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Post-Traumatic Stress Avoidance is Attenuated by Corticosterone and Associated with Brain Levels of Steroid Receptor Co-Activator-1 in Rats

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Abstract

Individuals with post-traumatic stress disorder (PTSD) avoid trauma-related stimuli and exhibit blunted hypothalamic-pituitary-adrenal (HPA) axis activation at the time of stress. Our laboratory has established a rodent model of stress that mimics the avoidance symptom cluster of PTSD. Rats are classified as ‘Avoiders’ or ‘Non-Avoiders’ post-stress based on avoidance of a predator-odor paired context. Previously, we demonstrated that Avoiders exhibit an attenuated HPA stress response to predator odor. We hypothesized that corticosterone administration prior to stress would reduce magnitude and incidence of avoidance of a stress-paired context. Furthermore, we predicted that Avoiders would exhibit altered expression of GR signaling machinery elements, such as steroid receptor co-activator (SRC)-1. Male Wistar rats ($n = 16$) were pre-treated with corticosterone (25 mg/kg) or saline and exposed to predator odor stress paired with a context, and tested for avoidance 24 h later. A second group of corticosterone-naïve rats ($n = 24$) were stressed (or not stressed), indexed for avoidance 24 h later, and killed 48 h post-odor exposure for analysis of phosphorylated GR, FKBP51, and SRC-1 levels in the paraventricular nucleus (PVN), central amygdala (CeA) and ventral hippocampus (VH), all brain sites that express high quantities of GRs and regulate HPA function. Rats pre-treated with corticosterone exhibited lower magnitude and incidence of avoidance. Predator odor exposure also reduced SRC-1 expression in the PVN and CeA of Avoiders, and increased SRC-1 expression in the VH of Avoiders. SRC-1 expression in PVN, CeA, and VH was predicted by prior avoidance behavior. These results suggest that blunted HPA stress response may contribute to stress-induced neuroadaptations in central SRC-1 levels and behavioral dysfunction in Avoider rats.

Keywords

Central amygdala; paraventricular nucleus; predator odor; ventral hippocampus

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Conflicts of Interest:

Dr. Gilpin is a consultant for Glauzer Life Sciences, Inc.

Introduction

Post-traumatic stress disorder (PTSD) manifests in a subset of individuals exposed to a traumatic stressor (1). A PTSD diagnosis is defined by presentation of three symptom clusters including intrusive recollection, hyper-arousal, and avoidance of trauma-related stimuli. Important for the present study, not all individuals that are exposed to trauma develop PTSD, a criterion for animal models of this disorder (2).

Stress-related disorders are often accompanied by dysregulation of the hypothalamic-pituitary-adrenal (HPA) stress axis, both at the time of stress and after the development of PTSD (3). While the paraventricular nucleus (PVN) is essential for appropriate initiation and termination of the stress response, the limbic system can also influence the HPA axis (4–6). Limbic forebrain structures such as the central amygdala (CeA) and ventral hippocampus (VH) regulate HPA responses to emotional stress (6). Like the PVN, the CeA and VH are rich in glucocorticoid receptors (GR) (5, 6). Extreme hyper- or hypo-reactivity of the HPA axis can be maladaptive and can contribute to the development of psychiatric disorders that include depression and PTSD (7, 8). Individuals with PTSD exhibit blunted HPA activity immediately after the traumatic event (9, 10), blunted basal 24 h urinary cortisol concentrations collected at 9 am and enhanced negative feedback post-stress (11). Previously, we reported that rats that exhibit persistent (>6 weeks) avoidance of a predator odor-paired context exhibit significantly attenuated ACTH and corticosterone levels immediately following traumatic stress and that low corticosterone at the time of stress is highly predictive of later avoidance (12).

Full GR activation at the transcriptional level requires numerous accessory proteins. For example, the FK506-binding protein-51 (FKBP51; *fkbp5* gene) regulates HPA negative feedback by preventing nuclear translocation of the GR complex (13). Humans with PTSD exhibit attenuated peripheral *fkbp5* gene expression, suggesting that FKBP51 may represent a promising target for treatment of traumatic stress disorders (13–15). Additionally, transcriptional co-regulators such as steroid receptor co-activator-1 (SRC-1) are involved in gating the transcriptional activity of nuclear receptors. SRC-1 is highly expressed in the brain and regulates GR-mediated corticotropin releasing factor (CRF) gene transcription (16). Finally, GR phosphorylation at various sites regulates GR trafficking and transcriptional activity. GR phosphorylation is initiated by glucocorticoid binding to the receptor, and is also modulated by the activity of various kinases (17, 18): specifically, GR phosphorylation on serine 232 facilitates GR nuclear trafficking and transcriptional activity (18, 19).

The purpose of these studies was two-fold. Because corticosterone/cortisol levels are blunted in Avoider rats and PTSD humans shortly after stress, we first aimed to examine the effect of corticosterone treatment on avoidance of a context paired with predator odor. We hypothesized that administration of corticosterone prior to stress would increase HPA activity at the moment of the stressor and decrease the magnitude and incidence of avoidance of a predator odor-paired chamber. We also wanted to measure the expression of GR elements in the brains of Avoider rats because altered corticosterone levels may affect HPA feedback processes. We hypothesized that predator odor stress would alter expression

and/or phosphorylation of GR machinery in a brain region-specific manner in Avoiders relative to Non-Avoiders and unstressed Controls.

Methods

Animals

Adult male Wistar rats (Charles River, Kingston, NY) (250–275 g) were pair-housed and fed a standard rat diet (Purina Rat Chow, Ralston Purina, St. Louis, MO) *ad libitum*. Rats were exposed to a 12h light/12h dark cycle (lights off at 8am). Rats were handled on a daily basis and given one week to acclimate to the colony room prior to initiation of experimental procedures. A total of 40 animals were used in these studies (Experiment 1 n = 8 vehicle-treated n = 8 corticosterone-treated; Experiment 2: n = 6 controls, n = 8 Non-avoiders, n = 12 Avoiders). Animal procedures were approved by the Institutional Animal Care and Use Committee of the Louisiana State University Health Sciences Center (LSUHSC) and were in accordance with the National Institute of Health guidelines.

