippocampus (Barbazanges et al., 1996; Koehl et al., 1999). As in the present study, these effects were all observed in adult offspring of dams that had been stressed during the final week of pregnancy, suggesting that the changes are life-long and could thus alter the response to subsequent chronic or traumatic stress in adulthood.

Table 2. Open field exploration and social interaction

	Controls	PNS alone	CAPS alone	PNS/CAPS
Open field line crossings	84.5 $\pm$ 14.76	74.3 $\pm$ 15.88	80.4 $\pm$ 12.28	83.45 $\pm$ 16.27
Open field center zone time (s)	8.23 $\pm$ 2.05	9.07 $\pm$ 1.51	11.47 $\pm$ 3.49	4.32 $\pm$ 1.2
Social interaction time (s)	67.3 $\pm$ 7.85	70.07 $\pm$ 10.16	58.67 $\pm$ 5.58	65.07 $\pm$ 10.99

Data expressed as mean $\pm$ SEM, $n = 10$ –12/group.

Both PNS and CAPS also impacted behavioral responses to fear conditioning and extinction, although in different ways, and rats exposed to PNS plus CAPS had the greatest behavioral deficits. First, PNS/CAPS rats displayed slightly elevated freezing at the end of fear conditioning, due primarily to a failure to decrease freezing on the fourth trial. We have frequently observed that after a certain number of tone-shock pairings during fear conditioning, rats can start to shift to a more active coping response, exhibiting active escape behavior and less passive freezing, which was just beginning to occur on tone 4 in the other groups. Thus, the pattern of response seen on the last conditioning trial may indicate that the PNS/CAPS rats persisted in maintaining a more passive coping response to the mild acute stress.

Next, the detrimental effects of adult stress on extinction were manifest as a delay in the rate of extinction. PNS alone modestly elevated the overall level of freezing, whereas CAPS-treated rats had a significantly slower rate of extinction, and this was enhanced by prior PNS exposure. It is noteworthy that on the extinction training day, despite clear differences in the rate of extinction learning, all groups eventually achieved extinction by the end of the session. Having thus achieved extinction, there were no residual effects of CAPS treatment alone on the retention of extinction 1 day later. However, during testing for the retention of extinction, the PNS-treated rats, particularly the combined PNS/CAPS-treated rats, continued to display elevated levels of freezing, which was evident even on tone 1, and was maintained across trials, but with no differences in the extinction rate constants on the retention day. Thus, even though all groups reached equivalent levels of extinction on the training day, PNS-treated rats maintained an elevated level of fear on the following day. These results suggest that severe adult stress exposure alone can have a transient detrimental effect on the rate of extinction, which may be evident in the short-term consequences of traumatic stress exposure, but that these deficits can eventually be resolved with sufficient extinction training, until full extinction is achieved. However, a predisposing history of PNS exposure not only exacerbated the detrimental effect of adult stress on the process of extinction, but in itself it also induced a modest but persistent propensity to exhibit enhanced fear, seen as an increase in freezing during both extinction learning and retention, thus acting as a vulnerability factor.

A similar effect of PNS was reported previously (Markham et al., 2010). In that study, PNS-treated male rats displayed less freezing during conditioning, but more freezing during extinction training and retention compared to control male rats. The different effects observed during conditioning as compared to the present study may be due to differences in the PNS protocol. In the Markham study, PNS involved a variable stress procedure, whereas we used a repeated homotypic stressor (immobilization). Interestingly, in the Markham study, no effects of PNS were seen in females, in either conditioning or extinction. Thus, the factors involved in PNS-induced vulnerability

may be particularly relevant to human males facing combat exposure.

Impairments in the rate of extinction of conditioned fear are relevant to human neuropsychiatric disorders in which conditioned fear is a prominent component, such as PTSD, panic disorder, and phobias. In these disorders, there is an inability to extinguish a fear reaction and reinforcement of the fear response. For example, PTSD patients show impairments in extinction (Blecher et al., 2007; Wessa and Flor, 2007) similar to those found in our study, and they have difficulty suppressing fear responses in the presence of safety signals, despite awareness of the safety signal and its meaning (Jovanovic et al., 2009). Further, exposure therapy, a form of extinction training, is effective for approximately 50% of PTSD patients (Bradley et al., 2005), although it is considered one of the most successful treatments for PTSD. In the present study, initial impairments in extinction were seen in the CAPS-treated rats, but successful “recovery” was maintained once extinction was achieved. This may reflect the success of exposure therapy in a proportion of trauma-exposed humans. On the other hand, PNS exposure induced a long-term vulnerability to adult stress, reflected by enhanced impairment of extinction learning and retention, and elevated fear even after extinction. This may reflect the fact that, in humans, certain vulnerable individuals remain impaired and/or resistant to exposure therapy. Further, the differences in the effects of CAPS alone versus PNS plus CAPS may reflect the transition from an acute and transient, perhaps even adaptive response to stress, into a long-term PTSD-like state in vulnerable individuals.

The neurochemical systems in which changes were observed after PNS may provide clues to potential mechanisms underlying the subsequent vulnerability to adult CAPS exposure, and the resulting impairments specifically in the extinction of conditioned fear. TH is the rate-limiting step in catecholamine synthesis. Chronic down-regulation of TH in the forebrain-projecting noradrenergic neurons in the LC, including the sole source of NE input to both PFC and hippocampus, could reflect a reduced capacity for sustained NE release in these forebrain targets in the face of chronic or severe stress. Reduction in brainstem TH, and presumably in NE release, could be one mechanism underlying impaired extinction learning and retention after PNS/CAPS. NE neurotransmission has been implicated in both fear conditioning and extinction. During conditioning, NE release in the amygdala is correlated with the retention of fear memories (Galvez et al., 1996; Quirarte et al., 1998; McIntyre et al., 2002), which is impaired by β -adrenergic receptor blockade (Fu et al., 2008). Likewise, NE levels increase in the mPFC in response to emotionally salient stimuli (Feenstra et al., 2001; Mingote et al., 2004; Hugues et al., 2007), and extinction retention is also impaired by blockade of β -adrenergic receptors, and enhanced by administration of yohimbine, an α_2 -adrenergic autoreceptor antagonist that increases NE levels, prior to extinction training (Mueller et al., 2008; Cain et al., 2004).

Glucocorticoids also affect learning and memory, although in a more complex manner (see de Quervain et al.,

2009). Acute CORT administration immediately after training in a novel object recognition task enhanced consolidation and recognition (Roosendaal et al., 2006). By contrast, injections of GR agonist into the hippocampus immediately before retention testing in a Morris water maze impaired spatial memory (Roosendaal et al., 2004), and both of these effects were dependent on convergent noradrenergic signaling. Further, either chronic low-dose CORT or acute GR antagonists given after the first extinction exposure impaired extinction (Gourley et al., 2009). Thus, chronically elevated CORT, together with GR down-regulation and reduced NE release capacity in PFC may have all contributed to impaired extinction after PNS/CAPS.

Monoamines, including NE, have been implicated in both the stress response and in stress-related affective disorders, and antidepressants that affect the monoamines, serotonin, and NE, are the most effective pharmacological treatments for depression and anxiety disorders. However, while drugs targeting NE are effective therapeutically, evidence suggesting dysregulation of noradrenergic signaling in mood and anxiety disorders is less consistent, including alterations in NE levels and adrenergic receptor expression in depression, and reduced NE metabolite levels during the depressive phase of bipolar disorder (see Muscettola et al., 1984; Schatzberg et al., 1989; Ressler and Nemeroff, 1999; Strawn and Geraciotti, 2008), although the profile is far from clear. It has been suggested that variability in such measures may be related to the expression of different symptoms, or to different subtypes of depressive disorder (Gold and Chrousos, 1999).

There is more convincing evidence of catecholaminergic dysregulation and increased response sensitivity in PTSD (Strawn and Geraciotti, 2008). Elevated plasma NE levels have been correlated with greater symptom expression (Yehuda et al., 1992; Lemieux and Coe, 1995). Peripheral NE release in response to traumatic reminders is enhanced (Blanchard et al., 1991), and administration of yohimbine induces symptoms in PTSD patients (Southwick et al., 1993). Increased peripheral NE activity is consistent with the modestly elevated TH levels we observed in the adrenal medulla of stressed rats. By contrast, a limited study of post-mortem brain tissue from soldiers showed an approximate 50% reduction in the number of LC-NE neurons in the probable-PTSD group compared to controls (Bracha et al., 2005). A similar profile was seen in suicide victims, suggesting reduced NE signaling capacity in depression (Arango et al., 1996). These central changes are similar to the reduction of brainstem TH in PNS rats in the present study, and may thus be more indicative of reduced stress coping capacity and a predisposition to stress vulnerability than to the overt expression of stress-induced illness per se.

