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Knockdown of Steroid Receptors in the Central Nucleus of the Amygdala Induces Heightened Pain Behaviors in the Rat

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Abstract

Background—Previously we demonstrated that exposure of the central nucleus of the amygdala (CeA) to elevated corticosterone (CORT) induces nociceptive behaviors that are reversed by glucocorticoid and/or mineralocorticoid (GR/MR) receptor antagonism. Here we test the hypothesis that in a cholesterol (CHOL)-implanted control rat, selective knockdown of GR/MR in the CeA would, via a corticotropin-releasing factor (CRF)-mediated mechanism, replicate the nociceptive behaviors produced by elevated amygdala CORT.

Methods—Micropellets of CHOL or CORT were stereotaxically placed on the dorsal margin of the CeA. Cannulae were implanted into the CeA for the delivery of vehicle or oligodeoxynucleotides (ODN) of either antisense (ASO) or random sequences (RSO) targeting GR or MR. Visceromotor behavioral response quantified visceral sensitivity in response to colonic distension, while von Frey filaments assessed somatic sensitivity. Receptor expression was determined with qRT-PCR.

Results—In CHOL implanted controls, knockdown of GR in the CeA increased both colonic and somatic sensitivity, whereas selective knockdown of MR in the CeA induced colonic hypersensitivity without affecting somatic sensitivity. CRF expression in the CeA was increased in CHOL-implanted rats treated with GR or MR ASO and resembled the augmented CRF expression seen in the CORT-implanted rats.

Conclusions—This is the first study to demonstrate that decreasing either GR or MR within the CeA is sufficient to induce visceral hypersensitivity whereas somatic hypersensitivity developed after only GR knockdown. The loss of either GR or MR was associated with an

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increased CRF expression, and may represent a common mechanism for the development of CeA-mediated nociceptive behaviors.

Keywords

glucocorticoid receptor; mineralocorticoid receptor; amygdala; oligodeoxynucleotide; rat; nociception

1. INTRODUCTION

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder that affects between 3–32% of the population of Western countries (reviewed by (Choung and Locke, 2011)). The hallmark symptom of IBS is chronic abdominal pain or discomfort, associated with altered motility (Longstreth et al., 2006). However, clinical studies have found that a subset of IBS patients (26–65%) have also been diagnosed with fibromyalgia, suggesting generalized pain hypersensitivity in this patient population (reviewed by (Riedl et al., 2008)). While the origin of the disorder is unknown, the altered pain perception in the absence of a measurable peripheral biomarker suggests a central origin for the syndrome. In support of a central mechanism, multiple brain imaging studies of IBS patients have demonstrated abnormal (either increased or decreased) brain activity in visceral and emotional processing areas in response to colonic distension (Bonaz et al., 2002; Naliboff et al., 2003; Wilder-Smith et al., 2004; Elsenbruch et al., 2010; Seminowicz et al., 2010). Additionally, IBS patients hyper-secrete cortisol (CORT) in response to direct corticotropin releasing factor (CRF) infusion (Dinan et al., 2006) or colonic distension (Chang et al., 2009), suggesting a dysfunctional regulation of the hypothalamic-adrenal-pituitary (HPA) axis.

The expected role for CORT (corticosterone in rodents) released as the end product of the HPA axis is to inhibit further stress responses through feedback inhibition of CRF at the paraventricular nucleus of the hypothalamus (PVN) as well as other stress-modulatory brain regions including the prefrontal cortex and hippocampus (reviewed by (Lupien et al., 2009)). The feedback inhibition is accomplished through CORT binding to its two receptors, the high affinity mineralocorticoid receptor (MR) and the low affinity glucocorticoid receptor (GR) (Sapolsky et al., 1983; Reul and de Kloet, 1985). Under basal conditions, MR mediates the actions of CORT, however, stress-induced CORT secretion rapidly saturates MR receptors, which allows for GR binding to mediate the rest of the response to stress (Reul and de Kloet, 1985; De Kloet and Reul, 1987). However, CORT binding within the amygdala can facilitate the HPA axis (Bhatnagar and Dallman, 1998) by increasing CRF expression in the both the amygdala and PVN (Makino et al., 1994). The amygdala can be divided into neuroanatomical subnuclei based on the receptor expression and connections to other brain regions. In particular, the central nucleus of the amygdala (CeA), unlike other subnuclei, highly expresses both MR and GR (Sapolsky et al., 1983; Reul and de Kloet, 1985). Additionally, the CeA integrates responses to stressful stimuli through modulation of behavior and visceral, autonomic responses, such as breathing, heart rate, urination and defecation (Gray, 1993; Gray and Bingaman, 1996; Schulkin et al., 1998; Swanson and Petrovich, 1998). Thus, the CeA is a key brain region that could regulate both stress and

abnormal visceral responses and has been demonstrated to have abnormal activation to colonic distension in IBS patients (Naliboff et al., 2003; Wilder-Smith et al., 2004).