Conditioned Place Aversion

Rats were exposed to predator odor and indexed based on avoidance of the predator-odor paired context as previously described by our laboratory (20). A conditioned place aversion (CPA) paradigm was utilized in which predator odor was paired with a distinct environment. The conditioning apparatus consisted of two boxes (36 cm length × 30 cm width × 34 cm height) separated by a small triangle. One conditioning chamber had circle visual cues on the walls and circle tactile cues on the floor. The other chamber had striped visual cues on the walls and grid tactile cues on the floor. Guillotine doors between chambers confine animals to individual chambers on neutral and odor conditioning days, but are removed to allow free exploration of both chambers during pre- and post-conditioning tests. Briefly, rats were subjected to a 5-minute pre-test to explore two distinct chambers differing in visual and tactile cues. On the second day, rats were confined to one chamber (neutral environment) for 15 min. On the third day, rats were confined to a second chamber for 15 min with a bobcat (*Lynx rufus*; Maine Outdoor Solutions; Hermon, Maine) urine-soaked sponge placed under the floor of the chamber. On the fourth day, rats underwent a 5 min video-recorded post-test in which they were allowed to freely explore the two chambers. Following odor exposure, the chambers were cleaned with Quatricide and the room deodorized with Pure Ayre. Control rats were treated identically to odor-exposed rats but the sponges did not contain bobcat urine. Both rats in a single cage experienced the same treatment on the same day. Avoidance was calculated as a difference in time between the postconditioning time spent in odor-paired context and pre-conditioning time spent in odor-paired context. Rats were classified as 'Avoiders' if they displayed a >10 sec decrease in time spent in odor-paired chamber (20).

Drugs

In Experiment 1 (see below), 1 hour before predator odor exposure, rats were injected subcutaneously with either saline (NaCl: 0.9%) or corticosterone (25 mg/kg), a dose previously reported to reduce anxiety-like behavior following stress (21). Corticosterone

acetate (Sigma Aldrich, St. Louis, MO) was prepared by sonication in 1% Tween 80 solution in normal saline and injected in a volume of 2 ml/kg.

Western Blot

We measured individual levels of total GR, phosphorylated GR (serine 232), FKBP51, and SRC-1 in brain regions involved in HPA regulation including the CeA (AP: -1.92, ML: 4.4, DV: 8.2), PVN (AP: -1.72, ML: 0.0, DV: 8.5), and VH (AP: -5.04, ML: -5.2, DV: 7.2). Rats were sacrificed under light isoflurane anesthesia followed by decapitation. Brains were quickly excised and flash frozen in cold isopentane. Regional brain tissue punches were obtained from frozen coronal brain slices (500 μ m) using 14–17 gauge punch needles guided by The Rat Brain Atlas of Paxinos and Watson (22). Brain punches were homogenized as previously described (Edwards et al., 2013) by sonication in lysis buffer (1% SDS solution containing sucrose, HEPES, EGTA, and EDTA) containing Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktails II and III (Sigma, St. Louis, MO). Following homogenization, samples were heated for 5 minutes at 100°C and protein content was measured using the Lowry method (Bio-Rad, Hercules, CA). Samples were stored in 10 μ g aliquots at -80 °C until Western analysis. Protein samples (10 μ g) were subjected to SDS-polyacrylamide gel electrophoresis on 8% acrylamide gels by using a Tris/Glycine/SDS buffer system (Bio-Rad), followed by electrophoretic protein transfer to polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Piscataway, NJ, USA). Membranes were blocked for 1 hour in 5% non-fat milk at room temperature. The following day, membranes were incubated overnight at 4 °C in primary antibody (see Table 1 for primary antibody concentrations) in 2.5% non-fat milk recognizing the glucocorticoid receptor phosphorylated at rat serine 232 (Antibody #4161; Cell Signaling, Danvers, MA, USA), total GR (Antibody #: PA1-511A ThermoFisher, Waltham, MA) FKBP51 (Antibody #: 46002; AbCam; Cambridge, MA) or SRC-1 (SRC-1 (128E7) Rabbit mAb #2191; Cell Signaling Danvers, MA, USA) (23–25). Membranes were washed and labeled with species-specific, peroxidase-conjugated secondary antibody (1:10K; Bio-Rad) for 1 h at room temperature. Blots were then treated with chemiluminescent substrate (SuperSignal West Pico; Thermo Scientific, Rockford, IL, USA) for 30 seconds, and exposed to autoradiography film (Denville Scientific Inc., Metuchen, NJ) for optimum amount of time in a Hypercassette (Amersham Biosciences, Buckinghamshire, England). The exposed autoradiography film was developed using Konica Medical Film Processor, model: SRX-101A. Following chemiluminescence detection (SuperSignal West Pico; Thermo Scientific, Rockford, IL, USA), blots were stripped for 20min at room temperature (Restore; Thermo Scientific) and were re-probed with tubulin (1:100K; Santa Cruz Biotechnology; Santa Cruz, CA) for normalization purposes. Developed films were scanned as tiff files and the protein bands were quantified using Image J.

Experimental Protocols

All rats were subjected to one week of handling procedures prior to initiating the experimental protocols. Rats were conscious and unrestrained for the duration of experiments. All experimental procedures were conducted at 9 am near the start of the dark cycle.

Experiment 1. Effect of corticosterone pre-treatment on avoidance—

Previously, our laboratory has demonstrated that Avoider rats exhibit blunted levels of corticosterone following predator odor exposure (26). This study aimed to assess whether corticosterone administration prior to predator odor exposure would impact the magnitude and incidence of avoidance (Fig. 1). Rats were subjected to the CPA paradigm described above. On neutral conditioning day, all rats received a vehicle injection one hour prior to conditioning. On the day of predator odor exposure, rats were randomly assigned to “vehicle” (n=8) or “corticosterone” (n=8) groups. Subcutaneous injections of either vehicle or corticosterone were administered one hour prior to predator odor exposure. Rats were indexed for avoidance 24 hours post-stress and classified as ‘Avoiders’ or ‘Non-Avoiders’.

