



## Stress-induced visceral pain in female rats is associated with epigenetic remodeling in the central nucleus of the amygdala

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### ABSTRACT

Stress and anxiety contribute to the pathophysiology of irritable bowel syndrome (IBS), a female-predominant disorder of the gut-brain axis, characterized by abdominal pain due to heightened visceral sensitivity. In the current study, we aimed to evaluate in female rats whether epigenetic remodeling in the limbic brain, specifically in the central nucleus of the amygdala (CeA), is a contributing factor in stress-induced visceral hypersensitivity. Our results showed that 1 h exposure to water avoidance stress (WAS) for 7 consecutive days decreased histone acetylation at the GR promoter and increased histone acetylation at the CRH promoter in the CeA. Changes in histone acetylation were mediated by the histone deacetylase (HDAC) SIRT-6 and the histone acetyltransferase CBP, respectively. Administration of the HDAC inhibitor trichostatin A (TSA) into the CeA prevented stress-induced visceral hypersensitivity through blockade of SIRT-6 mediated histone acetylation at the GR promoter. In addition, HDAC inhibition within the CeA prevented stress-induced histone acetylation of the CRH promoter. Our results suggest that, in females, epigenetic modifications in the limbic brain regulating GR and CRH expression contribute to stress-induced visceral hypersensitivity and offer a potential explanation of how stress can trigger symptoms in IBS patients.

### 1. Introduction

Irritable bowel syndrome (IBS) is a female-predominant brain-gut disorder that affects up to 30% of the global population. IBS patients experience chronic abdominal pain due to heightened visceral sensitivity and abnormal bowel habits. The persistence of abdominal pain is the primary reason why IBS patients seek medical care (Enck et al., 2016; Oshima and Miwa, 2015). However, due to our incomplete understanding of the underlying mechanism(s) of IBS, effective treatment options are lacking, which often results in inadequate pain management.

Clinical evidence suggests that repetitive daily stress contributes significantly to the pathophysiology of IBS (Hertig et al., 2007; Surdea-Blaga et al., 2012). Furthermore, chronic stress during recovery from an enteric infection is often involved in the transition from a non-patient to IBS patient (Spence and Moss-Morris, 2007). Furthermore, patients often report that stress can trigger or worsen IBS symptoms (Surdea-Blaga et al., 2012). Exposure to stress activates a neuroendocrine response mediated by the hypothalamic-pituitary adrenal (HPA) axis. The mediators of the HPA axis include

corticotrophin-releasing hormone (CRH) secreted by the paraventricular nucleus of the hypothalamus, adrenocorticotrophic hormone (ACTH) secreted by the pituitary gland and cortisol (CORT), which is released by the adrenal gland. When circulating CORT levels are low, CORT binds preferentially to the high-affinity mineralocorticoid receptor (MR). However, during episodes of high HPA axis activity and high CORT levels, CORT can also bind the low-affinity glucocorticoid receptor (GR). In IBS patients, HPA axis (re)activity is often disturbed, leading to higher basal levels of CRH and CORT (Patacchioli et al., 2001; Tache et al., 1999), and abnormal ACTH and CORT production when undergoing a CRH challenge (Dinan et al., 2006; Fukudo et al., 1998; Kano et al., 2017).

The underlying molecular mechanisms to explain how an altered stress response contributes or directly leads to abdominal pain in IBS patients, remain to be uncovered. Brain imaging studies in IBS patients revealed abnormalities in the corticolimbic brain, a collection of brain nuclei which are involved in both the facilitation of the stress response and pain processing. For instance, during provocative rectosigmoid mechanical distension, the prefrontal cortex, anterior cingulate cortex,

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insula and amygdala become overactive in IBS patients (Bonaz et al., 2002; Weaver et al., 2016). The importance of these brain regions in the regulation of visceral pain has been illustrated in several animal models of IBS patients (Gao et al., 2006; Gibney et al., 2010; Hubbard et al., 2016; Wang et al., 2013). In our laboratory, we have recapitulated the important role of the amygdala in stress-induced visceral hypersensitivity in several experimental models. For instance, the stereotaxic placements of CORT micropellets on the central nucleus of the amygdala (CeA) is sufficient to induce visceral hypersensitivity in rats (Myers and Greenwood-Van Meerveld, 2007). In another preclinical model, chronic water avoidance stress (WAS), which attempts to mimic chronic psychological stress, induced visceral hypersensitivity (Myers and Greenwood-Van Meerveld, 2012). On the molecular level, prolonged or repeated exposure to CORT leads to a decrease in GR expression and an increase in CRH expression in the CeA (Tran and Greenwood-Van Meerveld, 2012). In the CeA, GR acts as a negative regulator of CRH expression (Johnson and Greenwood-Van Meerveld, 2015), which prevents the increase in CRH that is necessary to induce visceral hypersensitivity (Johnson et al., 2015). A potential mechanism, through which chronic stress can lead to persistent changes in gene expression and consequently visceral hypersensitivity, is the modulation of the epigenome of the CeA. In support, we previously found in male rats that exposing the CeA to CORT micropellets increased binding of Sirtuin-6 (SIRT-6), a histone deacetylase, at the GR promoter, leading to deacetylation of histone 3 lysine 9 and ultimately the decrease of GR mRNA and protein levels. These CORT-induced epigenetic changes were reversed after direct administration of trichostatin A (TSA) into the CeA, which also prevented visceral hypersensitivity (Tran et al., 2015). In another study, we showed that intracerebroventricular administration of TSA prevented WAS-induced visceral hypersensitivity (Tran et al., 2013). In other animal models of visceral hypersensitivity, administration of the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA), prevented visceral hypersensitivity induced by maternal separation, 17 $\beta$ -estradiol injections or forced swim stress (Cao et al., 2015, 2016; Moloney et al., 2015).

Growing evidence suggests that HPA activity and pain sensitivity differ between the sexes (Bangasser and Wiersielis, 2018; Berkley et al., 2006; Wiesenfeld-Hallin, 2005). For example, neuropathic and inflammatory pain are characterized by sex-specific gene expression patterns (Cantu et al., 2019; Stephens et al., 2019), which may be caused by underlying sex-specific epigenetic mechanisms. In the current study, we aimed to investigate in female rats whether chronic stress affected the epigenome in a way similar to that previously reported in male animals (Tran et al., 2013, 2015). In the current study we tested two central hypotheses, i) we hypothesized that through epigenetic remodeling in the CeA, stress changes the expression of the GR and CRH, which lead to visceral hypersensitivity, and ii) we hypothesized that modulating the epigenome of only the CeA with HDAC inhibitors would be sufficient to prevent stress-induced visceral hypersensitivity.

## 2. Materials and methods

### 2.1. Experimental design

Before the start of any experimentation, all female rats were acclimated to the animal facility (7 days) and laboratory (7 days). In order to investigate the effects of chronic stress on visceral sensitivity and potential epigenetic changes in the CeA, animals ( $n = 36$ ) were exposed to WAS or SHAM stress ( $n = 18$ /group) for 1 h each day for 7 consecutive days. Twenty-four hours after the final stressor, colonic sensitivity was assessed on one cohort or the CeA and hippocampus were isolated for molecular assays (qPCR or ChIP-qPCR) in another cohort (Fig. 1A).

