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Visceral analgesia induced by acute and repeated water avoidance stress in rats: sex difference in opioid involvement

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Abstract

Background—Chronic psychological stress-induced alterations in visceral sensitivity have been predominantly assessed in male rodents. We investigated the effect of acute and repeated water avoidance stress (WAS) on the visceromotor response (VMR) to colorectal distension (CRD) and the role of opioids in male and cycling female Wistar rats using a novel non-invasive manometric technique.

Methods—After a baseline VMR (1st CRD, day 0), rats were exposed to WAS (1h/day) either once or for 4 consecutive days, without injection or with naloxone (1 mg/kg) or saline injected subcutaneously before each WAS session.

Key Results—The VMR to CRD recorded on day 1 or 4 immediately after the last WAS was reduced in both females and males. The visceral analgesia was mainly naloxone-dependent in females but naloxone-independent in males. In non-injected animals, on days 2 and 5, VMR was not significantly different from baseline in males while females exhibited a significant VMR increase at 60 mmHg on day 5. Basal CRD and CRD on days 1, 2 and 5 in both sexes without WAS induced similar VMR.

Conclusions and Inferences—When monitored non-invasively, psychological stress induces an immediate post-stress visceral analgesia mediated by an opiate signaling system in females while naloxone-independent in males, and hyperalgesia at 24 h after repeated stress only in females. These data highlights the importance of sex-specific interventions to modulate visceral pain response to stress.

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Keywords

colorectal distension; defecation; estrus cycle; manometry; naloxone; sex difference; stress-related visceral analgesia; water avoidance stress

INTRODUCTION

Stress has been recognized as an important factor in the pathophysiology of functional gastrointestinal disorders such as irritable bowel syndrome (IBS).¹ In particular, stress modulates visceral pain responses in rodents^{2,3} and participates in the exacerbation of symptoms in IBS patients.⁴ Existing evidence indicate that females are more responsive to stress^{5,6} and epidemiologic studies indicate a female predominance in IBS patients.⁷ The classical method to assess visceral sensitivity in rodents relies on monitoring the visceromotor response (VMR) to colorectal distension (CRD) by recording the abdominal contractions through electromyographic (EMG) signals captured by electrodes chronically implanted into the abdominal musculature.⁸ Using EMG recording, exposure to acute and repeated psychological stress in the form of water avoidance stress (WAS) is reported to induce visceral hyperalgesia in rats and mice.^{9–12} However, using a non-invasive method based on manometry and allowing animals to be group housed, we recently showed in male mice and rats that repeated WAS (rWAS) induced a strong reproducible analgesia immediately after the end of the stress period, while animals surgically equipped with EMG electrodes and single housed thereafter developed visceral hyperalgesia in response to rWAS.^{10,13} This suggests that the VMR to CRD after exposure to rWAS is influenced by pre-existing conditions induced by methods used to monitor visceral sensitivity in male rodents.¹⁰

Mechanisms underlying stress-related visceral analgesia in the non-invasive setting are still to be investigated. The ability of a variety of stressors to activate endogenous analgesic systems, a phenomenon referred to as stress-induced analgesia has been extensively delineated in the somatic pain field.^{14,15} In particular, there is evidence of opiate-dependent and -independent pathways recruited differentially according to the modalities of stress procedures, the pain test used and the genotype of the animal under investigations.^{14–16} While mild psychological stress-induced somatic analgesia in naïve animals engages the opioidergic system^{14,17–19} it was recently shown in rats that were previously exposed to pain (surgery, inflammation) that endogenous opioids can also paradoxically be associated with the development of stress-induced somatic hyperalgesia via the recruitment of N-methyl-D-aspartic acid-dependent pronociceptive systems.²⁰ Interestingly, postsurgical somatic hyperalgesia following hind paw incision has been found to be associated with long-lasting visceral hypersensitivity in male rats.²¹ This, coupled with our recent observations in mice suggest that studies assessing the influence of a stressor in animals previously exposed to surgery for EMG electrodes implantation may have been biased by surgery-associated factors, including postsurgical treatments and single housing vs no surgery.¹⁰ Lastly, despite the well known sex specific differences in stress responses and pain modulation, particularly at the opioidergic system level,^{14,17–19,22,23} most of the preclinical studies on stress and visceral sensitivity thus far have been largely performed in male rodents only.^{24,25}