Experiment 2. Effects of predator odor stress on levels of central glucocorticoid receptor (GR)-related proteins—The aim of this study was to characterize the effects of predator odor stress on changes in total GR, phosphorylated GR (pGR), FKBP51, and SRC-1 protein levels in the PVN, CeA, and VH (Fig. 1). Rats (n = 24) were subjected to the CPA paradigm described above. Rats were indexed for avoidance 24 hours post-stress and classified as ‘Avoiders’ or ‘Non-Avoiders’. Forty-eight hours post-stress rats were sacrificed under light isoflurane anesthesia by decapitation. The PVN, CeA, and VH were isolated from coronal brain slices (500µm) using 14–17 gauge punches for subsequent determination of protein levels via Western analysis. Brain punches were analyzed separately for each rat.

Statistical Analysis

Data are shown as mean \pm SEM with the number of rats indicated in the figure legends. Data were analyzed using Sigma Stat statistical software. In Experiment 1, avoidance data in Avoiders and Non-Avoiders were analyzed with a t-test. Population distributions were analyzed by Chi-squared test. In Experiment 2, molecular data were analyzed by one-way ANOVA. Pearson’s correlations were used to determine relationships between behavioral and molecular outcomes. *Post hoc* analysis with Student Neuman–Keuls test was used when appropriate. In all experiments, statistical significance was set at $p < 0.05$.

RESULTS

Corticosterone pre-treatment decreases avoidance

Rats were indexed for avoidance 24 h post-odor exposure. Figure 2 shows the distribution of Non-Avoiders and Avoiders following pretreatment with either saline (Figure 2A) or corticosterone (Figure 2B) 1 h prior to odor exposure. A lower percentage (25%) of rats pre-treated with corticosterone prior to stress were classified as Avoiders, $\chi^2(1, N = 16) = 4.00$, $p = 0.045$ relative to saline-treated rats (75% Avoiders). Figure 2C shows that significantly fewer rats pre-treated with corticosterone exhibit significantly less avoidance of the predator odor-paired context when compared to saline-treated rats, $t(26) = 4.837$, $p < 0.001$, and that corticosterone-treated Avoiders exhibit less extreme avoidance scores than vehicle-treated Avoiders.

Predator odor stress decreases SRC-1 protein levels in the PVN

The goal of Experiment 2 was to examine the effect of predator odor stress on total GR, phosphorylated GR (P-GR), FKBP51, and SRC-1 levels in Non-Avoiders and Avoiders in brain regions that receive HPA feedback and regulate HPA axis activity.

In the PVN, there were no differences in GR, pGR, or FKBP51 protein levels (Figure 3A-3C). As shown in Figure 3D, a one-way ANOVA yielded a significant effect of group (stress history), $F(2, 16) = 3.593, p = 0.05$, on SRC-1 content in the PVN. There was also a trend ($p=0.06$) toward a decrease in SRC-1 in Avoiders when compared to Non-Avoiders. This effect was more clearly resolved when we examined individual associations between PVN SRC-1 levels and avoidance of the predator odor stress-paired context. Figure 3E shows that PVN SRC-1 was strongly and negatively correlated with avoidance in all stressed rats ($r^2 = 0.58; p = 0.006$).

In the CeA, there were no differences in GR or pGR protein levels (Figure 4A-4B). As shown in Figure 4C, a one-way ANOVA yielded a significant effect of group (stress history), $F(2, 27) = 5.425, p = 0.01$, on FKBP51 protein levels in the CeA. Post-hoc analysis revealed a significant decrease in FKBP51 protein content in Avoiders when compared to control rats ($p = 0.01$). As shown in Figure 4D, a one-way ANOVA also yielded a significant effect of group (stress history), $F(2, 23) = 4.378, p = 0.03$, on SRC-1 protein levels in the CeA. *Post hoc* analysis revealed a significant decrease in SRC-1 protein in Avoiders when compared to Non-Avoiders ($p = 0.02$). In accordance with this group effect, Figure 4E shows that CeA SRC-1 content was trended to negatively correlated with avoidance in all stressed rats ($r^2 = 0.22; p = 0.06$), an effect just short of statistical significance.

In the VH, there were no differences in GR, pGR, or FKBP51 protein across groups post-stress (Figure 5A-5C). As shown in Figure 5D, a one-way ANOVA yielded a significant effect of group (stress history), $F(2, 19) = 7.502, p = 0.005$, on SRC-1 protein levels in the VH. *Post hoc* analysis revealed a significant increase in SRC-1 protein in Avoiders when compared to Control rats ($p = 0.004$) and Non-Avoiders ($p = 0.02$). Figure 5E shows that VH SRC-1 content was strongly and positively correlated with avoidance in all stressed rats ($r^2 = 0.54; p = 0.002$).

DISCUSSION

Here, we report that corticosterone administration prior to stress decreases the magnitude and incidence of avoidance of a predator odor-paired chamber. Furthermore, we report that predator odor stress differentially regulates GR machinery in Avoiders relative to Non-Avoiders and unstressed Controls. Previous findings from our laboratory indicate that Avoiders exhibit markedly attenuated circulating ACTH and corticosterone concentrations immediately following predator odor stress (12). Interestingly, blunted ACTH/corticosterone levels at the time of stress predict subsequent avoidance, suggesting that HPA dysregulation may play a role in the later emergence of stress-related symptoms. In the current study, pre-treatment with corticosterone decreased the percentage of rats that met the Avoider criterion and reduced the magnitude of avoidance in rats that continued to show avoidance. Because GR signaling is implicated in stress-related psychiatric disorders, we examined changes in

phosphorylated and total GR levels, as well as alterations in other GR co-factors such as FKBP51 and SRC-1, which are important for nuclear translocation and subsequent activation of GR target genes. Our analysis focused on brain regions critical for glucocorticoid regulation of central and systemic stress signaling including the PVN, CeA, and VH. Of the targets investigated, we observed altered SRC-1 levels in the PVN, CeA, and VH of Avoider rats. These data suggest that attenuated SRC-1 activity in the PVN and CeA and augmented SRC-1 function in the VH may contribute to stress-related behaviors. This is especially interesting because SRC-1 regulates CRF gene expression

Prior studies report inconsistent cortisol levels in individuals with PTSD, but the general consensus is that PTSD humans exhibit blunted cortisol response to traumatic stress and lower basal levels following stress (9, 10, 27). Early studies by Mason et al. reported significantly lower 24 hour urinary cortisol levels measured at 9 am in veterans with PTSD relative to veterans without PTSD (28). This study was later confirmed by others examining cortisol changes during the diurnal cycle showing overall lower cortisol levels (during the afternoon (12–8 pm) in women with PTSD (29, 30). Previously, we reported that rats that exhibit persistent avoidance of a predator odor-paired chamber display lower ACTH and corticosterone levels immediately following stress.