To investigate the effects of histone deacetylase inhibition in the CeA on stress-induced visceral hypersensitivity, animals ( $n = 72$ ) were randomly divided into four cohorts: i. SHAM + VEH, ii. SHAM + TSA, iii. WAS + VEH, and iv. WAS + TSA ( $n = 18$ /group). All animals underwent

stereotaxic bilateral implantation of cannula. Animals recovered for 7 days after which rats were exposed to SHAM or WAS for 7 consecutive days. Vehicle (VEH) or TSA was infused stereotaxically into the CeA directly after each SHAM or WAS session. Twenty-four hours after the final CeA infusion, colonic sensitivity was assessed or the CeA and hippocampus were isolated for molecular assays (Fig. 2A).

In order to investigate whether the epigenetic mechanisms of stress-induced visceral hypersensitivity are persistent, female animals ( $n = 36$ ) were exposed to WAS or SHAM stress ( $n = 18$ /group) for 1 h each day for seven consecutive days. Twenty-one days after the final stressor, colonic sensitivity was assessed or the CeA and hippocampus were isolated for molecular assays (qPCR or ChIP-qPCR) (Fig. 3A).

### 2.2. Animals

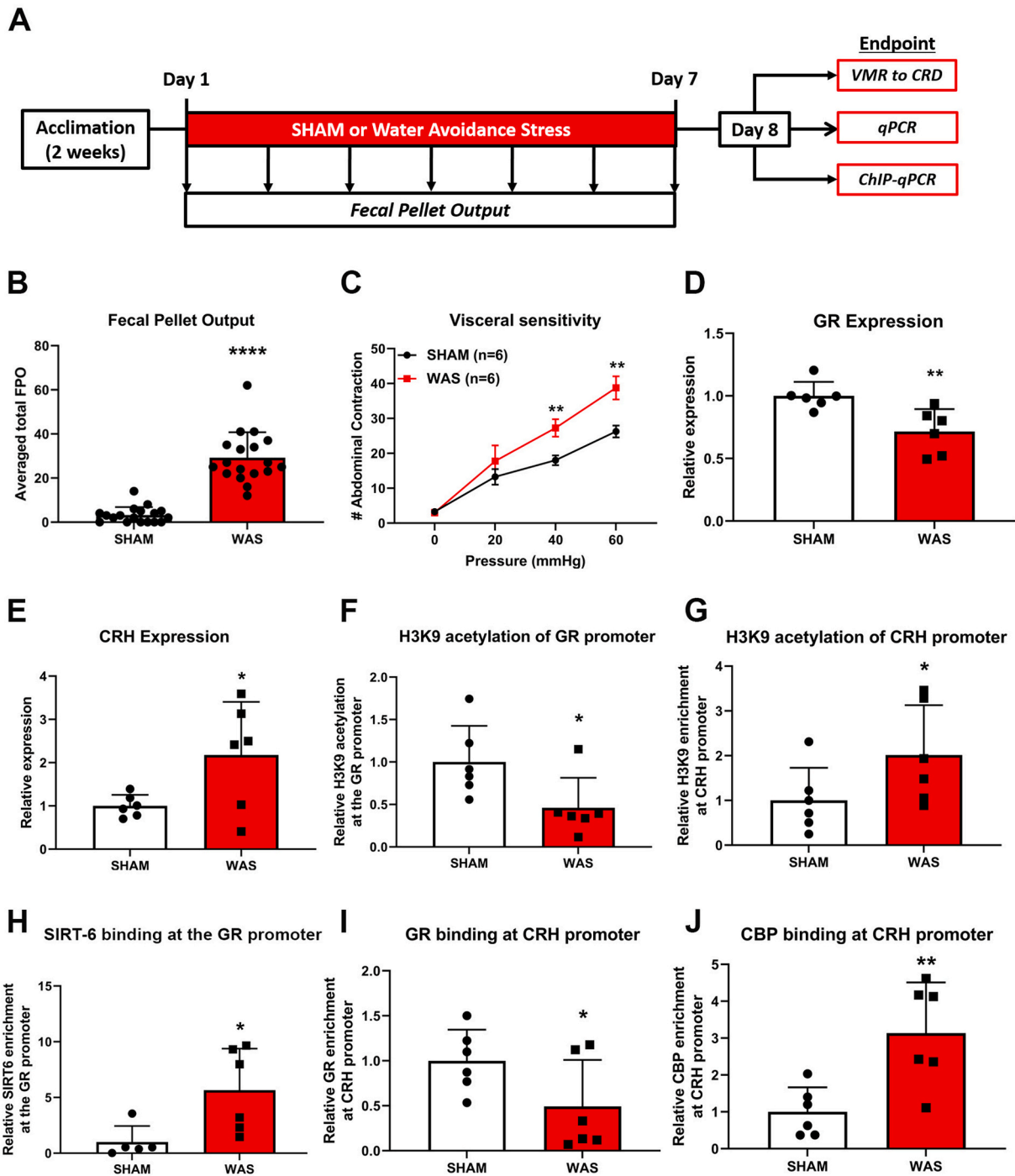
Experiments were performed in 144 female Fischer-344 rats (78–84 days old, 151–165 g on arrival), purchased from Charles River Laboratories (Wilmington, MA). Rats were double housed at 21 °C on a 12 h light/dark cycle (7:00 a.m.: 7:00 p.m.) with ad libitum access to food (5053 Irradiated PicoLab Rodent Diet; Dietlab; St. Louis, MO) and water. Rats were left undisturbed in the animal facility for 7 days and were acclimated to the experimental areas and experimenters for an additional 7 days. Rats that had undergone surgery were single housed for the remainder of the study. All protocols and procedures were in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the University of Oklahoma Health Sciences Center (18-057-SSAFH).