Based on the above, the aims of the present studies were (1) to characterize the influence of acute WAS and rWAS immediately or 24 h after the last exposure to the stressor on the VMR to CRD using a recently validated, novel non-invasive method based on manometric measures in naïve rats,¹³ (2) to determine potential sex differences in acute WAS or rWAS-induced alterations in visceral sensitivity immediately and 24 h after the end of WAS, and

(3) to evaluate the mechanisms involved in visceral response to stress, with a special focus on the role of opioid receptors in both male and female rats.

MATERIALS AND METHODS

Animals

Adult male and cycling female Wistar rats (6–7 weeks old; 240–300 g and 175–220g, respectively, Harlan Laboratory, Indianapolis, IN, USA) were group-housed (2–4/cage) and maintained under standard conditions of illumination (12:12 h light/dark cycle, lights on at 6:00 am) with water and chow (Purina®) *ad libitum* (total isoflavones: 574.5 aglycone equiv.) and enrichment (plastic tubes) in each cage. They were acclimated to the animal facility for 1 week before starting the experiments which were conducted during the light phase. Experiments followed NIH guidelines according to protocol # 11084-03 approved by the Institutional Animal Care and Use Committee of the Veteran Affairs Greater Los Angeles Healthcare System under the auspice of the Office of Laboratory Animal Welfare - Assurance of Compliance (A3002-01).

Vaginal Smear

Female rats were briefly anesthetized with isoflurane (~3 min, 3% in O₂) and the stage of estrous cycle was determined daily between 8:00 and 10:00 am over 2 cycles. Gentle vaginal wash/lavage was made with 100 µL of saline by using a pipette and wet smears were examined under microscope.^{26,27} The cell samples were classified into a particular phase of the estrous cycle according to the following identification criteria found in the samples: proestrus: presence of a majority (greater than 80%) of round, nucleated epithelial cells; estrus: a majority of cornified epithelial cells; metestrus: large amount of leukocytes and some round and cornified epithelial cells; diestrus: the absence of cells, although there were small amounts of all 3 cell types (leukocytes, round and cornified epithelial cells) present.^{26–29} Only rats with at least two consecutive regular 4-day cycles were included in the data analysis.

Water avoidance stress

Water avoidance stress was performed as described before.³⁰ It consisted of placing rats (males: 242–317g; females: 175–217g) individually for 1 h either once (acute WAS) or daily for 4 consecutive days (rWAS) between 8 and 10 am, on a rectangular platform (5.8-cm length × 5.8-cm width × 6.0-cm height for males, 5.0-cm length × 5.0-cm width × 6.0-cm height for females, AMAC box M series #510C and #522C+#754C, respectively, AMAC Plastic, Petaluma, CA) affixed in the center of a container (26.7-cm length × 48.3-cm width × 20.3-cm height, R20 rat cage, Ancare, Bellmore, NY) and filled with room temperature water (25°C) up to 1 cm from the top of the pedestal. To avoid variation in the stress response linked to a more comfortable position on the pedestal of female rats that have a smaller body weight than males, we used platforms of different sizes for males and females such that the ratio of body weight/surface was around 7–9 g/cm² for both sex. Non-stressed rats were kept in their home cage and handled daily (5 min). Rats body weight (bw) was monitored daily before each stress session.

Measurement of defecation and visceral pain

Fecal pellet output—In rats subjected to WAS, fecal output was monitored as the total number of pellets expelled for the 1-h period of stress exposure on a single exposure or each day for rWAS. For rWAS, mean 1-h output was calculated by averaging the total number over the 4 days. To account for weight differences between male and female rats, defecation was expressed per 100g bw. Basal defecation of rats was determined in separate groups of

animals at the same time of the day during which stress experiments were performed. Defecation was monitored in animals kept for 1-h in their home cage, after a quick bedding change.