Collectively, these data suggest that cortisol-based therapy prior to or following stress exposure may decrease susceptibility to the eventual development of PTSD. Clinical studies have shown that administration of low-dose cortisol following stress reduces symptoms associated with PTSD, including avoidance (31). Additional studies have reported that administration of glucocorticoids as part of a treatment for septic shock decreases the development of PTSD, in particular through decreasing traumatic recollections (11, 31–33). Here, we report that corticosterone pre-treatment prior to predator odor exposure decreases the percentage of rats classified as “Avoiders” and significantly reduces avoidance of the predator odor-paired chamber. Although corticosterone itself can be reinforcing (34) it was previously shown to be incapable of producing a place preference (35). These data suggest that low corticosterone levels in Avoiders post-stress reflect blunted HPA response to stress, although this does not preclude the possibility that negative feedback is also altered in Avoider rats. One potential mechanism for this blunted HPA stress response is altered balance of excitatory and inhibitory inputs to parvocellular neurons in the PVN, a focus of ongoing studies.

Recent evidence suggests that glucocorticoids affect memory consolidation and may be a factor in the decreased avoidance following corticosterone treatment. The effects of stress on memory consolidation largely depend on the timing of the stress exposure and/or glucocorticoid administration. Stress exposure prior to learning can result in both enhanced (36, 37) and impaired (38, 39) memory consolidation. Studies have shown that glucocorticoid administration within a short time frame *after* learning will facilitate memory formation (39). In our studies, pretreatment with corticosterone decreased avoidance behavior. It is possible that reduced avoidance reflects impaired memory following corticosterone treatment. In addition to timing of stress exposure, other factors such as the timing of glucocorticoid treatment, when testing occurs and how aversive the task is may contribute to the discrepancies among the findings. For example, studies have shown that

post-training corticosterone treatment increases memory consolidation in a water-maze spatial task but impairs memory consolidation when the task becomes more aversive (40). Future studies will examine how glucocorticoids affect memory consolidation in Avoiders and Non-Avoiders using traditional fear conditioning procedures.

While the HPA axis is controlled primarily by the PVN, upstream limbic circuitry (e.g., amygdala and hippocampus) involved in emotional processing and memory formation can also influence the HPA stress response (5). For example, electrical stimulation of the amygdala increases circulating corticosterone concentrations, suggesting positive feed-forward regulation of the HPA axis (6, 41, 42). Conversely, the hippocampus is a classic negative feedback site that inhibits HPA axis activity (4, 6, 43): hippocampal lesions increase corticosterone levels following stress (43, 44). More specifically, the ventral hippocampus is the primary mediator of hippocampal inhibition of HPA axis activity (6, 43). Because the PVN, CeA, and VH each express high levels of glucocorticoid receptors (6), we examined predator odor stress effects on GR machinery elements of these brain regions in Avoider and Non-Avoider rats.

Individuals with PTSD exhibit enhanced negative feedback as evidenced by greater glucocorticoid suppression following a dexamethasone suppression test (de Kloet et al., 2008). Here, we examined predator odor stress effects on proteins mediating GR function in PVN, CeA, and VH of Avoider and Non-Avoider rats relative to unstressed Controls. FK506-binding protein of molecular weight 51 (FKBP51) is part of a protein complex that controls cellular localization of GRs. Binding of FKBP51 to HSP90 of the GR complex prevents nuclear translocation, GR phosphorylation, and subsequent transcription of target genes (15). Attenuated FKBP51 expression in circulating peripheral blood mononuclear cells is associated with enhanced glucocorticoid-mediated negative feedback, which is consistent with enhanced HPA negative feedback mechanisms in individuals with PTSD (13, 15). Furthermore, four single nucleotide polymorphisms (SNPs) in the *fkbp5* gene are associated with risk for developing PTSD. Here, we observed a significant decrease in FKBP51 protein levels in the CeA of Avoider rats when compared to Controls, but no changes in FKBP51 in the PVN or VH.

An additional co-regulator important for GR-mediated gene transcription is SRC-1 (16, 45). SRC-1 is highly expressed in the brain (46). SRC-1 is involved in CRF gene expression in response to glucocorticoids and stress-related stimuli and may play a role in the differential HPA response in Avoiders and Non-Avoiders (16). SRC-1 negatively regulates CRF gene expression in the hypothalamus but positively regulates gene expression in the CeA. This distinction potentially results from differential expression of SRC-1 splice variants, SRC-1a and SRC-1e (16). Studies by de Kloet et al. demonstrate that the PVN, CeA, and VH all contain both variants, but in different amounts. The PVN has high expression of SRC-1a (a negative GR regulator) and very low, or even undetectable, expression of SRC-1e (a positive GR regulator), while the CeA and VH both contain relatively equal expression of each (46). Thus, selective activation of these splice variants may lead to distinct GR-mediated effects on CRF gene expression in these regions, even in the absence of changes in nuclear pGR or GR levels. Here, we show differential protein levels of total SRC-1 in the PVN, CeA, and VH of rats that exhibit avoidance following stress. In the PVN and CeA, we report decreased

SRC-1 levels in Avoiders that are negatively correlated with avoidance (in all stressed rats). In contrast, we find increased SRC-1 levels in the VH in Avoiders that are positively correlated with avoidance (in all stressed rats). While the SRC-1 antibody we employed does not distinguish between 1a and 1e isoforms, our results suggest that stress alters central SRC-1 expression in stress-susceptible individuals, perhaps as a result of the initial blunted HPA response to traumatic stress (47). These results are in partial agreement with past findings that acute restraint stress decreases SRC-1 protein levels in the hypothalamus of both male and female rats but increases SRC-1 levels in the hippocampus of female rats only (Lachize et al., 2009). Future studies will examine differences in SRC-1a and SRC-1e isoforms in these brain regions and their respective contributions to post-stress behavioral change in Avoiders and Non-Avoiders.