### 2.3. Stereotaxic bilateral implantation of cannula into the CeA

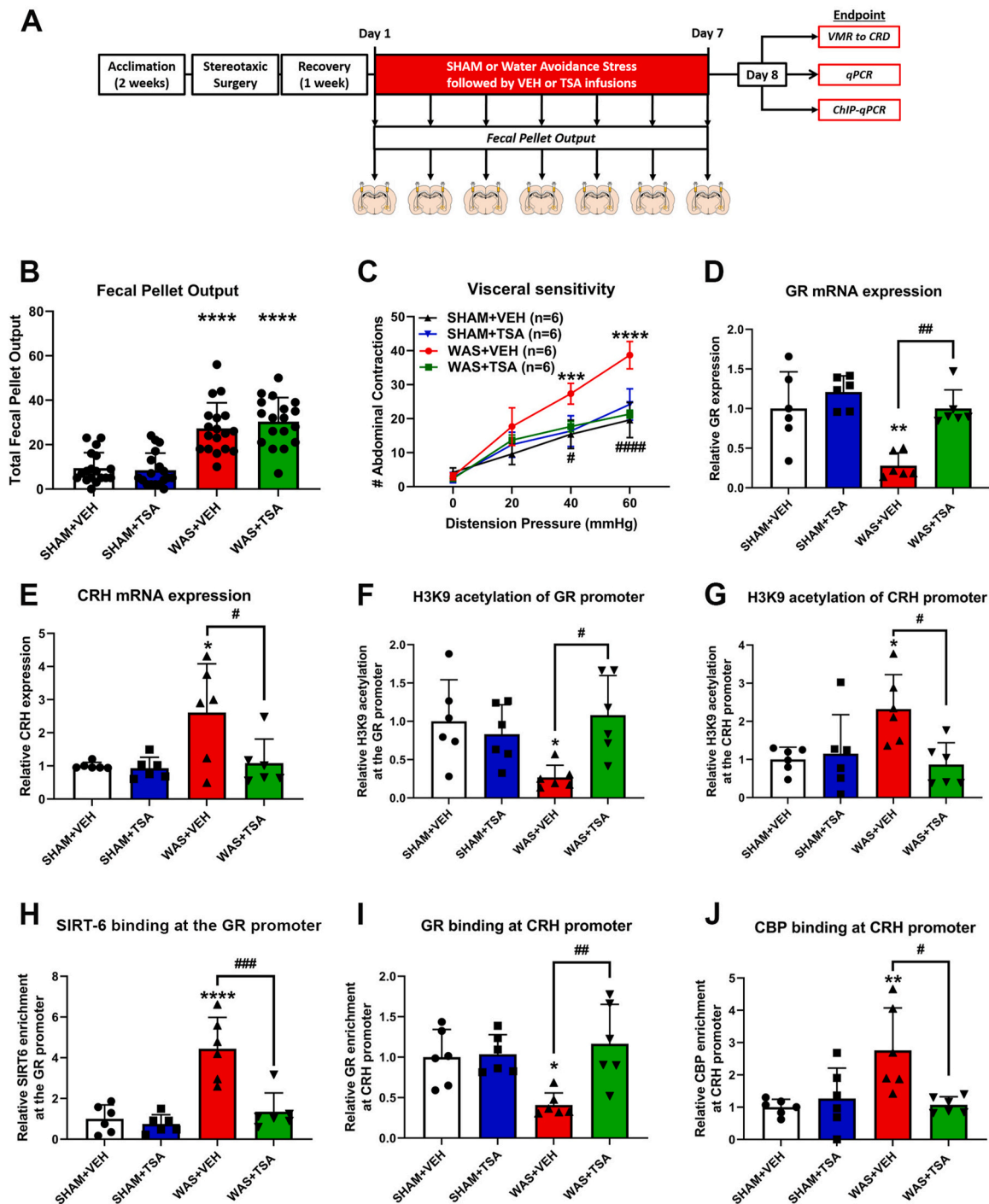
The procedure for stereotaxic bilateral cannulation has been described in detail elsewhere (Johnson and Greenwood-Van Meerveld, 2015; Johnson et al., 2015). Briefly, rats were anesthetized with 2–5% isoflurane with compressed air (Henry Schein Animal Health, Dublin, OH, USA) and received Carprofen (5 mg/kg subcutaneous) before being placed in the stereotaxic frame. An incision was made along the midline of the skull and cleared from the underlying fascia. A stainless steel mounting screw was placed in each quadrant. Bregma was marked and used as the reference point to determine the position of the CeA, based on the stereotaxic coordinates obtained from Paxinos and Watson (bregma  $-2.5$  mm, medial/lateral  $\pm 4.2$  mm and anteroposterior  $-7.0$  mm from dura). 26-gauge guide cannulas (Plastics One, Inc, Roanoke, VA) were bilaterally implanted on the dorsal margin of the CeA. Each guide cannula was secured to the mounting screws using dental cement (Stoelting, Wood Dale, IL, USA). 2–3 drops of 0.5% bupivacaine were administered to each quadrant, and removed after 2 min. The wound was closed with wound clips (9 mm, Kent Scientific Corporation, Torrington, CT, USA). After surgery, rats were single-housed in order to prevent cage mates from damaging the implanted cannulas. Rats were allowed to recover for 7 days and received subcutaneous Carprofen (5 mg/kg) injections for the first 2 days of recovery.

### 2.4. Drugs and infusions

Trichostatin A (TSA) was purchased from Tocris Bioscience (Minneapolis, MN, USA). TSA was dissolved in 0.1% dimethyl sulfoxide in artificial cerebrospinal fluid (Tocris Bioscience) to a concentration of 100 ng/ $\mu$ l. Immediately after WAS or SHAM stress, rats were anesthetized with 2% isoflurane and positioned within the anesthesia mask. The dummy cannulas were removed and replaced with matched injector cannula (Plastics One), attached to a microsyringe (Hamilton, Reno, NV, USA) and UMP3 injection pump (World Precision Instruments, Sarasota, FL, USA). Over a period of 5 min, a total volume of 0.5  $\mu$ l of TSA or vehicle was dispensed through each cannula at a flow rate of 0.1  $\mu$ l/min. The injection cannula was held in place for 2 additional minutes to ensure complete diffusion, after which it was removed and replaced by