Assessment of visceral pain response to CRD—This was assessed using the noninvasive manometric method that we have recently developed and validated for use in mice and rats.^{10,13,31} Briefly, a PE50 catheter was taped 3.5 cm caudal to the pressure sensor of a miniaturized pressure transducer catheter (SPR-524 Mikro-Tip catheter; Millar Instruments, Houston, TX). A custom-made balloon (2 cm wide × 5 cm long),^{31,32} prepared from an infinitely compliant polyethylene plastic bag was tied over the catheter at 1 cm below the pressure sensor with silk 4.0 (Henry Schein Inc., Melville, NY). At the beginning of each experiment, each “balloon-pressure sensor” was calibrated at known pressures of 0, 20, 40 and 60 mmHg using a barostat (Distender Series II, G&J Electronics Inc, Toronto, Canada), and voltage output was converted to pressure using a digital analog convertor (Micro1401, Cambridge Electronic Design, Cambridge, UK) and Spike 2 software (CED, Ltd., Cambridge). On the day of the experiment, rats were briefly anesthetized with isoflurane (3% in O₂) and the lubricated “balloon-pressure sensor” catheter was introduced into the colorectum such that the distal end of the balloon was positioned at 1 cm from the anus and the catheter was secured to the tail with tape. Each animal was placed in a Bollman cage, to which they had been habituated for 3 consecutive days prior to the experiment (1h/day), covered with a light tissue blanket and left to rest for 30 min before the CRD procedure. Each balloon was connected to the barostat and the miniaturized pressure transducer to a preamplifier (model 600; Millar Instruments, Houston, TX). The intracolonic pressure (ICP) signal was acquired using CED Micro1401/SPIKE2 program. The CRD protocol consisted of two CRDs at 60 mmHg to unfold the balloon, immediately followed by two consecutive series of graded phasic distensions to constant pressures of 10, 20, 40 and 60 mmHg (20 s duration, 4 min inter-stimulus interval within and between series). A similar CRD paradigm has been used previously to assess visceral pain-related responses in rats.^{13,31}

Data analysis—The phasic component of the intracolonic pressure (pICP) was extracted from the ICP signal recorded by applying the “DC Remove” Process in Spike 2 with a time constant of 1 s, to exclude the slower, tonic changes in ICP resulting from colonic smooth muscle activity, and by applying the “root mean square amplitude” process with a time constant of 1 s to the resulting trace. The VMR was defined as the increase in the area under the curve of pICP during CRD over the mean value of pre- and post-distension 20 s periods and was quantified using the “modulus” process in Spike 2. As each CRD pressure was repeated twice, the pre, during and post CRD values were averaged for each pressure. To examine the pressure-response relationship and adjust for inter-individual variations of the signal,³³ ICP amplitudes were normalized for each rat to the highest pressure (60 mmHg) in the 1st set of baseline CRD. This value served as 100% response (control) in the baseline period of data collection before exposure to WAS or treatment. The VMR to the consecutive CRDs was expressed either as % from their baseline values or mean change from the baseline response (Δ VMR in %) at different pressures of distension as validated in our previous studies.^{10,31,34}

Experimental protocols

Experiments (WAS and CRD) were performed in the morning, between 8 am and 12 pm each day to avoid variations due to the circadian rhythm. Body weight and fecal pellet output were monitored daily before and after each WAS session respectively. Vaginal smears were taken in each female before the intracolonic insertion of the balloon.