In summary, the present study reports that corticosterone pre-treatment prior to predator odor stress decreases avoidance associated of a stress-paired context. Additionally, co-activators and co-factors involved in GR translocation and gene transcription are altered in brain regions critical for HPA axis regulation, including the PVN, CeA, and VH. Changes in SRC-1, in particular, in these regions were highly predictive of avoidance behavior, identifying a potential link between abnormal HPA responses to stress and subsequent stress-related behavioral pathology. Future studies will more precisely characterize SRC-1 mediation of post-stress behaviors in Avoiders vs. Non-Avoiders and unstressed controls.

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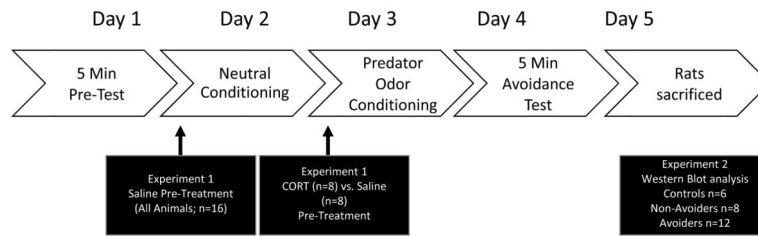


Figure 1.

Schematic of Experimental Design. Rats underwent the conditioned place aversion (CPA) paradigm to index for avoidance of predator odor-paired chamber. In Experiment 1, all rats received a subcutaneous vehicle injection prior to neutral conditioning. Rats were randomized to receive either vehicle or corticosterone pre-treatment prior to odor exposure. In Experiment 2, rats did not receive injections prior to conditioning and were sacrificed at 48 h post-stress for biochemical analysis.

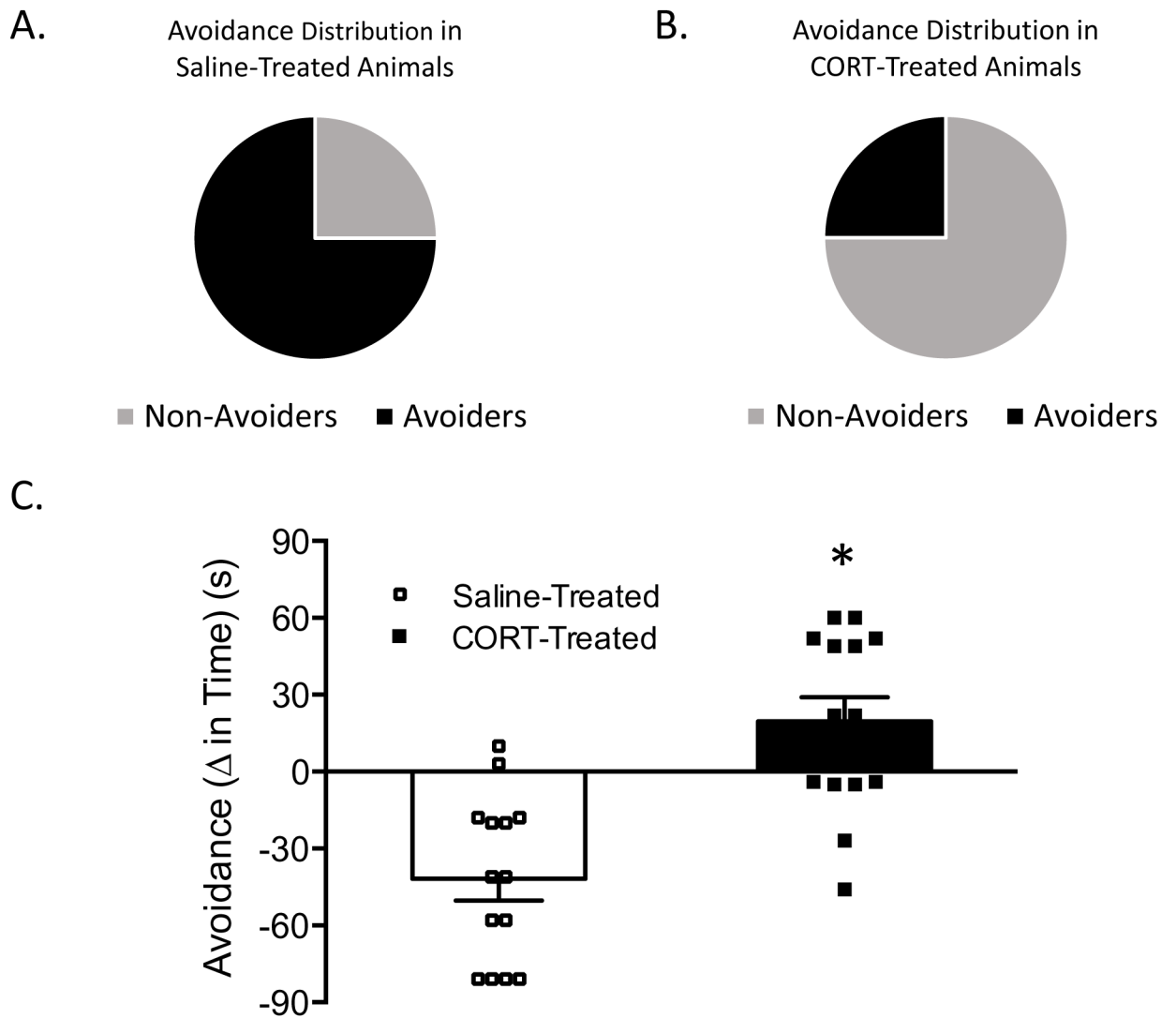


Figure 2.

A. Avoidance distribution in Saline-treated rats. Non-avoiders represented in grey and Avoiders represented in black. **B.** Avoidance distribution in corticosterone-treated rats. Non-avoiders represented in grey and Avoiders represented in black. **C.** Avoidance (time preconditioning test to post-conditioning test time spent in predator odor-paired chamber) following pre-treatment with either saline (white bar; n=8) or corticosterone (black bar; n=8). Data are presented as mean \pm SEM and were analyzed using a t-test. * indicates $p < 0.05$ vs. Saline-treated rats.