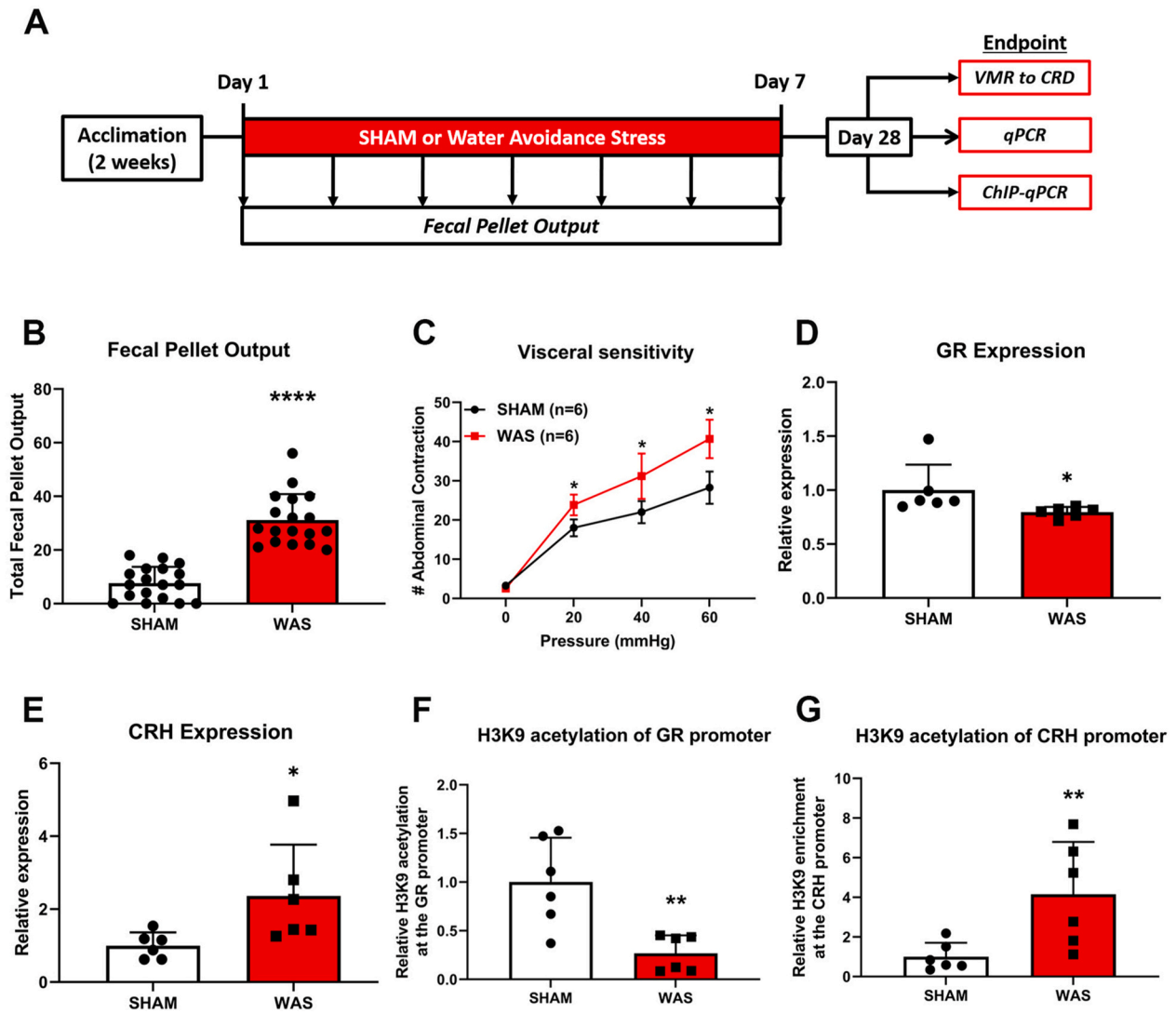


**Fig. 1.** Exposure to chronic WAS causes visceral hypersensitivity and is associated with epigenetic changes at the GR and CRH promoters in the CeA. (A) Experimental design. (B) Animals exposed to WAS (n = 18) had a higher total fecal pellet output than SHAM (n = 18) animals, which indicated they did not habituate to the daily stressor. (C) Twenty-4 h after chronic WAS exposure, visceral sensitivity at 40 and 60 mmHg was increased in female rats. (D) Twenty-4 h after chronic WAS exposure, real-time PCR analysis showed that GR expression was reduced, whereas (E) CRH expression was increased in the CeA. Chromatin was isolated from the CeA and immunoprecipitated with antibodies directed against either acetylated histone 3 lysine 9 (H3K9), the histone deacetylase (HDAC) Sirtuin-6 (SIRT-6), GR and the histone acetyl transferase (HAT) CREB-binding protein (CBP), followed by real-time PCR analysis for the GR and CRH promoter regions. (E) Twenty-4 h after chronic WAS exposure, chromatin immunoprecipitation assays revealed that H3K9 acetylation at the GR promoter was decreased, (F) while H3K9 acetylation at the CRH promoter was increased. Twenty-four hours after chronic WAS exposure, (H) the HDAC SIRT-6 was enriched at the GR promoter, (I) GR binding to the CRH promoter region was reduced, (J) the HAT CBP was enriched at the CRH promoter. Each experimental group contained six animals. All data are represented as mean + SD. Statistical differences in visceral sensitivity was assessed with a Two-Way Repeated Measurements ANOVA followed by Bonferonni post-hoc analysis. Statistical differences in fecal pellet output, gene expression and ChIP-qPCR assays were assessed using an unpaired *t*-test. Significance is expressed as \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001 vs SHAM.



**Fig. 2.** Infusions of HDAC inhibitor TSA directly in the CeA prevented WAS-induced visceral hypersensitivity and WAS-induced epigenetic changes. (A) Experimental design. VEH or TSA was administered directly after each daily SHAM or WAS exposure. Visceral sensitivity was assessed 24 h after the final infusion, or the CeA was isolated for molecular analysis. (B) Female rats exposed to WAS, receiving TSA infusions (n = 18), did not have a different fecal pellet output when compared to females that received VEH infusions (n = 18) The total fecal pellet output of WAS animals was higher than SHAM animals, indicating that WAS animals did not habituate to the stressor. (C) TSA infusions in the CeA prevented the development of WAS-induced visceral hypersensitivity. Animals that received TSA showed fewer abdominal contractions at 40 mmHg and 60 mmHg when compared to the WAS + VEH group. TSA did not affect visceral sensitivity of the SHAM group. The CeA was isolated from animals that had not undergone the visceral sensitivity assay. Real-time PCR analysis showed that TSA infusions prevented the WAS-induced decrease in GR mRNA expression (D) and the WAS-induced increase in CRH mRNA expression (E). Chromatin was isolated from the CeA and immunoprecipitated with antibodies directed against either acetylated H3K9, SIRT-6, GR and CBP, followed by real-time PCR analysis for the GR and CRH promoter regions. Infusions of TSA in the CeA directly after WAS prevented the WAS-induced decrease in H3K9 acetylation at the GR promoter (F) and the WAS-induced increase in H3K9 acetylation at the CRH promoter (G). TSA infusions reduced WAS-induced SIRT-6 binding at the GR promoter (H), increased GR binding at the CRH promoter (I) and decreased CBP binding at the CRH promoter (J). Each experimental group contained six animals. All data are represented as mean + SD. Statistical differences in visceral sensitivity was assessed with a Two-Way Repeated Measurements ANOVA followed by Bonferonni post-hoc analysis. Statistical differences in fecal pellet output and ChIP-qPCR assays were assessed using an unpaired t-test. Significance is expressed as \*p < 0.05, \*\*p < 0.01, \*\*\*\*p < 0.001 vs SHAM + VEH; #p < 0.05, ##p < 0.01, ###p < 0.001 vs WAS + VEH.





**Fig. 3.** Visceral hypersensitivity and epigenetic changes persists up until 21 days after the end of chronic WAS. (A) Experimental design. Visceral sensitivity was assessed 21 days after the final WAS exposure, or the CeA was isolated for molecular analysis. (B) Female animals exposed to WAS (n = 18) had higher total fecal pellet output than SHAM (n = 18) animals, which indicated they did not habituate to the daily stressor. (C) Twenty-one days after the final WAS exposure, female rats that had been exposed to WAS showed an increased number of abdominal contractions at 20, 40 and 60 mmHg when compared to SHAM controls. (D–E) Twenty-one days after the final WAS session, real-time PCR analysis revealed that GR expression was decreased and CRH expression was increased in the CeA of female rats exposed to WAS. Chromatin was isolated from the CeA and immunoprecipitated with antibodies directed against acetylated H3K9, followed by real-time PCR analysis for the GR and CRH promoter regions showed that changes in histone 3 lysine 9 acetylation at the GR promoter was reduced (F) and at CRH promoter was increased (G), 21 days after the last WAS session. Each experimental group contained six animals. All data are represented as mean + SD. Statistical differences in visceral sensitivity was assessed with a Two-Way Repeated Measurements ANOVA followed by Bonferonni post-hoc analysis. Statistical differences in fecal pellet output and ChIP-qPCR assays were assessed using an unpaired *t*-test. Significance is expressed as \**p* < 0.05, \*\**p* < 0.01, \*\*\*\**p* < 0.0001 vs SHAM.

the dummy cannula.

### 2.5. Water avoidance stress (WAS)

Chronic psychological stress was induced by exposing the animals to WAS for 1 h/day for 7 consecutive days. Rats in the WAS group were placed on a platform (8 × 8 × 8 cm) mounted in the center of a white semi-transparent plastic container (50 × 35 × 33 cm) filled with fresh, room temperature water to 1 cm below the surface of the platform. Rats in the SHAM stress group were placed in similar containers without water. For all rats, exposure to SHAM or WAS started between 9:00 a.m. and 10:00 a.m. each day. Rats were left undisturbed for 60 min, after which they were returned to their home cages. The number of fecal pellets expelled during the 60 min of WAS or SHAM stress was recorded as a measure of autonomic output.