We have previously demonstrated, in a rodent model, that targeted CeA activation with micropellets of CORT induces not only colonic hypersensitivity to colorectal balloon distension but also decreased threshold to withdraw from a somatic stimulus (Greenwood-Van Meerveld et al., 2001; Myers et al., 2007; Myers and Greenwood-Van Meerveld, 2007; Myers and Greenwood-Van Meerveld, 2010a), that persist for up to 28 days after manipulation (Myers and Greenwood-Van Meerveld, 2010b). Additional pharmacological studies determined that both GR and MR non-redundantly regulated the expression of the phenotypes (Myers and Greenwood-Van Meerveld, 2007; Myers and Greenwood-Van Meerveld, 2010a), such that both GR and MR regulated colonic hyperalgesia, while colonic allodynia was dependent upon MR and somatic sensitivity required only GR. These changes in behavior were concurrent with a decrease in GR expression and an increase in CRF expression within the CeA in rats with CORT micropellets at both 7- and 28-days post-implantation (Tran and Greenwood-Van Meerveld, 2012). However, a key unanswered question from our previous studies was if down-regulation of either GR or MR within the CeA would be sufficient to reveal a selective role for each receptor in the regulation of nociceptive behaviors, or if a stressor was necessary to produce the behavioral effects. Thus, the hypothesis for this study was that selective knockdown of GR or MR in the CeA, in the absence of the CORT micropellet, induces the development of colonic and somatic hypersensitivity.

2. MATERIALS AND METHODS

2.1. Animals

Adult male Fischer 344 rats (250–300 g) from Charles River (Wilmington, MA, USA) were housed under standard conditions (12 hr light cycle, lights on at 6:00 AM) in AAALAC approved animal care facilities at the University of Oklahoma Health Sciences Center. Upon arrival, rats were acclimated for at least 7 days to the animal facility. To reduce stress associated with experimentation, rats underwent a second 7-day period of acclimatization to the experimental environment. The University of Oklahoma Health Sciences Center and the Oklahoma City Veteran Affairs Medical Center Institutional Animal Care and Use Committee approved the use of animals in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8th edition, 2011) after verifying that the experiments were designed to minimize animal discomfort, used the minimum number of animals to produce statistically valid results, and considered whether non-animal alternatives were available.

2.2. Stereotaxic Surgeries

2.2.1. Micropellet Implantation—The procedure used in this study has been previously described (Myers and Greenwood-Van Meerveld, 2007; Myers and Greenwood-Van Meerveld, 2010a). Briefly, using stereotaxic coordinates (bregma: -2.5 mm, medial/lateral: ± 4.2 mm, -7.0 mm from dura), rats were randomly assigned to receive bilateral

micropellets of CHOL or CORT on the dorsal margin of the CeA. Buprenorphine was administered for pre- and post-surgery analgesia.

2.2.2. Cannula Implantation—The procedure used for cannulae implantation has been described previously (Johnson et al., 2012). In brief, after the micropellet implantation, bilateral 26-gauge cannulae (Plastics One, Inc., Roanoke, VA, USA) were implanted at the same stereotaxic coordinates to target the CeA. Dummy cannulae were placed to ensure patency of the cannulae for ODN infusions.

2.3. Oligodeoxynucleotide (ODN) Infusions

The sequences for the 18-mer endcapped phosphorothioate ODNs for GR and MR, verified by BLAST search and based on previous literature (Engelmann et al., 1998; Sakai et al., 2000; Wang et al., 2004; Wang et al., 2005; Ferrari et al., 2013), used in this study were: GR antisense ODN (ASO): 5'-TGG AGT CCA TTG GCA AAT-3', GR random sequence ODN (RSO): 5'-TGA AGT TCA GTG TCA ACT-3'; MR ASO: 5'-TTC CAT GTC TAG GCC TTC-3', MR RSO: 5'-CAT TTT GAA GGT TCC GGT-3'. All ODN were synthesized by Eurofins MWG Operon (Huntsville, AL, USA) and supplied as dried, salt-free stocks that were resuspended in artificial cerebrospinal fluid (150 mM NaCl, 3.0 mM KCl, 1.4 mM CaCl₂, 0.8 mM MgCl₂, 0.8 mM Na₂HPO₄, 0.2 mM NaH₂PO₄, pH 7.4) to a concentration of 6 µg/µl. For daily infusions, rats were anesthetized with 2% isoflurane inhalation, before using a 33-gauge injector, 0.5 mm longer than the guide cannula, to deliver 0.5 µl of ODN to each CeA (1 µl/rat/day) at a rate of 0.1 µl/min via a Hamilton microsyringe attached to an UltraMicroPump with a SYS-Micro4 controller (World Precision Instruments, Sarasota, FL, USA). Injectors were left in place for an additional 2 min to allow for diffusion of the ODN solution before being withdrawn.

2.4. Behavioral Studies

2.4.1. Assessment of Visceral Sensitivity—The response to isobaric colonic distension was recorded as previously described (Myers and Greenwood-Van Meerveld, 2007; Myers and Greenwood-Van Meerveld, 2010a; Johnson et al., 2012; Tran et al., 2012; Tran et al., 2014a). In brief, following an overnight fast, a 6-cm latex balloon (Trojan ENZ, Church & Dwight C. Inc., Princeton, NJ, USA) attached to a PE-205 catheter (Becton Dickinson and Company, Sparks, MD, USA), was inserted 11-cm into the proximal colon under isoflurane anesthesia (2% inhalation) and secured to the base of the tail with tape. ODN or VEH was then infused and animals were allowed to recover in their home cage. In conscious, freely moving rats, ~60 min after the infusion (~30–45 min after termination of anesthesia), the number of abdominal contractions to tonic, isobaric colonic balloon distension (20, 40, or 60 mmHg, presented in a random order; 10 min/distension, 10 min rest period (0 mmHg) between distension) was visually assessed to determine colonic sensitivity. The 10 min before the first distension (0 mmHg) was used to determine baseline colonic sensitivity. The total time for the distension protocol was 60 min from the start of the first distension.