The HPA axis and glucocorticoids have also been implicated in affective disorders. In some depressed patients, there is evidence of elevated basal cortisol secretion (Board et al., 1956; Gold et al., 1986; Souetre et al., 1988; Arborelius et al., 1999), elevated corticotropin releasing hormone in cerebral spinal fluid (Nemeroff et al., 1984), blunted diurnal rhythms (Souetre et al., 1988), and impaired negative feedback by dexamethasone (reviewed in Handwerger, 2009).

Likewise, anxiety disorders are also associated with alterations in HPA axis activity, but there is considerable inconsistency in the literature. Of particular relevance to the present study, HPA dysregulation has been reported in PTSD, although the exact nature of the dysregulation remains a matter of debate. Some studies have shown elevated basal urinary and plasma cortisol levels in PTSD patients compared to controls (Hoffman et al., 1989; Pitman and Orr, 1990; Lemieux and Coe, 1995), while others have shown lower urinary and plasma cortisol (Mason et al., 1986; Yehuda et al., 1990, 1993, 1995; Boscarino, 1996). Again, discrepancies may be related to the nature or duration of the trauma, or to the expression of specific symptoms (de Quervain et al., 2009; Handwerger, 2009).

Sensitized fear and impaired extinction may be indicative of a more general cognitive deficit related specifically to hypoactivity in the mPFC. Noradrenergic signaling in the mPFC is implicated in tasks requiring cognitive flexibility (Lapiz and Morilak, 2006; Aston-Jones et al., 1999), and extinction learning is a form of cognitive flexibility that is dependent upon the functional integrity of the mPFC (Morgan et al., 1993). Cognitive dysfunction, including cognitive inflexibility and perseveration, is an important component of stress-related psychiatric disorders, and individuals with depression, obsessive-compulsive disorder, or PTSD perform poorly on tests of executive function and cognitive flexibility (Sutker et al., 1995; Fossati et al., 1999; Koenen et al., 2001; Moritz et al., 2002; Kangaratnam and Asbjørnsen, 2007). Such cognitive deficits are often manifest in the form of negative biases, contributing to disordered thinking about self-worth, life stressors, and/or fear-provoking events (Coles and Heimberg, 2002; Elzinga and Bremner, 2002). It has been hypothesized that a hypoactive mPFC and associated cognitive deficits may not only contribute to the symptoms of affective disorders, but may also be a consequence of early-life stress exposure that creates a vulnerability to develop such disorders in response to later stress or trauma (Elzinga and Bremner, 2002).

The prenatal stress model of vulnerability shares some characteristics with genetic models of stress vulnerability, including Wistar-Kyoto (WKY) rats and high responder (HR) rats. WKY rats show a number of behavioral characteristics suggesting increased stress sensitivity, including heightened neophobia and depressive-like behaviors (Paré, 1994). Our laboratory has previously reported that WKY rats exhibit differences in both the expression and regulation of peripheral and central TH mRNA. Specifically, WKY rats showed attenuated TH induction and reduced NE release in the brain in response to acute stress under basal conditions, but greatly enhanced acute NE responses after chronic stress exposure, as well as increases in freezing behavior and acute HPA reactivity (Sands et al., 2000; Pardon et al., 2002, 2003; Ma and Morilak, 2004). Thus, noradrenergic dysregulation may contribute to stress-vulnerability in the WKY genetic model as well as that produced by PNS exposure in the present study. One difference is that prenatal stress induced basal changes in TH, whereas WKY rats did not differ in basal expression, but had attenuated acute stress-evoked induction of TH. In both models, the changes in TH expression may reflect reduced capacity for acute stress adaptation and coping, in-

cluding a preference for passive coping behaviors (i.e. freezing and/or immobility), which may be maladaptive in certain conditions.

HR rats are another rat model of stress vulnerability that share some neurobiological characteristics with the PNS-exposed rats, including elevated CORT, prolonged recovery of basal CORT levels post-stress (Piazza et al., 1991; Kabbaj et al., 2000), and decreased GR mRNA expression in hippocampus (Kabbaj et al., 2000). HR rats displayed increased locomotor responses to amphetamine (Piazza et al., 1989, 1991), as reported after PNS (Koenig et al., 2005), although we saw no changes in basal locomotion in the open field in the present study. However, HR rats have also been defined by high novelty-seeking behavior and enhanced exploration of anxiogenic environments (e.g. Kabbaj et al., 2000), whereas PNS rats have been reported to avoid such environments (Weinstock et al., 1992; Valle et al., 1997; Bosch et al., 2007; Fan et al., 2009). Thus, these models may produce different but overlapping behavioral and neurobiological manifestations that each may be informative for identifying mechanisms underlying individual differences in coping and stress vulnerability. The PNS/CAPS model exemplifies an experience-based stress vulnerability, which may help us to understand aspects of disorders such as PTSD, which present only after experiencing a severe stressor later in life.

Human psychopathologies are complex and multi-dimensional disorders, involving many brain systems and neural circuits. We would not suggest that PNS plus CAPS models all characteristics of any given affective disorder, nor does it replicate any human syndrome in its entirety in rats. Rather, it models key dimensions of many stress-related affective disorders, particularly those associated with fear and acute stress-reactivity. Moreover, it appears to model a vulnerability in specific neurobiological systems that can modulate the processes of fear conditioning and extinction. Thus, this model and others like it will allow a productive investigation of the potential mechanisms underlying long-term changes in the effectiveness of stress-coping capability, and of lifelong vulnerability to stress-induced psychopathology.

Acknowledgments—Funding for this work was provided to the STRONGSTAR Multidisciplinary PTSD Research Consortium by the Department of Defense through the U.S. Army Medical Research and Materiel Command, Congressionally Directed Medical Research Programs, Psychological Health and Traumatic Brain Injury Research Program award W81XWH-08-2-0118. We thank Dr. Milena Girotti and Dr. Brian Bingham for their assistance with the hormone assays, and with revision of the manuscript. We thank Ms. Kale Naegeli and Ms. Vanessa Martinez for technical assistance. We also thank Dr. Jim Mintz, Departments of Psychiatry and Epidemiology & Biostatistics, UTHSCSA, for his insights and suggestions on the statistical analyses. The views expressed in this paper are solely those of the authors and do not reflect an endorsement by or official policy of the Department of Defense or the U.S. Government.

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(Accepted 14 June 2011)
(Available online 22 June 2011)



Effects of chronic plus acute prolonged stress on measures of coping style, anxiety, and evoked HPA-axis reactivity

Megan K. Roth^{a,b,1}, Brian Bingham^{a,b,1}, Aparna Shah^{a,b,1}, Ankur Joshi^{a,b,1,2}, Alan Frazer^{a,b,c,1}, Randy Strong^{a,b,c,1}, David A. Morilak^{a,b,*,1}

^a Department of Pharmacology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, TX 78229, USA

^b Center for Biomedical Neuroscience, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, TX 78229, USA

^c South Texas Veterans Health Care Network, Audie L. Murphy Division, 7400 Merton Minter Drive, San Antonio, TX 78229, USA

ARTICLE INFO

Article history:

Received 8 March 2012

Received in revised form

22 June 2012

Accepted 16 July 2012

Keywords:

PTSD

Stress

Coping style

Anxiety

HPA-axis

ABSTRACT

Exposure to psychological trauma is the precipitating factor for PTSD. In addition, a history of chronic or traumatic stress exposure is a predisposing risk factor. We have developed a Chronic plus Acute Prolonged Stress (CAPS) treatment for rats that models some of the characteristics of stressful events that can lead to PTSD in humans. We have previously shown that CAPS enhances acute fear responses and impairs extinction of conditioned fear. Further, CAPS reduced the expression of glucocorticoid receptors in the medial prefrontal cortex. In this study we examined the effects of CAPS exposure on behavioral stress coping style, anxiety-like behaviors, and acute stress reactivity of the hypothalamic–pituitary–adrenal (HPA) axis. Male Sprague-Dawley rats were exposed to CAPS treatment, consisting of chronic intermittent cold stress (4 °C, 6 h/day, 14 days) followed on day 15 by a single 1-h session of sequential acute stressors (social defeat, immobilization, swim). After CAPS or control treatment, different groups were tested for shock probe defensive burying, novelty suppressed feeding, or evoked activation of adrenocorticotrophic hormone (ACTH) and corticosterone release by an acute immobilization stress. CAPS resulted in a decrease in active burying behavior and an increase in immobility in the shock probe test. Further, CAPS-treated rats displayed increases in the latency to feed in the novelty suppressed feeding test, despite an increase in food intake in the home cage. CAPS treatment also reduced the HPA response to a subsequent acute immobilization stress. These results further validate CAPS treatment as a rat model of relevance to PTSD, and together with results reported previously, suggest that CAPS impairs fear extinction, shifts coping behavior from an active to a more passive strategy, increases anxiety, and alters HPA reactivity, resembling many aspects of human PTSD.