### 2.6. Visceral sensitivity assessment

The detailed procedure for measuring visceromotor responses (VMR) to colorectal distention (CRD) in freely moving rats has been described previously (Myers and Greenwood-Van Meerveld, 2007; Greenwood-Van Meerveld et al., 2001). Visceral sensitivity was assessed 24 h or 21 days after final WAS or SHAM stress. The day before the VMR procedure around 3:00 p.m., rats were placed in a fasting cage (standard housing cage where the bedding was replaced with a wire mesh bottom, ad libitum water access) with ad libitum access to water. The following day, the VMR procedure was conducted between 10:00 a.m. and 2:00 p.m. Rats were anesthetized with 2–5% isoflurane. A 5-cm latex balloon catheter was inserted up to 11-cm from the anus and secured to the tail with surgical tape. Rats recovered from anesthesia for 30 min, before catheters were connected a Distender Series IIR Barostat (G&J

Electronics Inc., North York, Canada) for delivery of controlled, isobaric inflation of the balloon and CRD at randomized distensions of 0, 20, 40 and 60 mmHg. Each distention pressure was held for 10 min, during which the number of abdominal muscle contractions were counted. Between subsequent distention pressures, rats were allowed to recover for 10 min.

## 2.7. Colonic compliance

The volume-pressure relationship of the colon was measured after visceral sensitivity assessment to assess whether stress exposure or VEH/TSA infusions in the CeA affected colonic wall stiffness. A reduction in compliance would negatively affect the assessment of visceral pain. Colonic compliance data is presented in [Supplementary Fig. 1](#).

## 2.8. Brain isolation and tissue collection for molecular assays

In a separate cohort of rats that had not undergone visceral sensitivity assessment the brain was isolated, then tissue was collected 24 h or 21 days after the final WAS or SHAM stress. The procedure involved deeply anesthetizing the rats with 2–5% isoflurane before decapitation. As isoflurane is known to induce changes in immediate-early gene expression, decapitation, brain isolation and tissue collection were rapidly performed within 15–20 min of the start of anesthetization. The brain was isolated from the skull and placed in a precision coronal brain matrix (Braintree Scientific Inc., MA, USA). Two tissue slicing blades were placed at –2 mm and –4 mm from bregma. According to Paxinos and Watson brain atlas, this 2 mm slice should contain the CeA and hippocampus. The Paxinos and Watson brain atlas was used to identify the CeA and hippocampus. Animals that had undergone stereotaxic surgery showed the wound tracks of the cannula in this slice, indicating that cannulas were positioned correctly and providing an additional landmark for the isolation of the CeA. The CeA was collected using 1-mm precision micropunches (Braintree Scientific Inc.). The hippocampus was dissected from the same slides as the CeA. Tissue was flash frozen in liquid nitrogen and stored at –80 °C until processed.

## 2.9. Real time PCR

RNA was extracted from the CeA micropunches using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA extraction was followed by cDNA synthesis using the High-Capacity cDNA Reverse Transcription kit (ThermoFischer Scientific, Waltham, MA, USA). qPCR reactions were performed using TaqMan Fast Advanced Mastermix and TaqMan probes for rat NR3C1, rat CRH, and rat GAPDH as reference gene (all obtained from ThermoFischer Scientific). All reactions were performed in triplicate on the QuantStudio 5 (ThermoFischer Scientific) using the following protocol: initial activation (2 min, 50 °C); initial denaturation (2 min, 95 °C); 40 cycles of denaturation (1 s, 95 °C), annealing and extension (20 s, 60 °C). Differences between NR3C1 and CRH mRNA expression in the CeA of SHAM and WAS treated rats was calculated using the  $2^{-\Delta\Delta C_t}$  method and expressed as fold changes.

## 2.10. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed using the MAGnify ChIP assay kit (ThermoFisher Scientific). Chromatin was isolated from CeA micropunches and immunoprecipitated using one of the following antibodies: rabbit anti-acetyl-H3K9 (1:25, #9649, Cell Signal Technologies), rabbit anti-GR (1:25, #3360, Cell Signal Technologies), rabbit anti-CBP (1:25, #7425, Cell Signal Technologies), rabbit anti-SIRT6 (1:25, #ab62739, Abcam) or normal non-immune rabbit IgG antibody (1:100, ThermoFisher Scientific). One tenth of the lysate was reserved as input control. After reverse crosslinking, the rat GR and CRH promoter regions were subjected to qPCR amplification using QuantiFAST SYBR Green Mastermix (Qiagen, Valencia, CA, USA).

The GR promoter that was immunoprecipitated with H3K9 and SIRT-6 antibodies was amplified using the forward primer: 5'-TCAG-TATGTTTTCCACACTTGGAT-3' and reverse primer: 5'-TTTATCGCTCCTTGGTGAC-3'. The CRH promoter that was immunoprecipitated with H3K9 antibodies was amplified using the forward primer 5'-TTCCATTTTAGGGCTCGTTG-3' and the reverse primer 5'-CGACCCTTTCAGAAAGCAC-3', whereas the CRH promoter that was immunoprecipitated with GR and CBP antibodies was amplified using the forward primer 5'-GGTCAGTATGTTTTCCACACTTGG-3' and the reverse primer 5'-GCCTCTGCTCCTGCATAAAT-3'. qPCR reactions were carried out in triplicate on the QuantStudio 5 (ThermoFischer Scientific) with the following protocol: initial denaturation cycle (5 min, 95 °C), 40 cycles of denaturation (10 s, 95 °C), annealing and extension (30 s, 60 °C) and final hold at 4 °C. Relative quantification of binding was calculated by normalizing the immunoprecipitated DNA  $C_t$  values to the input DNA  $C_t$  values ( $\Delta C_t$ ) and transformed ( $2^{\Delta C_t}$ ) to show relative quantities.

## 2.11. Statistical analysis and experimental rigor

For behavioral and molecular assays, the sample size was calculated based on preliminary experiments carried out in our laboratory. These calculations indicated that six cycling female animals per treatment group were necessary to obtain significant results. Before the start of the experiments, female rats were randomly assigned to SHAM or WAS groups and WAS + VEH or WAS + TSA groups until each group contained 6 animals. We tested the normal distribution of our data using the Shapiro-Wilk normality test. When data showed a normal distribution, parametric tests were used for subsequent analysis. Stress-induced differences in visceral sensitivity were analyzed with a two-way repeated measurement analysis of variance (ANOVA), followed by a Bonferroni post-hoc test. Molecular assays were analyzed with a one-way ANOVA followed by a Bonferroni post-hoc test. Data were plotted (represented as mean  $\pm$  SD) and statistical analysis was performed with GraphPad Prism 8.0 (La Jolla, CA, USA). Results were considered significant if a p-value smaller than 0.05 was obtained.

Visceral sensitivity assays and molecular assays were performed by an experimenter blinded to previous treatment. The experimenter was unblinded for data analysis.