2.4.2. Assessment of Somatic Sensitivity—One hour after ODN or VEH infusion, withdrawal threshold for the plantar surface of the hind-paw was assessed with a von Frey

anesthesiometer (series 2390, IITC Life Science Inc., Woodland Hills, CA, USA), as previously described (Myers and Greenwood-Van Meerveld, 2010a; Myers and Greenwood-Van Meerveld, 2010b; Tran et al., 2012). The mean response of three withdrawal thresholds was used for data analysis.

2.5. Localization of Implants and Infusions

In the groups of animals used for gene expression, one hour after the final ODN or VEH infusion, individual rats were rapidly anesthetized with 5% isoflurane and decapitated (~3 min after leaving home cage). Brains were extracted and placed in a precision, coronal brain matrix (Braintree Scientific, Inc., Braintree, MA, USA) and a 1 mm slice targeting the CeA was removed. A 1 mm precision micropunch (Braintree Scientific, Inc.) was then used to isolate each CeA, which were then placed in RNAlater (Life Technologies, Grand Island, NY, USA) and stored at 4°C until processed. The location of the CeA was visually verified based on the structure of the hippocampus, the optic tract, and the tracts from the indwelling cannula. In the groups of animals used for behavioral studies, following the visceral sensitivity assessment, individual rats were anesthetized with 5% isoflurane and decapitated. Brains were extracted and snap-frozen in dry-ice chilled isopentane (Sigma Aldrich, St. Louis, MO, USA), and stored at -20°C until cryosectioned (50 µm sections) for localization of the micropellet based on the stereotaxic atlas (Paxinos and Watson, 2007), as previously described (Myers and Greenwood-Van Meerveld, 2007; Myers and Greenwood-Van Meerveld, 2010a; Johnson et al., 2012).

2.6. Quantitative Reverse Transcriptase-Polymerase Chain Reaction

A similar protocol for qRT-PCR was used as previously described (Tran et al., 2013). CeA samples from ODN or VEH infused rats that did not undergo behavioral studies were processed for total RNA using a PARIS kit (Life Technologies) per the supplied protocol. RNA quality and concentration was determined with an Experion system (Bio-Rad, Hercules, CA, USA) before being converted to cDNA with a QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA, USA). Triplicate samples and no template control (25 µl final volume) were used for qPCR using QuantiFast SYBR Green Master Mix (Qiagen). The following QuantiTect primers (Qiagen) were used: 28S rRNA (QT00199374), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (QT00199633), GR (QT00195251), MR (QT00183890), CRF (QT00183533). The reactions were performed with a StepOne Plus Real-Time PCR Thermal Cycler (Life Technologies) in fast mode per the protocol supplied with the primers. Melting curves confirmed single peaks at 87.7°C for 28S rRNA, 84.0°C for GAPDH, 81.0°C for GR, 82.1°C for MR, and 87.0°C for CRF; however, due to sample contamination (multiple-melting curves), two samples from the CHOL MR ASO group and one sample from the CHOL GR ASO group were excluded. GR, MR or CRF expression was normalized to the geometric mean (Vandesompele et al., 2002) of the 28S rRNA/GAPDH Ct and the expression in the CHOL VEH group was used for fold changes by the Ct method (Livak and Schmittgen, 2001).

2.7. Experimental Design

The experimental design for the study is illustrated in Fig. 1A. Rats were randomly assigned to treatment arm (expression only, or behavioral studies) and group (CHOL VEH, CHOL

GR RSO, CHOL GR ASO, CHOL MR RSO, CHOL MR ASO, CORT VEH) before surgery on day 0. Since the rats were monitored daily during the surgical recovery period (day 1–3), and with the daily infusion of ODN or VEH (day 4–7), the experimenter was not blinded to the treatment groups. All infusions took place between 9:00 AM and 12:00 PM, so that all behavioral experiments could be completed by 2:00 PM to avoid changes in circadian CORT secretion. Tissue for gene expression was collected by 11:00 AM. A summary of the post-mortem localization of the micropellets and the approximate region of the CeA micropunch is shown in Fig. 1B.

2.8. Statistical Analysis

All data were represented as the mean \pm SEM. Changes in the response to colonic distension were analyzed by two-factor (treatment \times pressure) repeated-measure analysis of variance (ANOVA). Changes in gene expression and somatic sensitivity were analyzed by one-factor (treatment) ANOVA. A Tukey's post hoc test was used to examine specific differences between group means. $P < 0.05$ was considered significant for all tests. Statistical tests and graphs were generated with GraphPad Prism (v6.0e, GraphPad Software, La Jolla, CA, USA). The final versions of the figures were arranged with GIMP ver. 2.8 (GNU Project, www.gimp.org).