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1. Introduction

Post-traumatic stress disorder (PTSD) is a disabling illness that occurs after exposure to a severe stress, e.g., a life-threatening event

Abbreviations: ACTH, adrenocorticotrophic hormone; CAPS, chronic plus acute prolonged stress; CORT, corticosterone; GR, glucocorticoid receptor; HPA, hypothalamic–pituitary–adrenal; mPFC, medial prefrontal cortex; NSFT, novelty suppressed feeding test; PD, postnatal day; PTSD, post-traumatic stress disorder; SPS, single prolonged stress.

* Corresponding author. Department of Pharmacology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, Texas 78229-3900, USA. Tel.: +1 210 567 4174; fax: +1 210 567 4300.

E-mail address: morilak@uthscsa.edu (D.A. Morilak).

¹ For the STRONG STAR Consortium.

² Present address: Center for Neuroscience, University of Pittsburgh, A210 Langley Hall, Pittsburgh, PA 15260, USA.

or witnessing such an event. PTSD is characterized by three classes of symptoms: re-experiencing, avoidance, and hyper-arousal (American Psychiatric Association, 2000). Re-experiencing involves intrusions of vivid memories and dreams, and even dissociations, related to the traumatic event. Avoidance of situations or stimuli that serve as reminders of the traumatic event may also be manifest as a general emotional and social detachment. Hyper-arousal is expressed as elevated anxiety, enhanced startle, irritability, sleep disturbance, and difficulty concentrating. Rape, physical attacks or abuse, threats with a weapon, and combat are some of the events typically associated with PTSD (Kessler et al., 1995). Chronic PTSD represents a significant health concern, not only because of the disabling nature of the symptoms, but also because of the long-term consequences on physical health, including higher rates of chronic disease, such as cardiovascular disease, diabetes, asthma, and obesity, as well as higher rates of

substance abuse (Centers for Disease Control and Prevention, 2006; Sareen et al., 2005). Chronic stress is also a risk factor, and possibly a causal factor, in the development of depressive and anxiety disorders (Breslau et al., 1999; Gilmer et al., 2005; Jordanova et al., 2007; Kendler et al., 1999; Koenen et al., 2007, 2002).

The complex nature of stress is particularly salient in wartime situations, in which there is a chronic state of environmental stress punctuated by intense, acute traumatic events. To model this, we developed a stress treatment that we have termed Chronic plus Acute Prolonged Stress (CAPS; not to be confused with the “CAPS” assessment used in human PTSD research). CAPS treatment combines 14-days of exposure to a chronic mild environmental stressor (chronic intermittent cold stress), followed on day 15 by a single session of intense acute stressors adapted from the Single Prolonged Stress (SPS) model (Yamamoto et al., 2009).

We have shown previously that CAPS treatment impaired fear extinction (Green et al., 2011), arguably an important component of human PTSD that may contribute to treatment resistance. For instance, PTSD patients show impairments in extinction (Blecher et al., 2007; Wessa and Flor, 2007), and they are incapable of suppressing fear responses in the presence of a safety signal, despite awareness of the safety signal and its meaning (Jovanovic et al., 2009). We also showed that CAPS treatment resulted in a down-regulation of glucocorticoid receptors (GR) in the medial prefrontal cortex (mPFC) (Green et al., 2011). This could have contributed to the impairments observed during extinction testing, as glucocorticoids are known to be involved in learning and memory, including fear and extinction learning (Gourley et al., 2009; de Quervain et al., 2009; Roozendaal et al., 2004, 2006). The mPFC, particularly the infralimbic cortex, is a key region involved in extinction learning (Milad and Quirk, 2002; Milad et al., 2004; Morgan et al., 1993; Quirk et al., 2000; Sutker et al., 1995). Furthermore, humans with PTSD display dysregulated hypothalamic–pituitary–adrenal (HPA) axis activity, although the nature of this dysregulation remains unclear (Boscarino, 1996; Hoffman et al., 1989; Lemieux and Coe, 1995; Mason et al., 1986; Pitman and Orr, 1990; Yehuda et al., 1995, 1993, 1990).

Having defined some key components of PTSD in this model, in the present experiments, we continued to explore the effects of CAPS on other measures of PTSD-like symptomatology, including coping style/defensive behavior and generalized anxiety, as well as acute HPA stress reactivity. In our previous study, we observed that CAPS, particularly when combined with early life stress, resulted in persistent freezing during fear conditioning, at a point when other rats were shifting to a more active escape strategy (rearing and jumping) (Green et al., 2011). Thus, using the shock probe-defensive burying test in the present experiment, we tested the hypothesis that CAPS would produce a shift from an active coping strategy (burying) to a passive coping strategy (immobility). Likewise, we examined if CAPS would increase anxiety-like behavior in the novelty suppressed feeding test (NSFT). Finally, we tested if CAPS treatment produced changes in the HPA response evoked by an acute stressor.

2. Experimental procedures

2.1. Animals

In total, 103 adult male Sprague-Dawley rats were used in these experiments. The rats were born in our animal facility, and after weaning, they were pair-housed with a same-sex littermate until postnatal day (PD) 46–60, depending on the experiment, at which time they were singly housed prior to starting the adult stress or unstressed control treatments. The rats were housed in Plexiglas cages (25 × 45 × 15 cm) on a 12/12 h light–dark cycle (lights on at 07:00) with food and water available *ad libitum*. In addition, for the social defeat procedure, 12 adult male Long-Evans rats (Harlan, Indianapolis, IN), weighing at least 400 g, were used as defeaters. They were housed, together with an ovariectomized female, in large

resident cages (60 × 60 × 35 cm) in a separate room on the same 12/12 h light cycle. All experiments were conducted during the light phase. All procedures were conducted according to NIH guidelines for the care and use of laboratory animals and were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio. All efforts were made to minimize animal pain, suffering or discomfort, and to minimize the number of rats used.

2.2. CAPS

CAPS treatment consisted of 2 weeks of chronic intermittent cold stress followed by a single 1-h session of acute prolonged stress on day 15. For cold stress, rats were transported in their home cage with food, water and bedding into a cold room at 4 °C for 6 h per day for 14 days. The acute prolonged stress on day 15 consisted of 20 min social defeat, followed immediately by 30 min immobilization, and then 10 min swim stress. For social defeat, the ovariectomized Long-Evans female was removed from the resident cage, and the test rat was placed in the cage with the resident Long-Evans male rat. Typically within 10–30 s, the resident would attack and defeat the smaller “intruder” Sprague-Dawley test rat. Once defeat occurred, defined by the test rat assuming a supine posture and the resident showing a dominant posture for at least 4 s, the test rat was placed under a wire mesh cage for 20 min, thus protecting it from further physical contact but allowing continued sensory exposure to the dominant rat. Immobilization involved taping the rat's torso and limbs gently but snugly in a prone position on a flat platform, allowing no movement, for 30 min. Finally, swim stress was accomplished by placing the rat in a cylindrical tank (30 cm diameter × 60 cm height) filled to a depth of 30 cm with water at approximately 23 °C. Control rats were handled briefly for approximately 30 s.

2.3. Experiment 1: shock-probe defensive burying test

CAPS treatment was initiated between PD 51–53 ($n = 9/\text{group}$). One day following the end of CAPS (or the comparable time for controls), rats were tested in the shock probe defensive burying test to evaluate potential shifts in active and passive behavioral coping strategies in response to acute stress. The rats were placed into a modified cage containing 5 cm of bedding, with a shock probe protruding 6 cm into one end of the cage. The probe was set to deliver 2 mA of current when the probe was touched. After the rat made contact with the probe and received a shock, the current was shut off and the 15 min test began. Behavior was recorded using a CCD camera mounted above the cage and stored to video files for offline scoring and analysis. The dependent measures analyzed were the amount of time spent immobile and the amount of time spent engaged in actively burying the probe. Burying was defined as behavior consisting of burrowing into the bedding with the snout and upper body, then plowing, pushing, or shoveling the bedding toward the probe, and also flicking or spraying bedding material toward the probe. Immobility was defined as a lack of movement other than that required for breathing (slight scanning movements of the head were permitted). Behavior clearly identified as resting behavior (e.g., laying on side, legs extended) was excluded from immobility measures. As a proportional measure of preferred response, the bury time ratio was calculated as (time spent burying)/(time spent burying + time spent immobile).