Paraventricular Nucleus

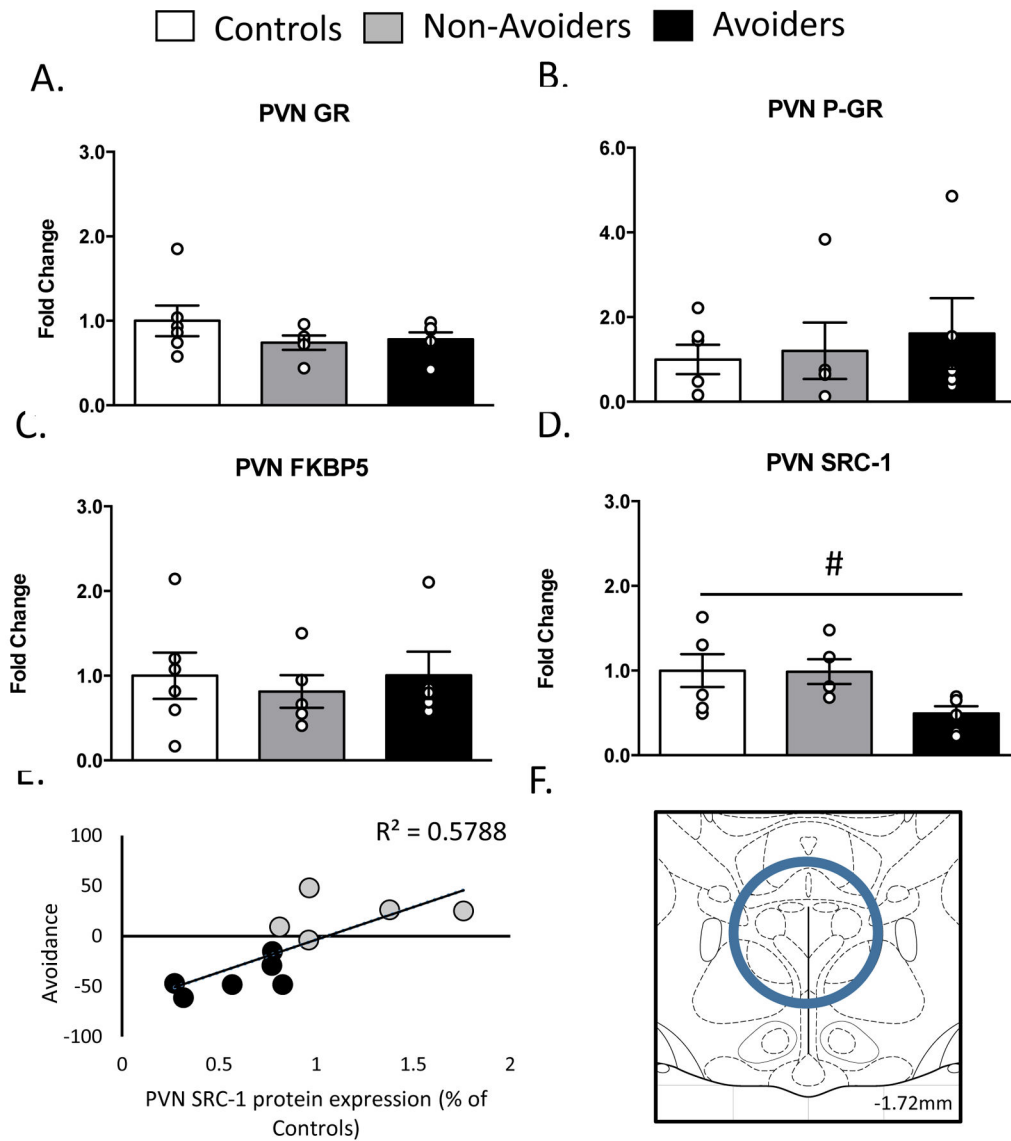


Figure 3.

Changes in Glucocorticoid Receptor (GR) machinery in the Paraventricular Nucleus (PVN; AP: -1.72, ML: 0.0, DV: 8.5). **A.** Total GR (% of Controls), **B.** pGR (expressed as a ratio of pGR/GR), **C.** FK506 Binding Protein 51 (FKBP51) (% of Controls) and **D.** Steroid Receptor Co-Activator (SRC)-1 (% of Controls) measured 48 h post-odor exposure in the PVN of Controls (white bars; n=6), Non-Avoiders (grey bars; n=8) and Avoiders (black bars; n=7). **E.** Scatter plot for individual rats (Avoider, black dots and Non-Avoider, grey circles; Experiment 2) shows avoidance of predator-paired context versus PVN SRC-1 protein expression 48 h post-stress. Rats that exhibited high avoidance of the predator-paired context 24 h post-odor exposure had lower PVN SRC-1 expression 48 h post-stress. **F.** Schematic

representation of PVN punch dissection. Data are presented as mean \pm SEM and were analyzed using a one-way ANOVA. # indicates treatment effect.