3. RESULTS

3.1. Effect of GR ASO in the CeA on Behaviors

Our previous studies demonstrated that implantation of a CORT micropellet on the CeA induced colonic and somatic hypersensitivity as well as anxiety-like behavior (Myers et al., 2007; Myers and Greenwood-Van Meerveld, 2007) concurrent with a decrease in GR expression (Tran and Greenwood-Van Meerveld, 2012). This series of experiments was designed to test whether a decrease in GR expression was sufficient to induce the changes in behavior in the absence of the CORT micropellet. As shown in Fig. 2A, analysis of the colonic sensitivity to distension demonstrated that the main effects of treatment ($F_{(3,20)}=21.44$, $p < 0.001$), pressure ($F_{(3,60)}=194.0$, $p < 0.001$), and the treatment:pressure interaction ($F_{(9,60)}=6.06$, $p < 0.001$) were all significant. Specifically, the CHOL GR RSO group had a similar response to the CHOL VEH group, while the CHOL GR ASO group was similar to the CORT VEH group, which displayed a hypersensitive response to colonic distension. There was also a significant main effect for treatment ($F_{(3,20)}=11.70$, $p < 0.001$) on somatic withdrawal threshold (Fig. 2B).

Comparison of the treatment means found that both the CORT VEH and CHOL GR ASO treated rats had a significantly reduced withdrawal threshold compared to CHOL VEH or CHOL GR RSO groups.

3.2. Effect MR ASO in the CeA on Behaviors

Based on our previous studies with implantation of the MR agonist, aldosterone, on the CeA that induced visceral hypersensitivity to distension (Myers and Greenwood-Van Meerveld, 2010a), we next investigated the effect of knockdown of MR in the CHOL-implanted rats on colonic and somatic hypersensitivity. The same CHOL VEH and CORT VEH groups, used

in Fig. 2., are presented throughout Fig. 3. The visceral sensitivity data demonstrated significant main effects of treatment ($F_{(3,20)}=26.48$, $p<0.001$), the repeated measure pressure ($F_{(3,60)}=217.4$, $p<0.001$), and a significant interaction ($F_{(9,60)}=4.96$, $p<0.001$). Fig. 3A shows that CHOL MR RSO treatment did not affect the response to balloon distension compared to the CHOL VEH group. In contrast, the CHOL MR ASO group demonstrated colonic hyperalgesia in response to the same distension pressures, similar to the hypersensitive response in the CORT VEH group. The significant main effect of treatment ($F_{(3,20)}=8.57$, $p<0.001$) on somatic sensitivity was due to the effect of the CORT VEH group. Post-hoc testing demonstrated that CHOL VEH, CHOL MR RSO and CHOL MR ASO groups had similar withdrawal thresholds (Fig. 3B).

3.3. Effect of ODN or VEH on GR, MR, or CRF Expression in the CeA

We have previously demonstrated that the CORT-implant on the CeA reduces expression of GR and increases expression of CRF (Tran and Greenwood-Van Meerveld, 2012) concurrent with the changes in colonic and somatic sensitivity (Myers and Greenwood-Van Meerveld, 2010b). Additionally, the ODN sequences we used in this study have been previously validated to produce changes in expression and/or behavior (Engelmann et al., 1998; Sakai et al., 2000). Thus, we used qRT-PCR to measure the fold change in expression of the GR, MR or CRF in the CeA following the completion of the daily infusions in rats not exposed to the behavioral studies. As shown in Fig. 4A, there was a significant main effect for treatment ($F_{(5,27)}=6.60$, $p<0.001$) on GR mRNA expression. Post-tests revealed that CHOL GR ASO treatment reduced GR expression to 0.20 fold of the CHOL VEH value. The effect of the GR ASO produced a similar decrease in GR expression as the CORT VEH group (0.26 fold). Neither MR RSO nor MR ASO effected GR expression. Analysis of MR expression (Fig. 4B) also resulted in a significant main effect for treatment ($F_{(5,27)}=7.92$, $p<0.001$). CHOL MR ASO reduced expression to 0.10 fold of CHOL VEH expression, which was comparable to the decrease in MR expression in the CORT VEH group (0.15 fold). The specificity of the ODN treatments was verified as the GR RSO and the GR ASO did not change MR expression. Fig. 4C graphs the effect on CRF mRNA expression, which had a significant effect of treatment ($F_{(5,27)}=6.99$, $p<0.001$). Both GR ASO and MR ASO in the CHOL-implanted rats increased CRF expression by 6.19 and 6.17 fold respectively, compared CHOL VEH group. Neither the GR RSO nor the MR RSO treatments changed CRF expression. As expected, CORT VEH treatment increased CRF expression 5.53 fold compared to the CHOL VEH.