Influence of acute and rWAS on visceral sensitivity in male and female rats—

Naïve male and cycling female rats were subjected to the 1st set of CRD on day 0, during which the baseline VMR was measured. Then, animals were divided into two groups. The first group (10 males and 11 females) was exposed to an acute 1h WAS only. The second group (13 males and 15 females) was exposed to rWAS (1 h/day for 4 consecutive days). A 2nd set of CRD was performed at 45–50 min after the WAS exposure on day 1 (1st group) or day 4 (2nd group). A 3rd set of CRD was performed 24 h after the last WAS exposure, on day 2 (1st group) or day 5 (2nd group), respectively.

Influence of repeated colorectal distensions on visceral sensitivity in control male and female rats—

According to the experimental protocol used in the stressed animals, in two groups of naïve male (n = 3–5/group) and female (n = 4/group) rats (each non-stressed and non-injected group), we performed baseline VMR (1st CRD on day 0), and additional CRDs on days 1 and 2 or on days 4 and 5, respectively.

Influence of naloxone on the modulation of visceral sensitivity induced by acute and rWAS in male and female rats—

The same protocol of CRD as above was used in separate groups of naïve male and cycling female rats exposed to acute vs rWAS. An additional step was implemented 10 min before each stress session, where animals were injected subcutaneously (sc) with saline (0.3 ml, pH~7.0) (9–12 males) or naloxone (1 mg/kg, pH~7.0) (9 males, 8–10 females), an opioid receptor antagonist with high antagonist activity against mu, kappa and delta opioid receptors.³⁵ In all rats, a 2nd set of CRD was performed 45–50 min after the end of WAS, on day 1 or 4, and a 3rd set of CRD 24 h after the last exposure to WAS on day 2 or 5, respectively. The dose of naloxone was chosen based on a previous report showing full blockade of morphine-induced visceral analgesia in rats.³⁶

Statistical Analyses

Statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Differences in weight gain over time in each group and between groups were analyzed using repeated measures one-way ANOVA followed by Dunnett's *post hoc* test. Comparison of the mean defecation under basal conditions and following 1 or 4 days of WAS between groups was made using one-way ANOVA followed by Bonferroni *post hoc* test and differences between males and females in each group was assessed by unpaired Student's t-test.

Statistical analyses examining the effects of acute and rWAS on VMR as well as the modulating influence of naloxone or saline were performed using the general linear mixed models for repeated measures in SAS 9.2. The general linear mixed models take into account the specification of a covariance structure for within-subject correlation over time yielding more precise estimates and standard errors. In the first two independent samples of animals exposed to acute or rWAS, the VMR was regressed on sex (male, female), distension parameters (10, 20, 40 and 60 mmHg) and days (0,1,2 or 0,4,5) and the interaction of each variable in a full factorial model with day 0 (baseline) as the time referent. The parameter estimates from the sex*distension*day interaction term represent effects of acute or rWAS at specific levels of distension on days 1/4 or days 2/5 (change in VMR responses from baseline to days 1/4 or day 2/5 for each distension in terms of standard deviation units). Within and between group changes were assessed using planned linear contrasts and interpreting significance at p<0.05. The influence of naloxone was tested by two additional general linear mixed models by adding treatment (naloxone, saline) as a factor designating the full factorial specification with all interaction effects as well as direct

comparisons between saline and naloxone treatments. Results are expressed as mean $\pm$ S.E.M, and p values < 0.05 were considered statistically significant.

RESULTS

Water avoidance stress and visceral sensitivity

Acute and rWAS induce immediate visceral analgesia in male and female rats while rWAS induces delayed visceral hyperalgesia in females only—Colorectal distension induces a stimulus intensity-dependent increase in the VMR of female and male rats as recorded by intraluminal colonic pressure (Fig. 1A). The baseline VMR to the 1st CRD at 10, 20, 40 and 60 mmHg was similar in naïve male and female rats (VMR AUC/min: 1.5 ± 1.4 , 21.5 ± 7.7 , 56.1 ± 12.4 and 70.5 ± 11.5 in males vs 4.0 ± 2.7 , 17.6 ± 5.9 , 64.8 ± 10.04 and 64.0 ± 6.6 in females respectively, $p > 0.05$). Two-way ANOVA showed a significant effect of pressure of CRD [$F(3, 76) = 30.22$, $p < 0.0001$] but no interaction between pressure and sex [$F(3, 76) = 0.3541$, $p = 0.7863$]. Comparisons were also performed according to the stage of the estrus cycle in females (Table 1), and 2-way ANOVA showed a significant effect of pressure of CRD [$F(3, 232) = 97.75$, $p < 0.0001$] but no interaction between pressure and estrus cycle stage [$F(9, 232) = 0.3374$, $p = 0.9618$].