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Central Amygdala

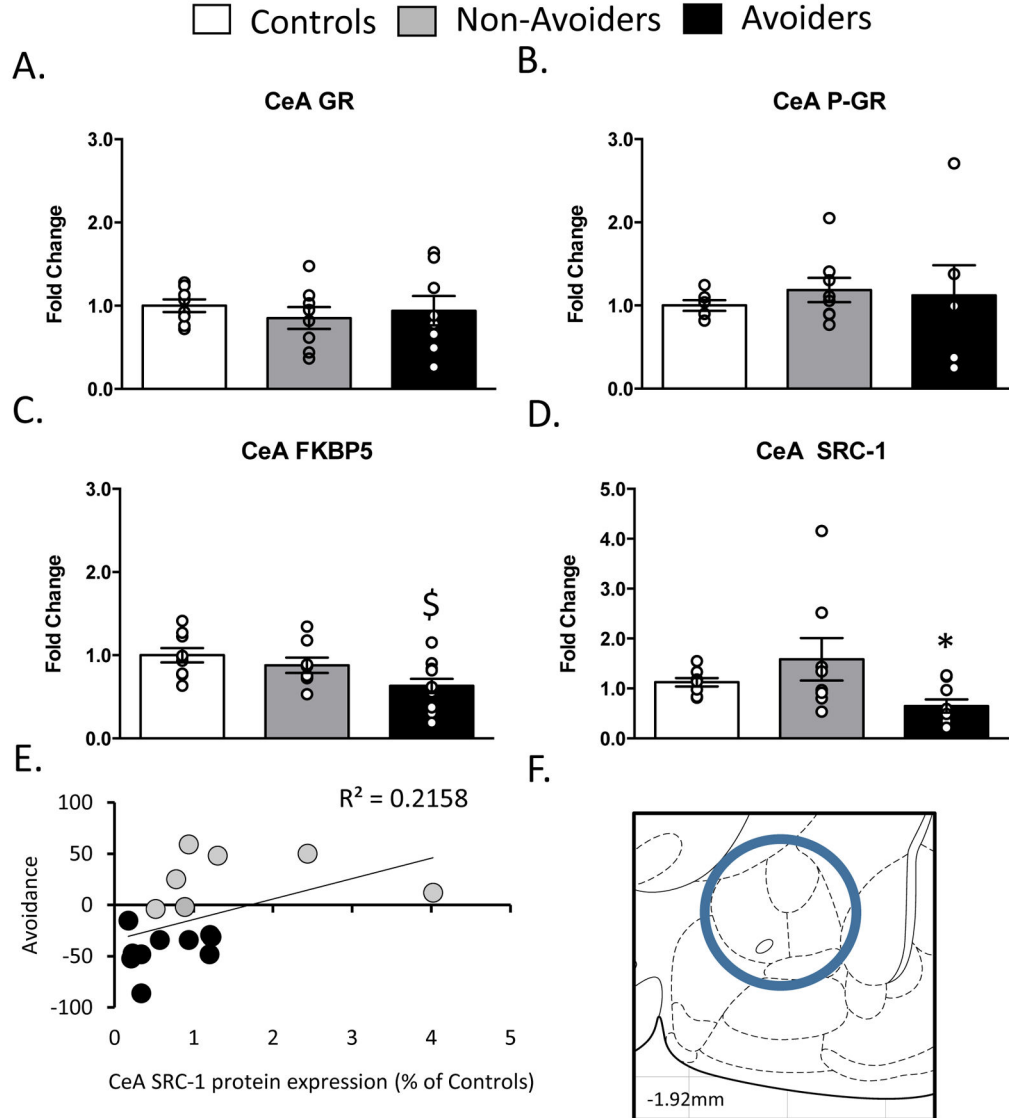


Figure 4.

Changes in Glucocorticoid Receptor (GR) machinery in the Central Amygdala (CeA; AP: -1.92, ML: 4.4, DV: 8.2). **A.** Total GR (% of Controls), **B.** pGR (expressed as a ratio of pGR/GR), **C.** FK506 Binding Protein 51 (FKBP51) (% of Controls) and **D.** Steroid Receptor Co-Activator (SRC)-1 (% of Controls) measured 48 h post-odor exposure in the CeA of Controls (white bars; n=6), Non-Avoiders (grey bars; n=8) and Avoiders (black bars; n=7). **E.** Scatter plot for individual rats (Avoider, black dots and Non-Avoider, grey circles; Experiment 2) shows change in preference for predator-paired context versus CeA SRC-1 protein expression 48 h post-stress. Rats that exhibited high avoidance of the predator-paired context 24 h post-odor exposure had lower CeA SRC-1 expression 48 h post-stress. **F.** Schematic representation of CeA punch dissection. Data are presented as mean ± SEM and

analyzed using a one-way ANOVA. \$ indicates $p < 0.05$ versus Control rats, * indicates $p < 0.05$ versus Non-Avoiders.