4. DISCUSSION & CONCLUSIONS

The goal of this study was to determine in the stress-naïve rat whether knockdown of GR or MR in the CeA was sufficient to induce colonic and somatic hypersensitivity. We have previously demonstrated that implantation of CORT micropellets on the dorsal margin of the CeA induced long-lasting colonic and somatic hypersensitivity (Greenwood-Van Meerveld et al., 2001; Myers et al., 2007; Myers and Greenwood-Van Meerveld, 2007; Myers and Greenwood-Van Meerveld, 2010b). We have also shown that following CORT-implantation into the CeA there was a decrease in GR expression and an increase in CRF expression in the CeA (Tran and Greenwood-Van Meerveld, 2012). However, the relationship between the

altered expression in the CeA and the increased nociceptive behaviors remained uncertain. Based on our previous data, two predictions were possible: 1) the changes in GR and CRF expression in the CeA should be sufficient to induce nociceptive behaviors in the absence of the CORT-micropellet, or 2) the changes in GR and CRF expression in the CeA are the result of the induced nociceptive behaviors. Based on the results of the targeted knockdown of GR or MR in the CeA of the CHOL-implanted rats, this study is the first to demonstrate that knockdown of either GR or MR is sufficient to induce visceral hypersensitivity to mechanical distension of the colon. Additionally, knockdown of GR in the CeA, but not MR, induced somatic mechanical allodynia. Finally, this is the first study to demonstrate that knockdown of both GR and MR in the stress-naïve CeA increased CRF expression, suggesting a common mechanism for the development of the nociceptive behaviors.

While not a natural stressor, the stereotaxic implantation of CORT micropellets attempts to model CeA dysfunction by pharmacologically isolating the CeA at a level of CORT exposure that mimics a stressor (Shepard et al., 2003). The CORT-implant model induces colonic and somatic hypersensitivity (Greenwood-Van Meerveld et al., 2001; Myers et al., 2007; Myers and Greenwood-Van Meerveld, 2010b), phenotypes that mimic some of the clinical features seen in IBS or fibromyalgia patients. Additionally, the changes in nociceptive behaviors were specific to the CeA (Myers et al., 2007; Tran et al., 2012). We have previously demonstrated that co-implantation of the CORT micropellet with either an antagonist of GR (mifepristone) or MR (spironolactone) reversed the nociceptive phenotypes (Myers and Greenwood-Van Meerveld, 2007), indicating non-redundant roles for GR and MR within the CeA in the regulation of stress-induced nociceptive behaviors. A subsequent study found a decrease in GR and no change in MR immunofluorescence following CORT-implantation (Tran and Greenwood-Van Meerveld, 2012), suggesting a complicated interaction between GR/MR receptor expression and behavior. Indeed, since GR has positive (GREs) and negative (nGREs) response elements, it is possible that stress-induced decreases in GR expression might shift the balance towards GREs, leading to increased nociceptive behaviors due to the loss of nGRE activity. Moreover, GR antagonists, in addition to direct inhibition of GREs, might also prevent the decrease in GR expression, thus preserving nGRE activity that inhibits nociceptive behaviors. In support of this hypothesis, mifepristone has been demonstrated to prevent decreases in GR expression in the PVN in a model of chronic HPA axis dysregulation (Hu et al., 2013). Additionally, a recent study demonstrated that mifepristone prevented increases in receptors negatively regulated by GR within the L6-S2 dorsal root ganglia (DRG) of rats exposed to chronic water avoidance stress (Hong et al., 2014).

In an attempt to clarify the role of both GR and MR in regulation of nociceptive behaviors, this study used targeted knockdown of the receptors in the CeA with specific ASO infusions in the stress-naïve, CHOL-implanted rat. The results of the colonic sensitivity assessment indicated that GR ASO caused an increased in abdominal contractions at all distension pressures (20–60 mmHg), while the MR ASO only increased the response to noxious distension (40 and 60 mmHg). Thus, there may be a subtle difference in the mechanism by which GR and MR modulate colonic sensitivity depending on whether the effect is produced by agonist activation (Myers and Greenwood-Van Meerveld, 2010a) or through knockdown of the receptors in the CeA. The finding that knockdown of GR or MR did not fully

reproduce the effect of the CORT micropellet on colonic sensitivity does not diminish the novel observation that disruption of GR or MR expression in the CeA is sufficient to change colonic sensitivity in an animal that had not been exposed to a stressor.

The effect of knockdown of GR or MR in the CHOL-implanted CeA on somatic withdrawal threshold more closely mirrors our previous findings from our pharmacological studies of CORT-, dexamethasone-, or aldosterone-implanted on the CeA (Myers et al., 2007; Myers and Greenwood-Van Meerveld, 2010a). This study demonstrated that GR ASO in the CHOL-implanted rats, but not MR ASO, induced a decrease in somatic withdrawal threshold that fully reproduced the effect of the CORT micropellet. Thus, there is a GR-mediated tonic inhibition of somatic sensitivity within the CeA that is disrupted by knockdown of the receptor. These results do not exclude a role for MR-modulation of somatic sensitivity outside of the CeA as reported in other models of inflammatory nerve injury (Gu et al., 2011; Dong et al., 2012).