## 3. Results

### 3.1. Stress induces visceral hypersensitivity and changes GR and CRH expression in the CeA

In order to measure chronic-WAS induced changes in visceral sensitivity and the WAS-induced changes in gene expression in the CeA, female rats were exposed daily for 7 consecutive days to 1 h of WAS or SHAM stress (See [Fig. 1A](#) for experimental design). Animals exposed to daily WAS had an elevated total fecal pellet output, when compared to SHAM animals ([Fig. 1B](#)), which indicated that these animals did not habituate to the stressor. We assessed visceral sensitivity 24 h after the final WAS session. Analysis of the VMR to CRD revealed a main effect of pressure ( $F$ ([Hertig et al., 2007](#); [Cantu et al., 2019](#)) = 374.3;  $p < 0.0001$ ) and a main effect of chronic WAS ( $F$ ([Enck et al., 2016](#); [Kano et al., 2017](#)) = 13.40;  $p = 0.0048$ ). Post hoc analysis revealed an increased number of abdominal contractions at distention pressures of 40 mmHg ( $p < 0.0009$ ) and 60 mmHg ( $p < 0.0001$ ) in female rats exposed to chronic WAS when compared to their respective SHAM control counterparts ( $n = 6$ /treatment group) ([Fig. 1C](#)). In order to determine whether chronic WAS changes GR and CRH expression in the CeA of female rats, we isolated the CeA from animals that did not undergo the colonic sensitivity assay. We measured GR and CRH expression after SHAM or WAS using qPCR assays ( $n = 6$ /treatment group). In these females, the expression of GR was decreased ( $t_{10} = 3.298$ ,  $p = 0.0040$ ), whereas the expression of CRH ( $t_{10} = 2.306$ ,  $p = 0.0219$ ) was increased

in the CeA of WAS animals when compared to SHAM animals (Fig. 1D and E).

### 3.2. WAS changes the epigenome at the GR and CRH promoters

GR and CRH are key genes involved in the regulation of the HPA axis and visceral nociception. Hence, we postulated that dysregulation of these genes in the CeA contributes to IBS pathophysiology. Epigenetic modifications such as histone tail acetylation are important regulators of gene expression. Decreases in histone acetylation are associated with decreased gene transcription. Therefore, changes in these epigenetic marks at the GR and CRH promoters may have caused the observed changes in mRNA expression. In order to test whether WAS induces changes in histone acetylation, we isolated the CeA from animals that did not undergo the colonic sensitivity assessment ( $n = 6/\text{treatment group}$ ). To determine whether WAS changes histone 3 lysine 9 (H3K9) acetylation at the GR and CRH promoters, we performed ChIP directed towards acetylated H3K9, followed by qPCR of the GR and CRH promoter regions. Our results show that H3K9 acetylation was significantly reduced at the GR promoter ( $t_{10} = 2.385$ ,  $p = 0.0191$ ) (Fig. 1F), but significantly increased at the CRH promoter ( $t_{10} = 1.869$ ,  $p = 0.0456$ ) (Fig. 1G) in the CeA of female rats exposed to chronic WAS, when compared to SHAM controls.

Our finding that WAS can decrease histone acetylation at the GR promoter and increase histone acetylation at the CRH promoter, prompted us to investigate which histone deacetylases (HDAC) and histone acetyltransferases (HAT) are involved. Here, we report that chronic WAS increased SIRT-6 binding at the GR promoter ( $t_{10} = 2.613$ ,  $p = 0.0141$ ) when compared to SHAM controls (Fig. 1H). When investigating the CRH promoter region, our ChIP-qPCR results revealed a decrease in GR binding after chronic WAS ( $t_{10} = 2.041$ ,  $p = 0.0342$ ) (Fig. 1I). As GR is a part of a complex that negatively regulates CRH expression, the loss of GR allowed other molecules to interact with the CRH promoter. We specifically investigated the presence of the HAT CREB binding protein (CBP), since a Cre-binding element is present in the CRH promoter. Our ChIP-qPCR results indicated that chronic WAS increased CBP interactions with the CRH promoter ( $t_{10} = 3.424$ ,  $p = 0.0033$ ) in the CeA of female rats (Fig. 1J).

### 3.3. Inhibition of histone deacetylases prevents WAS-induced visceral hypersensitivity

Having shown that WAS induces changes in histone acetylation at the GR and CRH promoter in the CeA, our next set of experiments was aimed at reversing these epigenetic changes, in an attempt to reverse stress-induced visceral hypersensitivity. Therefore, we investigated whether targeted administration of the HDAC inhibitor TSA directly into the CeA was sufficient to reverse the WAS-induced phenotype and the potential effects of TSA on the epigenetic regulation of GR and CRH (See Fig. 2A for experimental design). As shown in Fig. 2B, regardless of VEH or TSA infusions, animals undergoing WAS had a higher FPO than SHAM animals. We did not observe any differences between the FPO of animals belonging to the WAS + VEH and WAS + TSA group. We assessed colonic sensitivity of SHAM + VEH, SHAM + TSA, WAS + VEH and WAS + TSA animals ( $n = 6/\text{treatment group}$ ) 24 h after the final infusions. Analysis of VMR to CRD revealed a main effect of pressure ( $F_{(3,57)} = 353.9$ ;  $p < 0.0001$ ) and a main effect of treatment ( $F$  (Hertig et al., 2007; Tran and Greenwood-Van Meerveld, 2012) = 20.37;  $p < 0.0014$ ). Post hoc analysis showed that after TSA treatment in female rats exposed to chronic WAS, the number abdominal contractions at 40 mmHg ( $p = 0.0027$ ) and 60 mmHg ( $p < 0.0001$ ) was significantly lower than the WAS + VEH group and comparable to SHAM + VEH and SHAM + TSA controls (Fig. 2C).

In order to investigate the effects of TSA administration on the gene expression levels of GR and CRH and the epigenetic changes at the GR and CRH promoters, we isolated the CeA from SHAM + VEH, SHAM +

TSA, WAS + VEH and WAS + TSA animals ( $n = 6/\text{treatment group}$ ). When analyzing the mRNA expression of GR and CRH, we observed that TSA infusions after WAS increased GR expression ( $p = 0.0022$ ) and decreased CRH ( $p = 0.0297$ ) expression when compared to the WAS + VEH group. After TSA infusions, GR and CRH expression levels resembled expression levels of SHAM + VEH and SHAM + TSA animals. We did not observe any effects of TSA infusions on H3K9 acetylation levels or GR and CRH expression in the CeA of SHAM animals (Fig. 2D and E). Analysis of the ChIP-qPCR assays revealed that TSA infusions after WAS reversed the WAS-induced epigenetic changes at the GR and CRH promoters. When compared to the WAS + VEH group, TSA infusions reduced SIRT-6 binding ( $p = 0.0002$ ), and increased H3K9 acetylation levels ( $p = 0.0220$ ) at the GR promoter (Fig. 2E–F). In addition, TSA infusions after WAS increased GR binding ( $p = 0.0044$ ), decreased CBP binding ( $p = 0.0124$ ), and lowered H3K9 acetylation levels ( $p = 0.0198$ ) at the CRH promoter (Fig. 2G–I).