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Ventral Hippocampus

□ Controls ■ Non-Avoiders ■ Avoiders

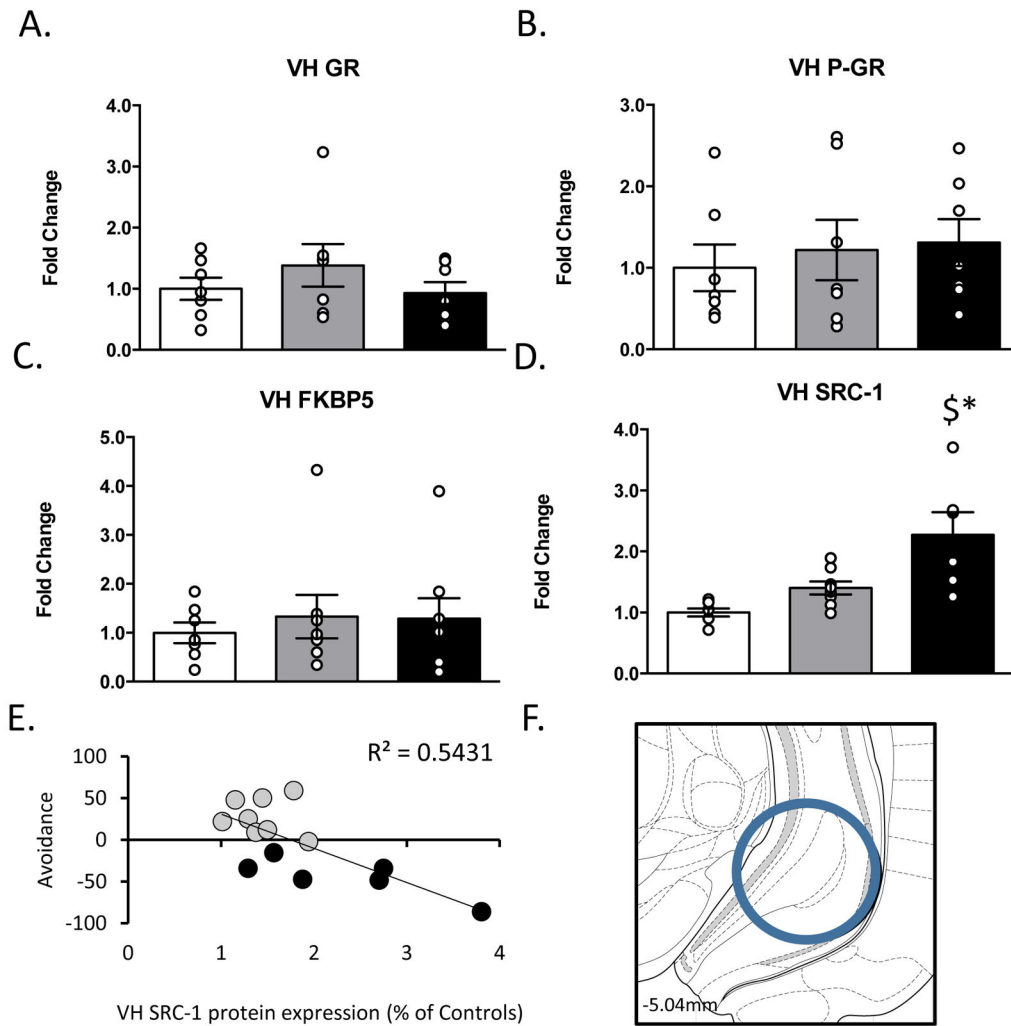


Figure 5. Changes in Glucocorticoid Receptor (GR) machinery in the Ventral Hippocampus (VH AP: -5.04, ML: -5.2, DV: 7.2) **A.** Total GR (% of Controls), **B.** pGR (expressed as a ratio of pGR/GR), **C.** FK506 Binding Protein 51 (FKBP51) (% of Controls) and **D.** Steroid Receptor Co-Activator (SRC)-1 (% of Controls) measured 48 h post-odor exposure in the VH of Controls (white bars; n=6), Non-Avoiders (grey bars; n=8) and Avoiders (black bars; n=7). **E.** Scatter plot for individual rats (Avoider, black dots and Non-Avoider, grey circles; Experiment 2) shows change in preference for predator-paired context versus VH SRC-1 protein expression 48 h post-stress. Rats that exhibited high avoidance of the predator-paired context 24 h post-odor exposure had higher VH SRC-1 expression 48 h post-stress. **F.** Schematic representation of VH punch dissection. Data are presented as mean \pm SEM and analyzed using a one-way ANOVA. \$ indicates $p < 0.05$ versus Control rats.

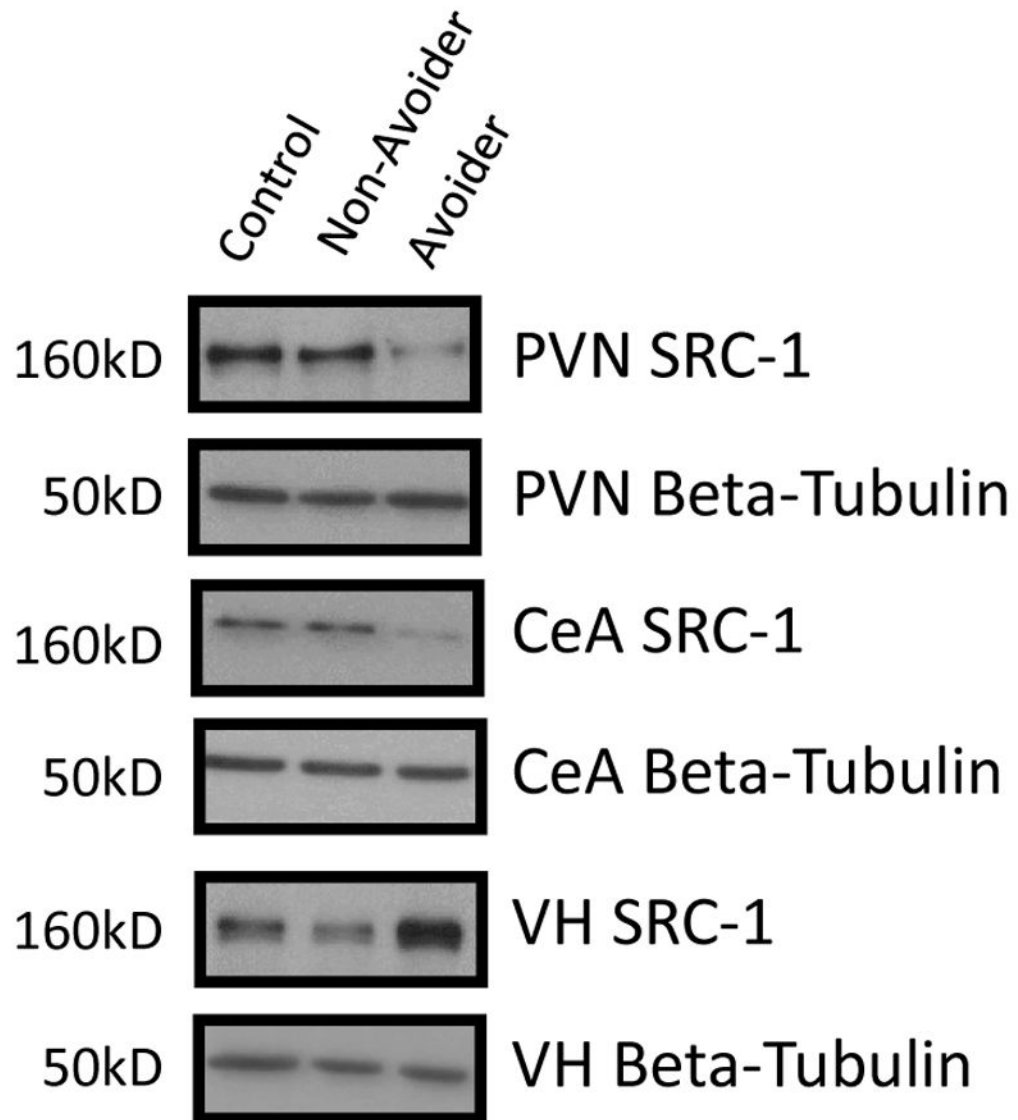


Figure 6. Representative western blot images for steroid receptor co-activator (SRC)-1 levels (molecular weight 160 kDa) in paraventricular nucleus (PVN), Central Amygdala (CeA) and ventral hippocampus (VH) in Control (C; n = 6), Avoider (A; n = 7) and Non-Avoider (NA; n = 8) groups.

Table 1

Primary antibody concentrations for Western Blot analysis of phosphorylated glucocorticoid receptor (P-GR), total glucocorticoid receptor (GR), FKBP51, and steroid receptor co-activator (SRC)-1 in the paraventricular nucleus (PVN), central amygdala (CeA) and ventral hippocampus (VH).

| | PVN | CeA | VH |
|----------------------|------------|------------|-----------|
| pGR (Ser 232) | 1:500 | 1:1000 | 1:1000 |
| GR | 1:1000 | 1:1000 | 1:1000 |
| FKBP51 | 1:20,000 | 1:10,000 | 1:20,000 |
| SRC-1 | 1:1000 | 1:1000 | 1:1000 |

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