2.4. Experiment 2: novelty suppressed feeding test (NSFT)

CAPS was initiated on PD 47 ($n = 14\text{--}15/\text{group}$). Following CAPS (or the comparable time period for controls), the rats were left undisturbed for 2 days. Beginning on the 3rd day, the animals were food deprived for 48 h (water was available *ad libitum*). The test was conducted on the 5th day post-CAPS, as described by Bodnoff et al. (1988), with minor modification. The rats were transferred to the behavior room and allowed 1 h to acclimate. The rats were then individually placed into a corner of an unfamiliar black Plexiglas open field (100 × 100 × 40 cm) facing the center where food pellets were placed. The latency to begin feeding and the amount of food consumed during the 12 min test were recorded. Latency to feed was defined as the time from when the rats were placed into the open field until they began to eat the pellets (not just approach or play with them). Following the test, the rats were returned to their home cage, where food consumption was monitored for another 30 min to determine if there were any changes in appetitive behavior. Food consumption was determined by subtracting the weight of any remaining food from the total weight of food placed in the open field and the home cage.

2.5. Experiment 3: evoked HPA responses to acute immobilization stress

CAPS was initiated between PD 46–60 ($n = 7\text{--}14/\text{group}$). Group assignments were matched to balance the range of ages at which CAPS was initiated across all groups. Three days prior to the acute prolonged stress (Day 12 of CAPS or the comparable time for controls), all rats underwent jugular catheterization surgery. Rats were anesthetized with a mixture of ketamine 43 mg/ml, acepromazine 1.4 mg/ml, xylazine 8.6 mg/ml, administered i.m. at 1.0 ml/kg, and a catheter comprised of silastic and PE50 tubing was inserted into the jugular vein, then passed subcutaneously and exteriorized via an incision at the back of the neck and plugged. Every

3rd day until testing the catheter was flushed with approximately 0.2 ml of sterile heparinized saline (50 IU/ml) to maintain patency.

Separate groups were tested 1 or 5 days following the termination of CAPS. On the test day, the rats were transported to a quiet room, the catheter was connected via a fluid filled line to a syringe for remote blood collection without disturbing the animal, and approximately 0.1 ml of heparinized saline was administered to ensure patency. The rats were then given 90 min to acclimatize after transport. For blood sampling, 0.4 ml of blood was withdrawn via the catheter and replaced with 0.4 ml of sterile saline. Two baseline blood samples were collected 15 min apart. The rats were then immobilized for 30 min as described in Section 2.2. Two blood samples were collected during the stress, one at 5 min after the onset of stress, and one at 30 min. Following the 30 min stress sample, the rats were returned to their home cages and allowed to recover, during which time 4 blood samples were collected at 15, 30, 60, and 90 min post-stress. Blood was collected into tubes containing 10 μ l of 0.5 M EDTA. Plasma was separated immediately by centrifugation at 10,000 rpm for 15 min at 4 °C, and stored at –80 °C until assayed.

Because the CAPS protocol includes a single 30-min immobilization stress, it was possible that any changes observed during the acute stress exposure on test day could be due to habituation or sensitization to the second presentation of immobilization stress, rather than an effect of CAPS specifically. Therefore, a control experiment was conducted in which 2 separate groups of rats ($n = 8–10$) were exposed to a single 30 min immobilization stress rather than the full CAPS procedure, 3 days after catheterization surgery. A third group was briefly handled but not immobilized. Then, 1 or 5 days later, all rats were exposed to an acute 30-min test immobilization, and blood samples were collected as above.

Plasma levels of ACTH and CORT were analyzed by radioimmunoassay. ACTH was determined from duplicate 100 μ l samples according to the manufacturer's instructions (MP Biomedicals, Orangeburg, NY). The detection limit was 6 pg/ml, and the inter-assay variability was 10%. CORT was measured in diluted plasma samples according to the manufacturer's instructions (MP Biomedicals). Detection limit was 8 ng/ml, and inter-assay variability was 8%.

2.6. Statistical analyses

For the shock-probe defensive burying data, differences in immobility time, active burying time, and bury-time ratio were analyzed by *t*-test. Likewise, in the novelty suppressed feeding test, differences between groups in latency to feed and amount of food consumed were analyzed by *t*-test. Plasma hormone measures were analyzed by 2-way analysis of variance (ANOVA; group $\times$ sample, with repeated measures). In all analyses, significance was determined at $p < 0.05$. After ANOVA, sources of any significant main effects or interactions were determined by analysis with the Newman–Keuls *post-hoc* test.

3. Results

3.1. Experiment 1: shock-probe defensive burying test

CAPS-treated rats displayed significantly less burying behavior than control rats (Fig. 1A, $t_{(16)} = 2.258$, $p < 0.05$) and significantly more immobility (Fig. 1B, $t_{(16)} = 2.963$, $p < 0.01$). Consequently, CAPS-treated rats displayed a significantly lower bury-time ratio than control rats (Fig. 1C, $t_{(16)} = 4.169$, $p < 0.001$). This reduction in bury ratio reflects a shift from a predominantly active behavioral coping strategy to a predominantly passive coping strategy.

3.2. Experiment 2: novelty suppressed feeding test

CAPS-treated rats displayed a significantly longer latency to feed (Fig. 2A, $t_{(27)} = 2.532$, $p < 0.05$). CAPS reduced weight gain during the treatment, as these rats had lower mean body weight (281.5 ± 5.3 g) than controls (298.0 ± 5.6 g) prior to testing (Fig. 2B, $t_{(27)} = 2.135$, $p < 0.05$), as expected after chronic cold stress. Nonetheless, there was no difference in the amount of food consumed during the test period (Fig. 2C, $t_{(27)} = 1.54$, $p > 0.05$), and CAPS-treated rats consumed slightly more food than controls in their home cage (Fig. 2D, $t_{(27)} = 2.451$, $p < 0.05$). The fact that CAPS-treated rats consumed equivalent amounts of food during the test, and more food than controls in the home cage indicates that the increase in latency to feed was not due to a reduction in appetite, but to an increase in anxiety in the novel environment.