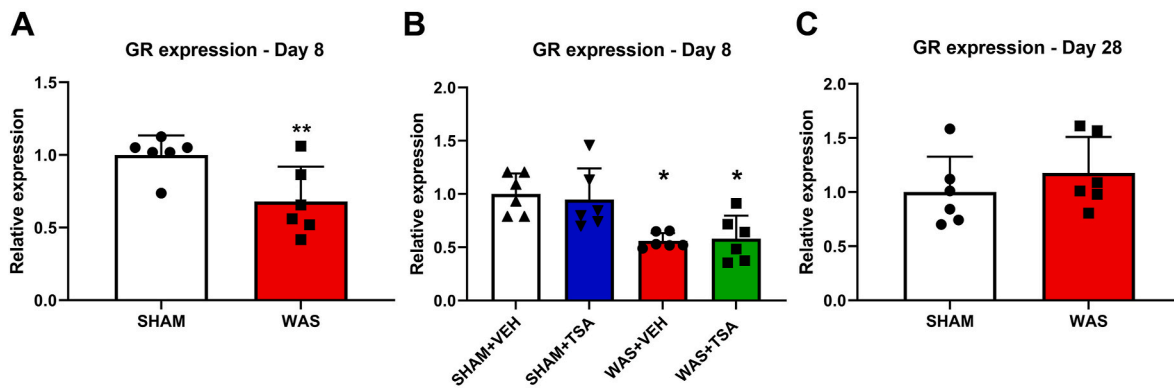
### 3.4. WAS-induced visceral hypersensitivity persists

Here, we investigated whether daily exposure for 1 h to WAS for 7 days induced persistent visceral hypersensitivity in female rats despite removal of the stressor (See Fig. 3A for experimental design). Females that were exposed to WAS had a higher total fecal pellet output than SHAM controls (Fig. 3B). When visceral sensitivity was assessed 21 days later, the analysis of VMR to CRD revealed a main effect of pressure ( $F$  (Hertig et al., 2007; Cantu et al., 2019) = 176.0;  $p < 0.0001$ ) and a main effect of chronic WAS ( $F$  (Enck et al., 2016; Kano et al., 2017) = 34.34;  $p = 0.0002$ ). Post hoc analysis revealed an increased number of abdominal contractions at distention pressures of 20 mmHg ( $p = 0.0315$ ), 40 mmHg ( $p = 0.025$ ) and 60 mmHg ( $p = 0.0017$ ) in female rats exposed to chronic WAS when compared to female rats exposed to SHAM ( $n = 6/\text{treatment group}$ ) (Fig. 3C). In order to investigate the underlying causes of persistent visceral hypersensitivity, we isolated the CeA from female animals 21 days after removal of the stressor ( $n = 6/\text{treatment group}$ ) and assessed the expression of GR and CRH using qPCR. We observed that, 21 days after WAS, GR expression was significantly decreased ( $t_{10} = 2.037$ ,  $p = 0.0325$ ), whereas CRH expression was significantly elevated ( $t_{10} = 2.292$ ,  $p = 0.0224$ ) (Fig. 3D–E).

Evidence suggests that epigenetic marks can be maintained in the absence of the initial triggering event; therefore, changes in H3K9 acetylation could have been responsible for the persistent changes in GR and CRH expression. In order to investigate the persistence of changes in histone acetylation at the GR and CRH promoters, we isolated the brain and collected the CeA then performed ChIP-qPCR assays ( $n = 6/\text{treatment group}$ ). We observed that H3K9 acetylation at the GR promoter was still decreased ( $t_{10} = 3.643$ ,  $p = 0.0023$ ), and H3K9 acetylation at the CRH promoter remained increased H3K9 ( $t_{10} = 2.826$ ,  $p = 0.0090$ ) (Fig. 3G–H) in female rats.

### 3.5. WAS-induced changes in hippocampal GR expression are not persistent

Having observed persistent changes in GR expression in the CeA of female rats exposed to chronic WAS, which were reversible with TSA infusions, we next aimed to verify whether these changes are specific to the CeA or extend to other brain regions involved in the HPA axis. When performing qPCR on hippocampal tissue, isolated 24 h after WAS, we observed a decrease in GR expression ( $t_{10} = 3.064$ ,  $p = 0.0091$ ) (Fig. 4A). The stereotaxic administration of TSA to the CeA did not affect GR expression in the hippocampus. GR expression of the WAS + TSA group was not statistically different from WAS + VEH group ( $t_{10} = 1.370$ ,  $p = 0.1003$ ) (Fig. 4B). To determine whether GR expression also remained lower in the hippocampus, we assessed GR expression 21 days after WAS. Our qPCR results showed that at this time point, there was no difference in GR expression between SHAM and WAS animals ( $t_{10} = 0.9281$ ,  $p = 0.1876$ ) (Fig. 4C).



**Fig. 4.** Changes in GR expression in the hippocampus are transient and unaffected by TSA infusions in the CeA. Hippocampal tissue was collected from the same slice as the CeA, and at the same time when the CeA was isolated. Hippocampal tissue was analyzed to analyze GR expression using real-time PCR. (A) Twenty-four h after chronic WAS exposure, GR expression in the hippocampus of female rats exposed to WAS is decreased when compared to SHAM animals. (B) GR expression in the hippocampus, 24 h after WAS, is unaffected by TSA infusions in the CeA. (C) Twenty-one days after the final WAS exposure, GR expression of female rats that had been exposed to WAS is similar to SHAM animals. Statistical differences in gene expression were assessed using an unpaired *t*-test. Significance is expressed as \**p* < 0.01.

#### 4. Discussion

The novel aspect of the current study was that we were able to attenuate chronic-stress induced visceral hypersensitivity in female rats by modulating the epigenome of the CeA. Here, we showed how chronic stress affects this key region of the limbic brain, where changes in the epigenetic modifications at the GR and CRH promoters increase visceral sensitivity. Furthermore, our results indicated that targeting the CeA of stressed female rats with the HDAC inhibitor TSA prevented stress-induced visceral hypersensitivity. On the molecular level, HDAC inhibition prevented the decrease in GR expression during chronic stress, thereby maintaining the GR-mediated negative regulation of CRH expression.

We report that chronic WAS increased binding of the HDAC SIRT-6 at the GR promoter, and decreased H3K9 acetylation at the GR promoter in the CeA from female rats. Decreases in H3K9 acetylation are associated with gene repression and these epigenetic modifications were likely responsible for the decrease in GR mRNA expression in the CeA after chronic WAS. In our previous work in male rats, we showed that stress-induced decreases in GR mRNA expression are associated with decreased GR protein expression (Tran et al., 2015). Here, we observed that less GR was bound at its regulatory regions in the CRH promoter after WAS. The disruption of GR-mediated negative regulation may have allowed the HAT CBP to interact with the AP-1 regulatory element in the CRH promoter (Arias et al., 1994; Bannister and Kouzarides, 1995; Davies et al., 2011; Lee et al., 1996). As a result, CBP could have contributed to the increased H3K9 acetylation of the CRH promoter, we observed after WAS. Since increases in H3K9 acetylation are associated with increased gene expression, these epigenetic changes could have caused the increased CRH mRNA expression in the CeA of female animals after WAS. Taken together, the decrease in GR and the consequential increase in CRH in the CeA are important mediators of chronic stress-induced visceral hypersensitivity (Johnson et al., 2015).

Since stress-induced epigenetic changes in the CeA required an active process, in which multiple epigenetic regulatory enzymes are involved, intervening in this process should prevent stress-induced visceral hypersensitivity. We have demonstrated previously that central administration of the HDAC inhibitor TSA prevented WAS-induced visceral hypersensitivity in male rats (Tran et al., 2013, 2015). In this study, we showed that direct administration of TSA into the CeA is sufficient to prevent stress-induced visceral hypersensitivity in female rats. The beneficial effects of TSA are likely due to an active intervention in the nociceptive properties of the CeA, rather than damping the CeA's ability to facilitate HPA axis activity. Although we did not measure CORT levels following the WAS procedure, we observed that the fecal pellet output

from the WAS + VEH and WAS + TSA groups was similar. These findings suggest that in both groups there was a robust activation of the HPA axis. Increased fecal pellet output has been used previously to support activation of the HPA axis (Bradesi et al., 2005; Million et al., 2000). In support, Hong et al. (2011) showed that GR antagonism prevented the WAS-induced increase in fecal pellet output, indicating that the increase in fecal pellet output during WAS depended on the interaction between CORT and GR (Hong et al., 2011). We showed previously that both daily measured plasma CORT levels and fecal pellet output remain elevated in animals exposed to WAS (Prusator and Greenwood-Van Meerveld, 2016). Interestingly, TSA administration in the CeA of SHAM animals had no effect on SIRT-6 binding, histone acetylation of the GR promoter, GR mRNA expression or visceral sensitivity. This indicates that during chronic WAS, a signaling cascade is activated that promotes the active recruitment of SIRT-6 to the GR promoter. The ensuing histone deacetylation initiates a pathway leading to visceral hypersensitivity. Our results indicate that after TSA infusions, less SIRT-6 was bound to the GR promoter and H3K9 acetylation was increased at the GR promoter in the CeA of animals exposed to WAS. TSA administration, after each WAS session maintained, GR expression at normal levels in the CeA, favoring GR binding at the CRH promoter. As a result, CBP could not bind at, and promote the H3K9 acetylation of the CRH promoter. Taken together, TSA administration in the CeA prevented the decrease of GR expression and the loss of GR-mediated negative regulation of CRH expression.