On day 1, 45–50 min after the end of 1h-WAS session, female rats exhibited a significant decrease in the VMR to CRD compared to baseline at 40 and 60 mmHg (VMR: $52.9 \pm 11.5\%$ vs $111.8 \pm 24.6\%$ and $69.1 \pm 11.1\%$ vs $100.0 \pm 0.0\%$, $p < 0.0001$ and $p < 0.05$, respectively, Fig. 1B) while males showed a decrease in VMR to CRD only at the pressure of 40 mmHg (VMR: $47.6 \pm 8.6\%$ vs $88.6 \pm 10.7\%$, $p < 0.0001$, Fig. 1C). The magnitude of analgesia at 40 mmHg was similar in females and males ($43.7 \pm 10.1\%$ vs $40 \pm 14.3\%$, respectively, $p > 0.05$; Fig. 1B–C). The immediate visceral analgesia (significant decreased VMR below baseline in response to CRD 40 mmHg) developed in 82% of female and 90% of male rats subjected to an acute session of WAS. On day 2, 24 h after the single session of WAS, the VMR was no different from baseline values in females (Fig. 1B) and males (Fig. 1C).

In separate groups of animals exposed to rWAS for 4 days, on day 4, 45–50 min after the last session of 1-h WAS, there was a significant decrease in the VMR to CRD at 40 mmHg compared to baseline in both female and male rats (VMR: $34.7 \pm 5.8\%$ vs $99.2 \pm 7.4\%$ in females, $n = 15$; $p < 0.0001$; Fig. 1D; and VMR: $44.4 \pm 10.1\%$ vs $86.0 \pm 9.6\%$ in males, $n = 13$; $p < 0.001$; Fig. 1E). The immediate visceral analgesia developed in 93% of female and 85% of male rats subjected to rWAS. There was no sex difference in the analgesic response to rWAS for 4 days as monitored just after the last session (VMR: females: $60.2 \pm 9.4\%$ vs males: $35.3 \pm 19.4\%$, $p > 0.05$; Fig. 1D,E). On day 5, 24 h after the last 1-h exposure to rWAS, the VMR to CRD was significantly increased at 60 mmHg in female rats (VMR: $124.1 \pm 17.74\%$ vs $100.0 \pm 0.0\%$, $n = 15$; $p < 0.05$; Fig. 1D) while back to baseline values in males (Fig. 1E). There was no sex difference in the VMR as monitored 24h after the last session.

Repeated colorectal distensions did not influence visceral sensitivity in control male and female rats—When exposed to 3 repeated CRD (days 0, 1 and 2 or days 0, 4 and 5), males - days 0, 1 and 2 [$F(6, 28) = 1.611$, $p = 0.1810$] and days 0, 4 and 5: [$F(6, 41) = 1.531$, $p = 0.7691$] - or females - days 0, 1 and 2 [$F(6, 36) = 0.6202$, $p = 0.7127$] and days 0, 4 and 5: [$F(6, 36) = 0.1951$, $p = 0.9761$] - did not show any change in their VMR over time (Table 2).

Sex difference in the naloxone dependence of acute and rWAS-induced analgesia Saline pretreatment—In sc injected rats, at 45–50 min after the end of the 1st

session of WAS, female rats showed decreased VMR only at 60 mmHg ($p < 0.01$; Fig. 2A) while male rats did not show significant changes in their VMR compared to baseline ($p > 0.05$; Figs. 2B). On day 2, 24 h after the acute exposure to WAS and saline sc injection, female rats showed an increased VMR at both 40 and 60 mmHg compared to baseline ($101.6 \pm 22.5\%$ vs $67.8 \pm 11.2\%$, $p < 0.01$ and $138.2 \pm 14.9\%$ vs $100.0 \pm 0.0\%$, $p < 0.01$, respectively) (Fig. 2A), while males still did not show any change in their VMR to CRD (Fig. 2B).