In agreement with previous studies using the same ASO and RSO constructs for GR or MR (Engelmann et al., 1998; Sakai et al., 2000; Wang et al., 2004; Wang et al., 2005; Ferrari et al., 2013), the results of the qRT-PCR analysis of mRNA expression indicated that the ASO infusions selectively knocked-down only their targeted corticosteroid receptor within the CeA. The GR ASO infusions decreased GR mRNA expression by 67–88% of the CHOL VEH/GR RSO values, which was comparable to the expression in the CORT VEH group (66–80% decrease). Similarly, the MR ASO knocked-down MR mRNA expression by 87–93%, while the expression in the CORT VEH group was reduced by 81–88%. While none of the previous literature used the ASO constructs to directly examine the CeA, the decrease in GR and MR expression in the CHOL-implanted rats observed in our study was comparable to other literature (Han et al., 2014). Both the GR and MR ASO infusion also increased CRF expression within the CeA of the CHOL-implanted rats by 6.2 fold, which was similar to the increase in the CORT VEH group (5.5 fold), and was consistent with increased expression seen in previous studies of this model (Shepard et al., 2000; Tran and Greenwood-Van Meerveld, 2012; Tran et al., 2014b). In addition to the effect within the CeA, the CORT-implant model also increases CRF expression within the bed nucleus of the stria terminalis (BNST) and the PVN (Shepard et al., 2003; Shepard et al., 2006; Tran and Greenwood-Van Meerveld, 2012; Tran et al., 2012), providing evidence for a circuit by which increased CRF expression within the CeA can induce nociceptive behaviors. Since both GR and MR were decreased in the CeA by the CORT micropellet, whereas the GR ASO or MR ASO only decreased one of the receptors, there is the intriguing possibility that if both GR and MR ASO had been administered to CHOL-implanted animals, we might have been able to fully reproduce the hyperalgesic response to the 60 mmHg distension. Somatic nociceptive behaviors from the hindpaw are modulated by CRF through CRF-type 1 and -type 2 receptors within the CeA through a circuit through the parabrachial nucleus (Ji et al., 2007; Ji and Neugebauer, 2007; Fu and Neugebauer, 2008; Ji and Neugebauer, 2008). However, there is no evidence that the sub-region of the CeA that receives somatic nociceptive signals from the parabrachial nucleus expresses MR (Joels and Baram, 2009). As such, both the allodynic response to 20-mmHg distension and the decreased withdrawal threshold to von Frey filament may involve the parabrachial-CeA circuit, which may be insensitive to MR manipulation. In contrast the hyperalgesic responses to colonic distension are likely relayed

through other brainstem nuclei that express both GR and MR, such as the locus coeruleus (Joels and Baram, 2009). We have also demonstrated that targeted administration of CRF receptor antagonists into the CeA or the BNST inhibits colonic hypersensitivity induced by CORT-implantation or in a genetically hypersensitive rat model (Johnson et al., 2012; Tran et al., 2012; Tran et al., 2014a), suggesting that inhibition of the increased CRF expression in this study would be effective at inhibiting the nociceptive behaviors. Thus, a goal for future studies will be to further evaluate both the role of CRF-mediated mechanisms and the brain circuitry responsible for the colonic and somatic hypersensitivity induced by either GR or MR ASO in the CeA.

Another intriguing question for future studies is determining what additional underlying mechanism(s) may be responsible for sustained decreases in GR and sustained increases in CRF within the CeA. Epigenetic processes have been implicated in long-term changes in GR and CRF expression following early life adversity (Anacker et al., 2014; Reul et al., 2015). There is also evidence for epigenetic modifications of GR expression in other stress paradigms, including models of post-traumatic stress disorder (McGowan, 2013; Stankiewicz et al., 2013; Reul, 2014). In the CORT-implant model, we have recently demonstrated changes in histone acetylation are responsible for the prolonged decrease in GR expression and prolonged increase in CRF expression in the CeA (Tran et al., 2014b). Thus, future studies will evaluate whether a similar mechanism is responsible for the ASO-induced change in CRF expression demonstrated in this study.

Multiple clinical imaging studies have provided evidence for not only general changes in the central pain matrix but also specific changes within the amygdala of both IBS (Wilder-Smith et al., 2004; Berman et al., 2008; Elsenbruch et al., 2010; Seminowicz et al., 2010; Tillisch et al., 2011; Schmid et al., 2013) and fibromyalgia patients (Burgmer et al., 2009; Cifre et al., 2012; Jensen et al., 2012; Kim et al., 2013). Clinical studies have also demonstrated alterations in GR sensitivity and CRF reactivity in the patient population (Riedel et al., 2002; Dinan et al., 2006; McLean et al., 2006; Macedo et al., 2008; Chang et al., 2009; Geiss et al., 2012). Our study demonstrated that targeted knockdown of either GR or MR within the CeA in the stress-naïve rat induced both increased CRF expression and colonic hypersensitivity to distension, with increased somatic sensitivity modulated only by GR. This preclinical evidence provides further support for a central mechanism by which corticosteroid receptors and CRF may induce stress-mediated chronic pain disorders such as IBS.

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Abbreviations

ANOVA	analysis of variance
ASO	antisense oligodeoxynucleotide

BNST	bed nucleus of the stria terminalis
CeA	central nucleus of the amygdala
CHOL	cholesterol
CORT	corticosterone
CRF	corticotropin-releasing factor
DRG	dorsal root ganglia
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HPA	hypothalamic-adrenal-pituitary
IBS	irritable bowel syndrome
MR	mineralocorticoid receptor
nGRE	negative glucocorticoid response element
ODN	oligodeoxynucleotide
PVN	paraventricular nucleus of the hypothalamus
qRT-PCR	quantitative reverse transcription-polymerase chain reaction
RSO	random sequence oligodeoxynucleotide
SEM	standard error of the mean
VEH	vehicle

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Highlights

- Corticosterone on the central amygdala (CeA) induces colonic and somatic hypersensitivity.
- Corticosterone on the CeA decreases GR and MR expression.
- Knockdown of GR in the naïve CeA induces colonic and somatic hypersensitivity.
- Knockdown of MR in the naïve CeA induces colonic hyperalgesia.
- CRF expression is increased by knockdown of either GR or MR in the naïve CeA.