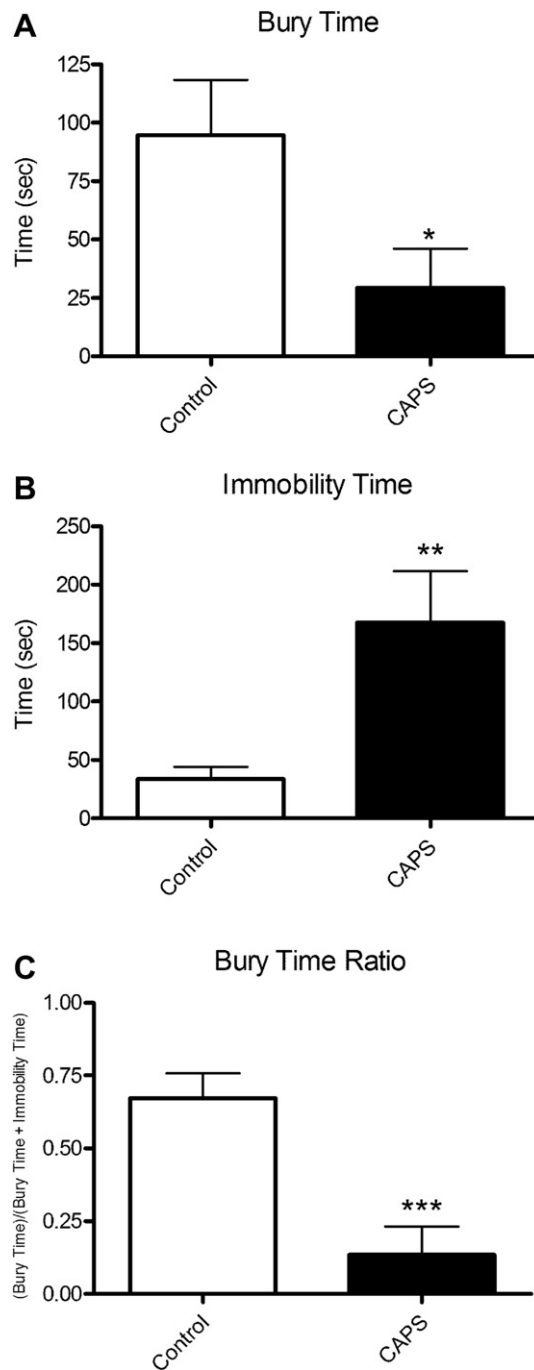


Fig. 1. Effect of CAPS on active defensive burying behavior and passive immobility in the shock-probe defensive burying test. A) On the shock-probe test, CAPS-treated rats displayed significantly less active burying behavior (29.33 ± 16.77 s) than unstressed control rats (94.67 ± 23.58 s). B) CAPS-treated rats displayed significantly more immobility (167.60 ± 43.98 s) in response to a single, brief mild shock than did the non-stressed controls (33.67 ± 10.39 s). C) Consequently, CAPS-treated rats had a lower bury ratio (0.13 ± 0.10) than control rats (0.67 ± 0.09), reflecting a shift from a predominantly active behavioral coping strategy (ratio > 0.5) in the control group to a predominantly passive strategy (ratio < 0.5) following CAPS treatment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data expressed as mean $\pm$ SEM, $n = 9$ /group.

3.3. Experiment 3: evoked HPA response to acute immobilization stress

Acute immobilization stress induced a significant increase in both ACTH and CORT ($F_{(7,168)} = 47.8$, $p < 0.0001$; $F_{(7,182)} = 62.05$, $p < 0.0001$, respectively; significance not indicated in Fig. 3 for

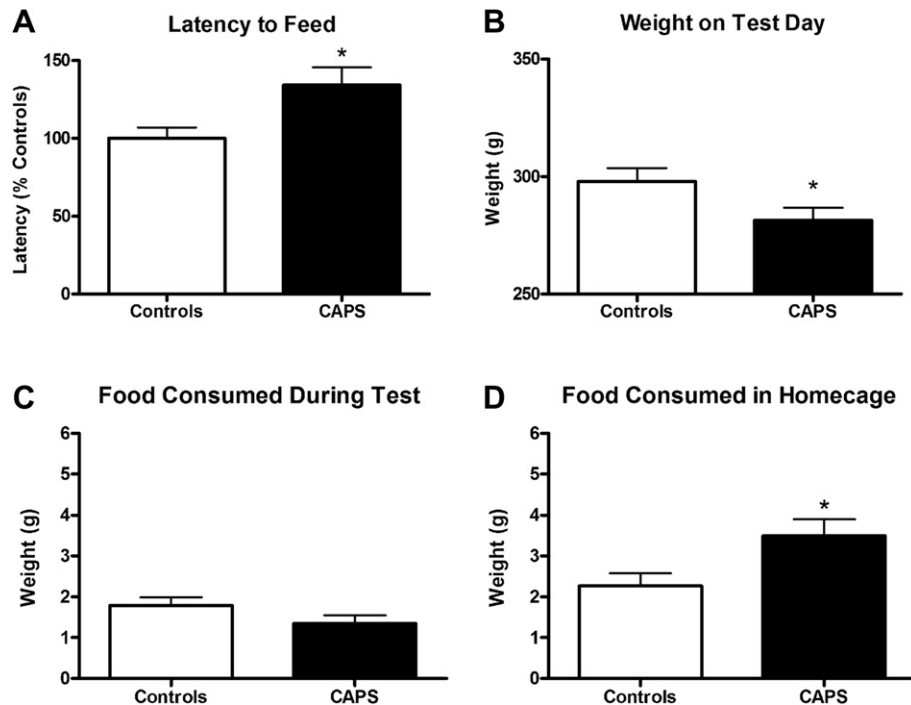


Fig. 2. Effect of CAPS on latency to feed in the novelty suppressed feeding test. A) CAPS-treated rats displayed a longer latency to begin eating ($134.3 \pm 11.4\%$ of controls, compared to $100.0 \pm 6.9\%$). B) As expected, CAPS-treated rats had lower mean body weight on test day (281.5 ± 5.3 g) compared to controls (298.0 ± 5.6 g). C) CAPS-treated rats and control rats consumed an equivalent amount of food during the test (1.35 ± 0.20 g and 1.79 ± 0.20 g, respectively). D) In fact, CAPS-treated rats displayed increased food consumption in the home cage after the test (3.5 ± 0.4 g) compared to control rats (2.3 ± 0.3 g), perhaps related to the reduction in body weight gain during CAPS treatment (panel B). Thus, the increase in latency to feed was not attributable to a loss of appetite, but to an increase in anxiety in the novel environment. * $p < 0.05$. Latency expressed as mean percent of controls $\pm$ SEM. Feeding expressed as mean $\pm$ SEM, $n = 14$ – 15 /group.

clarity). There was a significant main effect of Group on ACTH release (Fig. 3A, $F_{(2,24)} = 3.469$, $p < 0.05$) and a Group $\times$ Sample interaction ($F_{(14,168)} = 2.345$, $p < 0.01$). Specifically, CAPS-treated rats displayed a blunted ACTH response to acute stress. Post-hoc analyses revealed that the 1-day post-CAPS group was significantly different from the control group on the 5- and 30-min stress samples, and significantly different from the 5-day post-CAPS group on the 30-min stress sample. The effect on the 5-min stress sample for the 5-day post-CAPS group approached significance ($p = 0.0505$). Thus, the most robust effect of CAPS on the subsequent ACTH response to an acute immobilization stress occurred one day after the CAPS procedure, and by 5 days, the ACTH response was returning toward that seen in control rats. Although CORT levels in the 1-day post-CAPS group were slightly but consistently lower than in controls, this was not a significant reduction (Fig. 3B, $F_{(2,26)} = 1.542$, $p > 0.05$ for main effect of stress; $F_{(14,182)} = 1.228$, $p > 0.05$ for stress $\times$ sample interaction).

The control experiment, conducted to ensure that the blunted ACTH response in CAPS-treated rats was not due simply to habituation to a second exposure to immobilization stress, showed no effect of prior immobilization on ACTH release in response to the test immobilization stress (Fig. 4, $F_{(2,24)} = 1.162$, $p > 0.05$), nor was there a group $\times$ sample interaction ($F_{(14,168)} = 0.871$, $p > 0.05$). Because the effect of CAPS on the ACTH response in experiment 3 was seen only at 1-day post-CAPS, we also analyzed the results of the control experiment for day 1 only, excluding day 5. There was still no effect of prior immobilization ($F_{(1,17)} = 1.161$, $p > 0.05$) nor an interaction ($F_{(7,119)} = 1.224$, $p > 0.05$). Thus, these results suggest that the changes in evoked ACTH response following exposure to CAPS were due to the CAPS treatment specifically, and not due simply to a second exposure to immobilization. Because the effect of CAPS on CORT did not achieve significance in experiment 3, CORT was not analyzed in the control experiment.

4. Discussion

In the present set of studies, CAPS-treated rats displayed a shift from an active to a passive coping style, and an increase in anxiety-related behavior. These behavioral effects co-occurred with a blunted ACTH response to acute stress. In addition to these changes, we have previously reported that CAPS impaired fear extinction and reduced GR expression in the mPFC (Green et al., 2011).