Although, TSA is non-selective inhibitor for class I and class II HDACs (Drummond et al., 2005), recent reports have suggested that TSA can also directly inhibit SIRT-6, which is a member of the class III HDAC family (Wood et al., 2018; You and Steegborn, 2018). It is plausible that TSA interacted with SIRT-6 after WAS to prevent histone deacetylation, however, we cannot exclude that TSA inhibited multiple other HDACs, and thus influenced histone acetylation levels, at other genes in the CeA, which might have contributed to the attenuation of WAS-induced visceral hypersensitivity. In addition, TSA may have exerted its anti-nociceptive effects by intervening in the upstream signaling pathway, for instance, by interaction with NF- $\kappa$ B subunits that mediated NF- $\kappa$ B signaling. Therefore, we cannot completely rule out that any of these effects contributed to the attenuation of stress-induced visceral hypersensitivity. In order to investigate whether the epigenetic effects of stress-induced visceral hypersensitivity are mediated through (solely) SIRT-6 activity at the GR promoter, our future work will concentrate on specific SIRT-6 inhibition or knockdown in the CeA.

The goal of the current study was to focus on female animals in an attempt to recapitulate the female predominance of IBS. There is ample preclinical and clinical evidence for sex differences in pain sensitivity, therefore we investigated whether these differences were caused by



sex-specific epigenetic regulation of nociceptive genes in the CeA. Our results indicate that in female animals, stress-induced visceral hypersensitivity is associated with similar epigenetic modifications at the GR and CRH promoters in the CeA, as we previously reported in male animals (Tran et al., 2015). The similarities in epigenetic regulation of GR and CRH may be due to the highly conserved function and regulation of GR and CRH in both sexes. However, the existence of a similar epigenetic mechanism in the CeA does not imply that the level of, histone acetylation is identical between males and females. For example, the CRH promoter contains several estrogen and androgen response elements, and estrogen and testosterone have opposite effects on CRH transcriptional activity (Bao et al., 2006; Figueiredo et al., 2007; Lal-mansingh and Uht, 2008; Rubinow et al., 2005). In light of the fact that epigenetic mechanisms make genes more or less accessible for transcription, it is therefore possible that estrogen receptors could further boost CRH expression through the recruitment of additional HATs, whereas testosterone and androgen receptors have the opposite effect and dampen CRH expression. This would imply that in cycling females, the stage of the estrus cycle could affect CRH expression. Our future work, will aim to investigate the role of the gonadal hormones and how they may affect epigenetic remodeling of the CeA. Furthermore, since we limited our study to the epigenetic regulation of two key genes of visceral hypersensitivity, it remains a possibility that other pro-nociceptive genes in the CeA, known to be involved in visceral hypersensitivity, show a sex-specific (epigenetic) regulation in response to stress. In support, RNA-sequencing of the dorsal root ganglia (DRG), from male and female rats exposed to nerve injury, showed that there was still a significant overlap between gene expression patterns, but that some genes showed a sex-specific regulation (Stephens et al., 2019).

The sexual dimorphism in pain perception may also be explained by stress-induced epigenetic changes that occur outside of the CeA. For instance, chronic WAS causes epigenetic remodeling at the promoter regions of genes encoding tight junction proteins in the colonic epithelial cells leading to increased colonic permeability (Wiley et al., 2020). In addition, chronic WAS also increased CpG methylation at the GR and CNR1 promoters in the L6-S2 DRG, which ultimately lead to the increase of pro-nociceptive TRPV1 (Hong et al., 2015). Intrathecal administration of the HDAC inhibitor suberoylanilide hydroxamic acid after forced swim stress increased histone 3 acetylation in the spinal cord, leading to increased expression of mGluR2 and mGluR3 and attenuation of visceral hypersensitivity (Cao et al., 2016).

Taken together, our findings suggest that stress increases visceral nociceptive transmission through persistent epigenetic remodeling in key parts of the pain pathway. In contrast to the CeA, we discovered that changes in hippocampal GR expression did not persist up until 21 days after the last stressor. These divergent gene expression profiles may reflect the role of GR in visceral pain perception in these brain regions. Although chronic stress decreases GR expression in the hippocampus, it remains unknown whether this change also leads to pro-nociceptive remodeling in the hippocampus. So far, only neonatal noxious colorectal stimulation and maternal separation induce changes in respectively hippocampal NMDA and AMPA receptors that underlie visceral hypersensitivity in adulthood (Chen et al., 2014, 2017). Whether these changes are GR-mediated and occur in the hippocampus after chronic stress in adulthood, remains to be investigated. In addition, it is possible that early life stress and chronic stress in adulthood lead to visceral hypersensitivity through different mechanisms. In our previous work in a rodent model of early life stress, we uncovered a different epigenetic mechanisms in the CeA that was responsible for visceral hypersensitivity (Louwies and Greenwood-Van Meerveld, 2020). Early life stress induced a persistent upregulation of both GR and CRH in the CeA of adult female rats (Prusator and Greenwood-Van Meerveld, 2017), through an increase in H3K9 acetylation at both the GR and CRH promoter. Since negative CRH regulation by GR was lost, visceral hypersensitivity could only be attenuated by inhibiting HATs in the CeA of female rats (Louwies and Greenwood-Van Meerveld, 2020). Here, we showed that

attenuation of visceral hypersensitivity after repetitive daily stress in adulthood relies on re-establishing the negative regulation of GR on CRH expression.

In conclusion, chronic stress causes persistent epigenetic dysregulation and concomitant changes in gene expression in the CeA of female rats. These epigenetic changes at the GR and CRH promoters are responsible for stress-induced visceral hypersensitivity that were reversed after HDAC inhibition. Our data provides a novel insight into the potential molecular mechanisms underlying stress-mediated abdominal pain in IBS patients. Stress can shift the epigenome of the amygdala towards a pro-nociceptive state, which can facilitate pain perception in these patients. This knowledge may lead to the development of future therapies that can target the epigenome for the treatment of abdominal pain in IBS patients.

#### CRedit authorship contribution statement

**Tijs Louwies:** Performed experiments, Formal analysis, Writing – original draft, Writing – review & editing. **Albert Orock:** Performed experiments, Writing – review & editing. **Beverley Greenwood-Van Meerveld:** Designed the study, Writing – review & editing.

#### Declaration of competing interest

The authors have no conflicts of interest to declare.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.yjnstr.2021.100386>.

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