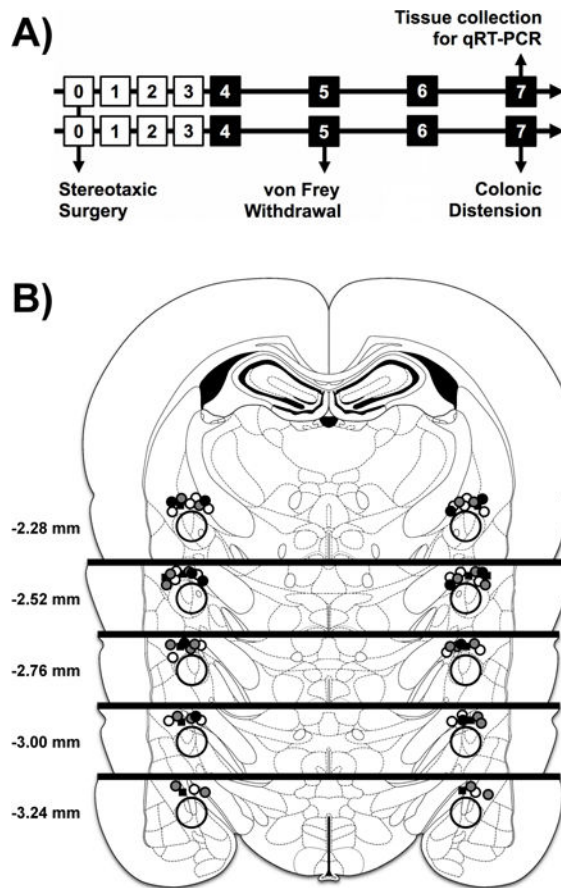


Fig. 1. Experimental Design and Localization of Implants/Infusions

A) Experimental design for ODN treatment and behavioral studies. The day of study is listed in the square. Black squares indicate ODN dosing 1 hr before a behavioral test. Separate groups of rats were used for behavioral studies and tissue collection for gene expression. $n=6$ for all groups. B) Localization of the micropellet implants on the dorsal surface of the CeA. Black squares are CORT implants with VEH treatment. Filled circles are CHOL implants, with black=VEH, white=RSO, gray=ASO. Distance is from bregma, based on the stereotaxic atlas (Paxinos and Watson, 2007). The larger, open circle represents the approximate location of the micropunch used for expression analysis.

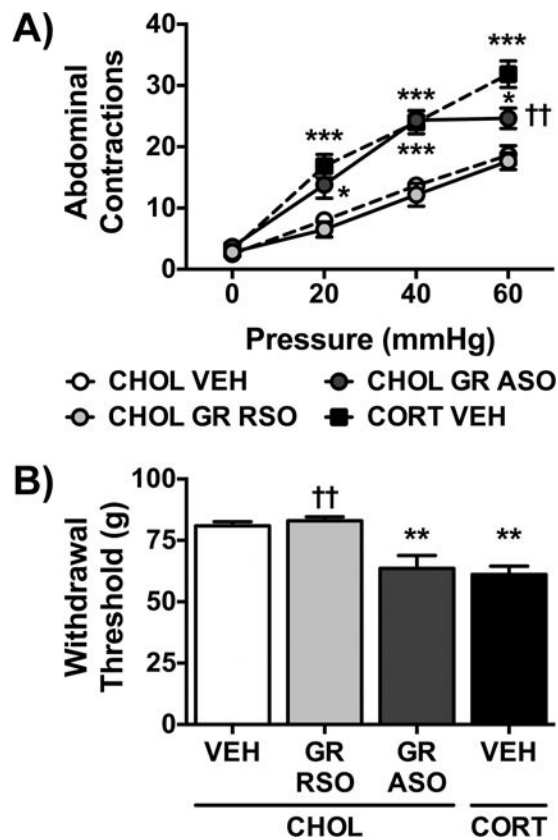


Fig. 2. Effect of Knockdown of GR in the CeA

A) The number of abdominal contractions in response to graded colonic distension was similar in the CHOL VEH and CHOL GR RSO groups. GR ASO in the CHOL-implanted rats induced a significant increase in colonic sensitivity to isobaric distension at all pressures tested. The response to colonic distension was similarly increased in the CORT-implanted rats treated with VEH. B) Somatic withdrawal threshold in CHOL-implanted rats treated with VEH was not affected by GR RSO administration. In contrast, GR ASO infusions in CHOL-implanted rats induced a significant decrease in withdrawal threshold that mirrored the decrease in the CORT VEH group. Data is represented as mean \pm SEM, $n=6$ /group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. CHOL VEH, †† $p < 0.01$ vs. CORT VEH, one- or two-factor ANOVA with- or with-out repeated measures, Tukey Post-hoc test.