4.1. Passive coping

In our previous report (Green et al., 2011), prenatal stress did not alter the preference for active coping relative to passive coping on the shock-probe defensive burying test (prior to CAPS exposure). However, after CAPS exposure, we noted a different behavioral profile during fear conditioning. After multiple tone–shock pairings, control rats begin to show a decrease in freezing, which appears to be due to a shift in behavioral response to the tone, away from the passive freezing response to more active escape behaviors, including rearing and jumping. In that study, rats exposed to prenatal stress and CAPS as adults did not display this shift. Rather, they continued to display high levels of freezing. While this observation is anecdotal, and behavior observed during fear-conditioning is not a validated measure of coping style, this led us to hypothesize that CAPS might produce a shift from active coping to a more passive coping strategy. This hypothesis was tested explicitly in the present study using the shock probe defensive burying test, in which rats can exhibit 2 qualitatively different types of behavioral responses to the shock probe in varying proportions—an active response (burying the probe) and a passive response (immobility). Control rats displayed a slight preference for active coping behavior (burying), whereas CAPS-treated rats displayed a substantial shift to a strong preference for

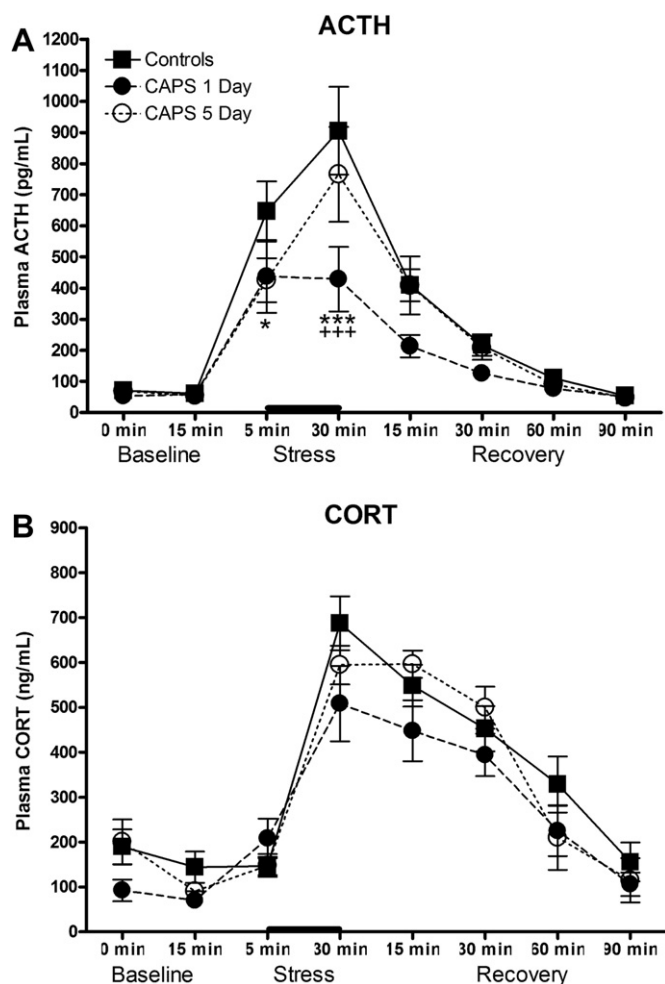


Fig. 3. Effect of CAPS on HPA stress reactivity in response to acute immobilization stress. A) CAPS-treated rats displayed a blunted ACTH response to a subsequent acute stressor (bar), particularly in the group tested on day 1 post-CAPS. B) There were no significant differences between groups on the acute CORT response to immobilization stress, although there was a modest decrease in the group tested on day 1 post-CAPS. * $p < 0.05$ 1-day post-CAPS vs controls, *** $p < 0.001$ 1-day post-CAPS vs controls, +++ $p < 0.001$ 1-day post-CAPS vs 5-days post-CAPS. Data expressed as mean $\pm$ SEM, $n = 7$ –14/group.

passive coping behavior (immobility). The increase in immobility cannot be explained by an overall decrease in locomotor activity, as we previously showed that CAPS-treated rats displayed no change in exploration in an open field (Green et al., 2011).

Coping style can mitigate the physiological impact of stress, and there is evidence from both animal and human research that active coping is more adaptive. Previous research has shown that when a rat is given the option to engage in an active coping response, such as chewing on a dowel during immobilization, the stress response is reduced (e.g., Hori et al., 2004; Ono et al., 2008). By contrast, when a rat is deprived of an active response option, such as removing the bedding during the shock probe test so the rat cannot bury, the physiological stress response is increased (Bondi et al., 2007). Likewise, rats that show a low bury response have higher CORT responses during the test (for review see Koolhaas et al., 1999).

A shift to immobility in the shock probe test resembles the “learned helplessness” phenomenon described in both the human and animal psychological literature. Passive responding and failure to engage in active coping responses has long been demonstrated in a number of animal models, originally in dogs (e.g., Seligman

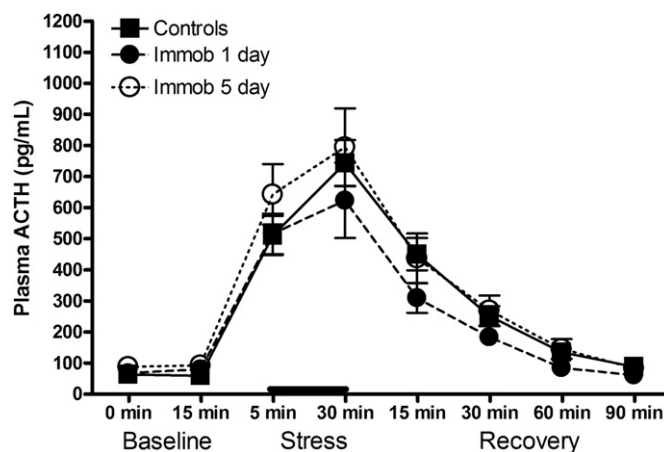


Fig. 4. Lack of effect of a single prior exposure to acute immobilization stress on the subsequent ACTH response to a second immobilization stress. There was no effect of prior immobilization on ACTH release evoked by a second immobilization stress (bar) administered either 1 or 5 days later, suggesting that the effect seen in Fig. 3 was due to CAPS exposure, specifically. Data expressed as mean $\pm$ SEM, $n = 8$ –10/group.

et al., 1968) and then rodents (e.g., Maier, 1984). The learned helplessness model shares some characteristics with the CAPS model. For example, rodents exposed to inescapable tail shock display less aggression in a shock-elicited aggression test (similar to our finding of reduced active burying in the shock-probe defensive burying test) and reduced intruder attack by alpha males (Maier, 1984). These rats also display cognitive deficits, including more errors in tests involving learning contingencies (Maier, 1984) and delayed contextual fear extinction (Baratta et al., 2007). Again, this is similar to our previous finding that CAPS treatment impaired fear extinction.

Passivity may contribute to maladaptive stress responses. Consistent with this, studies with animals and humans have shown that active, stimulus-based, or problem-oriented coping styles, as opposed to more passive, emotion-based, avoidant coping styles, buffer HPA activation in response to the stressor/stimulus, increase the ability to eliminate the threat, and improve long-term mental and physical health outcomes (for review see, Koolhaas et al., 1999; Olf et al., 2005). On the other hand, in humans, the negative thought patterns related to many affective disorders often contribute to the perception that there is no way out of a stressful situation and little or no control over one's situation and environment. Emotional withdrawal is a key symptom in the diagnosis of PTSD (American Psychiatric Association, 2000), and studies have shown that individuals previously exposed to traumatic events show greater levels of introversion, social isolation, and emotional blunting (e.g., Bunce et al., 1995). A predisposition for withdrawal may actually contribute to the development of PTSD, and greater symptom expression over time. For example, traumatized individuals who show a shift toward passive coping styles, including withdrawal, are more likely to develop PTSD at 3 months post-trauma (Gutner et al., 2006). Furthermore, men who were abused as children and display high levels of introversion and withdrawal are more likely to meet thresholds for clinical diagnosis of PTSD in adulthood (O'Leary, 2009). Similarly, individuals who report peritraumatic feelings of helplessness are more likely to develop PTSD (Beck et al., 2006; Hari et al., 2010; O'Donnell et al., 2010).

4.2. Anxiety

We previously examined the effect of CAPS on anxiety-related behavior in an open field and found no differences. In the present experiment, we examined potential anxiogenic effects of CAPS

using a more robust test of anxiety involving an approach-avoidance conflict, the novelty suppressed feeding test. In this test, food deprived rats must enter an anxiety-provoking environment to obtain food. Previous studies with the NSFT have shown that chronic stress results in longer latency to approach the food and begin eating, particularly in more passive, “low responder” rats (Stedenfeld et al., 2011), and that antidepressant treatment reduces the expression of anxiety-related behaviors in this test (Furmaga et al., 2011; Ibarguen-Vargas et al., 2009). Similarly, CAPS-treated rats displayed an increased latency to feed in the novel environment, reflecting greater anxiety, despite an increase in total food intake.

Anxiety is an important component of most animal models of human stress-related psychiatric disorders, as anxiety is a key element of such disorders, including PTSD. Further, PTSD is highly comorbid with other anxiety disorders, and also with depression (Kessler et al., 1995; Rush et al., 2005). These disorders are all notable for an extensive degree of overlap in symptomatology, including, for example, irritable mood, difficulty concentrating, and sleep disturbances. Further, antidepressants are also effective pharmacological treatment for many anxiety disorders (for review, see Morilak and Frazer, 2004). Thus, there are likely to be common neurobiological mechanisms and similar psychopathological processes underlying these shared symptoms.

4.3. Acute HPA stress-reactivity

CAPS treatment reduced the acute ACTH stress response, especially on day 1 after the termination of CAPS. The control experiment confirmed that this was not merely due to habituation to the prior exposure to immobilization stress on day 15 of CAPS treatment. There was also a slight but non-significant suppression of the CORT response to acute stress, and during the post-stress recovery period. It is not clear why the effect of CAPS on the acute CORT response was less robust than on the ACTH response. It may simply be due to differences in the temporal sensitivity of these measures. ACTH is rapidly and dynamically reactive. However, with CORT being slower to respond and slower to clear, a sample at any given time point represents a cumulative response. Thus, differences may have been obscured. On the other hand, it is possible that the adrenal glands may have been sensitized by the previous stress exposure, resulting in greater CORT release in response to ACTH, thus compensating in part for the reduction in evoked ACTH levels. Previous research has shown that chronic stress increases adrenal mass, which may contribute to such sensitization (e.g., Blanchard et al., 1998; Hauger et al., 1990). Another possibility may be related to intensity of the stress induced by immobilization. The HPA response to immobilization was very robust, and may have masked a modest difference between CORT responses in Control and CAPS-treated rats. It may be informative to employ a milder probe stimulus in future studies. Finally, it is important to note that the baseline CORT levels in this experiment were higher than those reported in our previous study (Green et al., 2011). This is likely attributable to differences in methodology. In the present study, rats were exposed to surgery, and then on the test day to handling and a novel environment, all of which can elevate baseline CORT levels, even with a period of acclimation. In the previous study, CORT levels were measured in trunk blood samples collected by rapid decapitation immediately after removal from their home cages.

The changes observed in acute HPA axis stress reactivity are interesting in light of the human PTSD literature. Evidence suggests that HPA activity is reduced in PTSD. However, the full HPA axis profile of individuals with PTSD is not clear, and there are many inconsistencies in the literature (for discussion, see de Kloet et al.,

2006). Some studies have shown urinary and plasma cortisol levels to be lower in PTSD patients compared to controls (Boscarino, 1996; Mason et al., 1986; Yehuda et al., 1995, 1993, 1990), and these hormone levels may be negatively correlated with symptom severity (Olff et al., 2006). Further, individuals with lower CORT levels at the time of the post-trauma emergency room visit are more likely to develop PTSD (Delahanty and Nugent, 2006). Reduced hormonal responses may be due to a sensitized negative feedback mechanism, as PTSD patients tend to show greater ACTH suppression by dexamethasone (e.g., Duval et al., 2004). The present results are in line with these findings.

Few animal models of stress have replicated the HPA-axis characteristics of human PTSD, as the typical effect of chronic stress exposure in rodent models is sensitization of the HPA response to acute stress, if any change is observed at all. Rimanoczy et al. (2003) showed that prenatal stress exposure to morphine resulted in a suppressed ACTH response to restraint in adulthood, while maintaining a normal CORT response, similar to the effect seen in our study. Similarly, the SPS model, from which the acute component of our CAPS model was adapted, enhanced HPA suppression in response to dexamethasone treatment (Yamamoto et al., 2009). Further, rats exposed to SPS also display a blunted CORT response to a subsequent acute stressor (Harvey et al., 2006).

By comparison, varying alterations in ACTH and/or CORT have been reported in studies employing the widely-used Chronic Variable Stress (CVS)/Chronic Mild Stress (CMS) model. Most have shown either no change or an increase in basal ACTH (e.g., Choi et al., 2008a,b; Kioukia-Fougia et al., 2002; Ostrander et al., 2006) and no change or an increase in basal CORT (e.g., Choi et al., 2008a,b; Christiansen et al., 2012; Ostrander et al., 2006; Wu and Wang, 2010). Blunting of circadian cycles has been reported (Christiansen et al., 2012). Changes in HPA response to acute stress challenge after CVS/CMS are variable. One study reported an increased ACTH response to acute restraint stress, but no change in CORT response (Choi et al., 2008b). In another, an increase in ACTH response to a mild novelty stress was seen 1 day after CVS, which returned to normal on day 4 post-CVS, followed by a decrease in ACTH response on day 7, returning to baseline by day 30 (Ostrander et al., 2006). As in the present study (and in Choi et al., 2008b), the CORT response did not match the ACTH response. There was no change in the CORT response one day post-CVS, a decrease at days 4 and 7, then a return to normal by day 30. By contrast, when this same group challenged with a systemic stressor (hypoxia), the effect was similar to that seen in the present study, a decrease in ACTH response one day post-CVS, which returned to normal on day 4, with no change in CORT. ACTH then increased on day 7 post-CVS, again with no comparable change in CORT. Other factors that can affect changes in hormonal response after stress are anhedonia-like traits (Christiansen et al., 2012) and strain differences (Wu and Wang, 2010). In most chronic stress models, regardless of the nature of the change in HPA response, it is important to note that, as in the present study, effects were transient, and changes in ACTH and CORT responses are often dissociated.

These results would suggest that an HPA regulatory process that blunts the ACTH response to a subsequent acute stressor emerges in response to chronic stress, then dissipates over time when the stress ceases. In humans with PTSD, even after termination of the primary stressor, the cognitive process of re-experiencing may become a secondary chronic stressor on its own, maintaining the dysregulatory process. Thus, animal models may be particularly useful in revealing mechanisms by which pathological processes after traumatic stress are initiated, and in identifying unique mechanisms by which HPA responses may be inhibited in PTSD, as opposed to the hyperactive HPA axis often seen in other chronic stress-related mood disorders, such as depression.

Despite the transient effect of CAPS on the HPA response to acute stress, behavioral effects were evident at all time points. We examined the effects of CAPS on shock-probe defensive burying behavior on day 1 post-stress, comparable to when we observed the greatest ACTH suppression. However, the need for food restriction in the novelty-suppressed feeding test, and the desire to avoid confounding stress with food deprivation, necessitated testing on day 5 post-stress. In both cases, at 1 day and 5 days post-stress, we observed behavioral effects of CAPS treatment. In general, then, it appears that although the HPA effects begin to recover by day 5, the behavioral effects are evident at day 1 (increased passive coping in shock probe defensive burying), day 2 (increased freezing in fear conditioning, Green et al., 2011), and still present at day 5 (increased anxiety in NSFT, and impaired fear extinction, Green et al., 2011).

4.4. Conclusion

Valid animal models of human psychopathology must be based on a theoretical framework that shares a fundamental aspect of the human disorder, and they must show behavioral and biochemical features that resemble those in the human disorder (Willner, 1986). One requirement for a diagnosis of PTSD is experience of a traumatic event (American Psychiatric Association, 2000). This was modeled by the CAPS treatment in the present study, involving a low-level chronic “state” of stress, followed by a highly salient and intense acute stress experience, which may model the kinds of experiences that initiate PTSD, particularly in combat veterans. Chronic stress is correlated with vulnerability to PTSD (Breslau et al., 1999; Koenen et al., 2007, 2002), and in combat situations, chronic stress, punctuated by acute traumatic events, is the norm. Further, once the trauma has been experienced, an exaggerated and persistent fear response is arguably the fundamental aspect of PTSD (American Psychiatric Association, 2000), and this may be prolonged by impairments in extinction learning (Blecher et al., 2007; Wessa and Flor, 2007; Jovanovic et al., 2009). In our previous report, we showed that CAPS exposure enhanced freezing during fear conditioning and impaired extinction. Further, in the present study, CAPS resulted in other PTSD-like symptoms, including: anxiety; a shift from effective active coping to less adaptive passive coping; and HPA axis dysregulation.