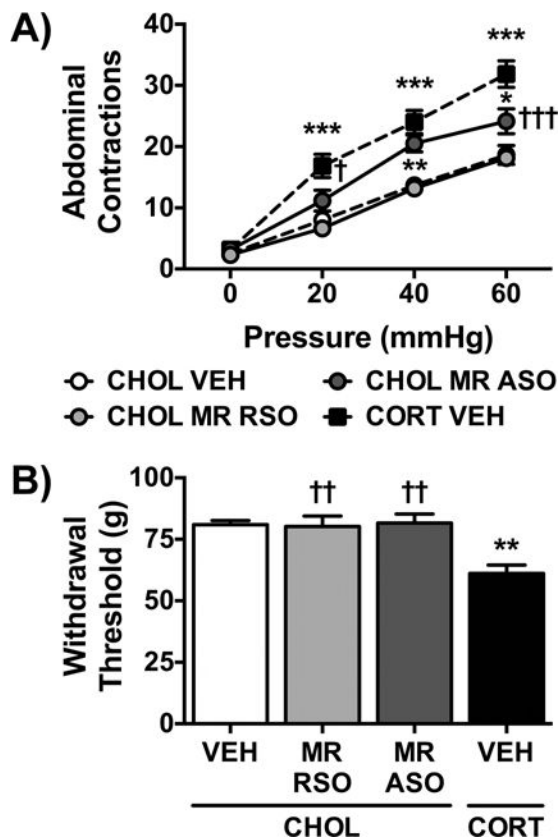
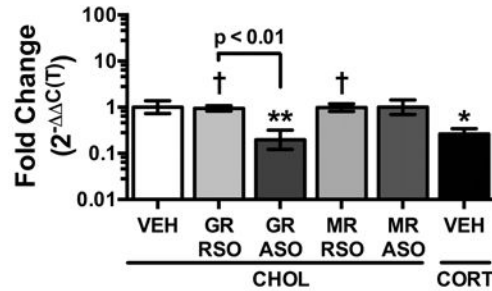


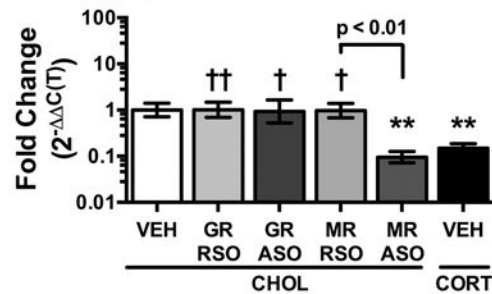
Fig. 3. Effect of Knockdown of MR in the CeA

A) Both the CHOL VEH and CHOL MR RSO groups had similar responses to colonic distension. MR ASO in the CeA induced a significant visceral hyperalgesia to colonic distension in rats with CHOL-implants, partially reproducing the visceral hypersensitivity induced by the CORT-implant on the CeA. B) MR RSO infusions in the CeA of CHOL-implanted rats did not change somatic sensitivity as compared to the CHOL VEH group. However, MR ASO also failed to change somatic withdrawal threshold, whereas there was a significant decrease demonstrated in the CORT VEH group. Data is represented as mean \pm SEM, $n=6$ /group. * $p < 0.05$, ** $p < 0.01$ vs. CHOL VEH, $\dagger p < 0.05$, $\dagger\dagger p < 0.01$, $\dagger\dagger\dagger p < 0.001$ vs. CORT VEH, one- or two-factor ANOVA with- or with-out repeated measures, Tukey Post-hoc test.

A) GR Expression



B) MR Expression



C) CRF Expression

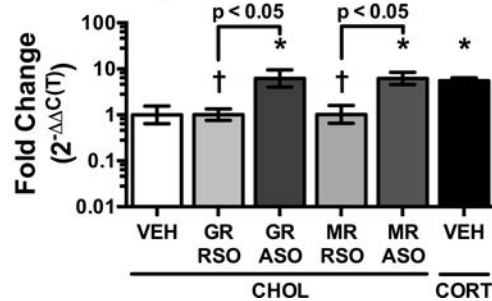


Fig. 4. Verification of Knockdown in the CeA

A) As expected, GR ASO infusions in the CeA of CHOL-implanted rats significantly reduced GR expression compared to either the GR RSO or VEH treatment. A similar reduction in GR expression was induced in the CORT VEH group. The specificity of the MR ODN was confirmed by the lack of change in GR expression in those groups. Neither MR RSO nor MR ASO changed GR expression. B) MR expression was significantly decreased following ASO infusion in the CHOL-implanted rats, while MR RSO did not have an effect. The CORT VEH group also demonstrated a significant reduction in MR expression. Neither GR ODN affected MR expression. C) An increase in CRF expression was measured in the CeA of CHOL-implanted rats following either GR ASO or MR ASO treatment. The increase in CRF expression in the ASO groups was similar to the elevated expression in the CORT-implanted group. Data is represented as mean \pm SEM, $n=6$ /group. * $p < 0.05$, ** $p < 0.01$ vs. CHOL VEH, † $p < 0.05$, †† $p < 0.01$ vs. CORT VEH, one-factor ANOVA, Tukey Post-hoc test.