The brain mechanisms that underlie these effects remain to be elucidated. CAPS is a combination of chronic metabolic stress (chronic cold) and a single session of intense acute stress that was adapted from the single-prolonged stress model (SPS; Yamamoto et al., 2009, 2010). Each of these components may have neurobiological consequences that contribute to the resulting phenotype. SPS has been shown to increase inhibitory avoidance, decrease extinction, and increase acoustic startle (Yamamoto et al., 2010; Ganon-Elazar and Akirav, 2012; Knox et al., 2012). This may be due, in part, to reduced excitatory neurotransmitter tone in the PFC and hippocampus, as SPS decreased glutamate and creatine in the PFC, and increased glycine transporter expression in the ventral hippocampus (Yamamoto et al., 2010; Knox et al., 2010). Chronic cold has been shown to impair cognitive flexibility and to decrease serotonin release in the orbital frontal cortex (Lapiz-Bluhm et al., 2009). Changes in prefrontal executive function could compromise the ability to regulate or select from among possible responses in fear- or anxiety-provoking situations. Chronic cold stress alone has been shown to sensitize the ACTH response to immobilization stress (Ma and Morilak, 2005), whereas SPS increased negative feedback inhibition of ACTH release (Liberzon et al., 1997), similar to the blunted ACTH response in the present study. Thus, the phenotype of CAPS-treated rats appears to be a combination of acute and chronic stress effects, perhaps involving changes in modulatory

neurotransmission in the prefrontal cortex, consistent with our previous observations of altered GR expression following CAPS treatment (Green et al., 2011). This further suggests that drugs that modulate monoaminergic transmission, glucocorticoid activity, or excitatory amino acid signaling may represent viable strategies for treatment and symptom management of PTSD. Interestingly, it was recently reported that the SPS-induced increase in glycine transporter expression in the hippocampus was normalized with repeated extinction training, perhaps identifying a mechanism by which therapeutically effective behavioral interventions can also mitigate the effects of chronic stress (Yamamoto et al., 2010).

In sum, the CAPS model may prove useful as a valid animal model with which to investigate neurobiological mechanisms underlying pathophysiological changes associated with PTSD, or mechanisms of novel therapeutic strategies for PTSD.

Acknowledgments

This work was supported by a NIMH National Research Service Award individual postdoctoral fellowship F32 MH090693 (MKR), NIMH research grant R01 MH053851 (DAM), Department of Veterans Affairs Office of Research and Development (AF, RS), and by funding provided to the STRONG STAR Multidisciplinary PTSD Research Consortium by the Department of Defense through the U.S. Army Medical Research and Materiel Command, Congressionally Directed Medical Research Programs, Psychological Health and Traumatic Brain Injury Research Program award W81XWH-08-2-0118. The views expressed in this paper are solely those of the authors and do not reflect an endorsement by or official policy of the Department of Defense or the U.S. Government.

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Poster

190. Early Life Experience: Prenatal

Location: Hall A-C

Time: Sunday, November 13, 2011, 8:00 am - 12:00 pm

Program#/Poster#: 190.09/UU4

Topic: E.05. Stress and the Brain

Support: Department of Defense CDMRP PH/TBI award W81XWH-08-2-0118 to the STRONGSTAR PTSD Research Consortium

Title: Potential role of corticosterone in the long lasting effects of prenatal stress

Authors: *B. C. BINGHAM, S. KADAPAKKAM, R. STRONG, D. MORILAK; Pharmacol., Univ. of Texas Hlth. Sci. Center- San Antonio, San Antonio, TX

Abstract: Early-life stress is a risk factor for the development of cognitive and emotional disorders later in life. We have previously demonstrated long-term, stable changes in central stress-responsive systems that have been implicated in such disorders, most notably the HPA axis and the brain noradrenergic system, in adult rats that had been exposed to prenatal stress (PNS). Specifically, PNS increased basal corticosterone (CORT) secretion, reduced glucocorticoid receptor (GR) protein levels in prefrontal cortex and hippocampus, and decreased expression of tyrosine hydroxylase (TH) mRNA in brainstem noradrenergic neurons, associated with increased vulnerability to the behavioral effects of a subsequent adult stress. Other PNS models suggest that CORT released during PNS may be a causal agent in the long-term dysregulation of these systems. Thus, in this study, we investigated the potential role of CORT in PNS-induced alterations in the stress response systems of the adult brain. In experiment 1, we first established a dose of CORT that produces plasma CORT levels in both the dams and fetuses that approximated those achieved during acute immobilization stress (1 hr/day from prenatal day E14 until birth). Acute immobilization increased plasma CORT in both the dams (1056 ng/ml) and fetuses (573 ng/ml). This response did not habituate over the week of daily stress treatment. Comparable CORT levels were measured in the fetuses 1 hr after acute administration of 5 mg/kg CORT (s.c.) to the mothers. Then, in experiment 2, pregnant Sprague-Dawley rats were either immobilized or given a daily injection of CORT (5 mg/kg, s.c.) instead of stress. The offspring were allowed to grow to adulthood undisturbed, at which point they were sacrificed and brain regions dissected, including the medial prefrontal cortex (PFC), dorsal hippocampus (Hpc), and a section of the rostral pons containing the locus coeruleus (LC). Both PNS and prenatal CORT treatment decreased GR protein levels in the adult PFC, and CORT also reduced GR levels in Hpc, compared to unstressed vehicle controls. However, by contrast with PNS, prenatal CORT treatment increased TH mRNA expression in the adult LC. Thus, these data suggest that CORT may be responsible for some of the lasting neurobiological effects of PNS, especially with regard to HPA dysregulation. However, other factors engaged by PNS appear more likely to affect the development and dysregulation of the stress-responsive brain noradrenergic system. Further studies, including selective blockade of CORT activity during PNS, are underway to further establish the functional role of CORT in the lasting neurobiological and behavioral sequelae of PNS.

Disclosures: B.C. Bingham: None. S. Kadapakkam: None. R. Strong: None. D. Morilak: None.

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Poster

190. Early Life Experience: Prenatal

Location: Hall A-C

Time: Sunday, November 13, 2011, 8:00 am - 12:00 pm

Program#/Poster#: 190.06/UU1

Topic: E.05. Stress and the Brain

Support: NIMH NRSA Post-Doctoral Fellowship F32MH090693

DoD CDMRP PH/TBI award W81XWH-08-2-0118 to the STRONGSTAR PTSD Research Consortium

Title: Effects of prenatal stress and combined chronic plus acute adult stress on anxiety-like behavior and evoked hpa axis activity in rats

Authors: *M. K. GREEN, A. JOSHI, A. SHAH, A. FRAZER, J. R. STRONG, D. A. MORILAK;
Pharmacol., Univ. Texas Hlth. Sci. Ctr. San Antonio, SAN ANTONIO, TX

Abstract: Stress is a risk factor for the development of affective disorders. However, not all individuals who experience either chronic or traumatic acute stress develop an affective disorder. Thus, other factors must confer a vulnerability to stress. We have previously shown that exposing rats to prenatal stress (PNS) alters the subsequent response to a severe chronic stressor in adulthood, increasing the expression of fear and impairing the extinction of conditioned fear. Further, PNS stably decreased the expression of tyrosine hydroxylase in the brainstem, elevated basal corticosterone secretion, and reduced glucocorticoid receptor expression in the medial prefrontal cortex and hippocampus. In the present study, we examined the influence of PNS as a vulnerability factor in producing stable changes in anxiety-like behavioral stress reactivity and evoked HPA axis activity after exposure to a model of traumatic adult stress. For prenatal stress, pregnant Sprague-Dawley rats were immobilized for 1 hr daily during the last week of pregnancy, and their male offspring were studied as adults. Half of the adult PNS-treated or control rats were then exposed to a combined chronic plus acute prolonged stress (CAPS) treatment, consisting of chronic intermittent cold stress (4°C, 6hrs/day, 14 consecutive days) followed on day 15 by a single 1 hr session of sequential acute stressors (social defeat, immobilization, cold swim). After CAPS or control treatment, anxiety-like behavior was assessed in different groups by measuring novelty suppressed feeding (NSF) behavior, open-arm exploration on an elevated plus maze (EPM, including both basal exploration and the acute response evoked by a brief immobilization stress), and ACTH and corticosterone release evoked in response to acute immobilization stress. CAPS increased the latency to feed in the NSF test, without affecting the amount of food consumed. The addition of PNS further enhanced this effect, although PNS had no effect on its own. In the EPM test, acute immobilization stress prior to testing reduced open arm exploration. However, neither PNS nor CAPS had an effect on their own, nor did they interact with the acute stress exposure to alter the evoked response on the EPM. CAPS treatment alone reduced the magnitude of the HPA response evoked by an acute immobilization stress. These results and those reported previously suggest that PNS and CAPS have independent and sometimes different effects on fear, extinction, anxiety and activity of the HPA axis. Further, for some but not all measures, PNS acts as a vulnerability factor, altering the basal status of central stress response systems and enhancing the effects of adult CAPS exposure.

Disclosures: M.K. Green: None. A. Joshi: None. A. Shah: None. A. Frazer: None. J.R. Strong: None. D.A. Morilak: None.