In separate groups of animals exposed to daily WAS for 4 days, 45–50 min after the last session of rWAS, compared to non-injected groups, both female and male rats injected sc with saline 10 min before each WAS exposure exhibited a visceral analgesic response to CRD that was present at both 40 mmHg and 60 mmHg in females (VMR: $40.4 \pm 6.9\%$ and $66.5 \pm 12.2\%$ vs $80.8 \pm 12.2\%$ and $100.0 \pm 0.0\%$ Fig. 3A) and males ($19.3 \pm 7.1\%$ and $71.4 \pm 7.1\%$ vs $59.9 \pm 9.6\%$ and $100.0 \pm 0.0\%$, Fig. 3B). On day 5, 24 h after the last WAS, the VMR to CRD was back to baseline in both females (Fig. 3A) and males (Fig. 3B). There was no sex difference in the analgesic response to rWAS in animals pretreated with sc saline before each WAS session.

Naloxone pretreatment—In females, when compared to saline sc injected groups, naloxone pretreatment (1 mg/kg, sc) prevents 1-h WAS-induced decrease in VMR to CRD at 60 mmHg as monitored within 45 min after WAS (Fig. 2A,C). This is shown by the VMR values that were significantly lower than basal at 60 mmHg in animals injected with saline (Fig. 2A) while in the naloxone-pretreated group, values did not differ from initial basal levels nor were significantly different from the value of saline-treated animals ($p < 0.05$). In male rats, naloxone did not affect the VMR and values were similar to baseline and to those of the saline treated group (Fig. 2B,D).

Interestingly, in female rats exposed to rWAS, compared to saline-injected females, naloxone pretreatment blocked the analgesic response at day 4 as shown by similar VMR compared to baseline and significant difference compared with saline-treated group. However the analgesia was still present at 60 mmHg and induced at 60 mmHg at day 5 (Fig. 3C) ($67.8 \pm 11.0\%$ vs $100.0 \pm 0.0\%$, $p < 0.01$). In contrast, naloxone pretreatment did not affect the analgesia in response to rWAS observed in male rats (Fig. 3D).

When assessed 24h post a single exposure to WAS on day 2, or rWAS on day 5, naloxone treatment significantly reduced the VMR to CRD at 40 and 60 mmHg compared to the group injected with saline in females ($p < 0.05$ and $p < 0.0001$, for day 2 and $p < 0.001$ each for day 5, respectively, Fig. 2C,3C). In contrast, naloxone and saline did not differ significantly in their influence on the visceral pain response of male rats 24h post acute WAS or rWAS. (Figs. 2D,3D).

In each experiment, female rats were spread across the different stages of estrus cycles (Fig. 2A,C, 3A,C).

Water avoidance stress and defecation

Sex difference in acute and rWAS-induced defecation—The mean basal defecation in female and male rats kept in home cages for the 1 or 4 day experimental period remained consistent (1.1 ± 0.4 vs 0.8 ± 0.1 and 0.7 ± 0.2 vs 0.6 ± 0.1 pellets/h/ 100g bw, respectively; $p > 0.05$; Fig. 4A–B). WAS exposure 1 h/day for 1 or 4 days increased the mean defecation score compared with values of female and male rats not exposed to stress (4.6 ± 0.5 vs 1.1 ± 0.4 and 3.2 ± 0.3 vs 0.7 ± 0.2 pellets/h/ 100g bw in females, $p < 0.01$ and $p < 0.001$; 2.1 ± 0.4 vs 0.8 ± 0.1 and 2.2 ± 0.1 vs 0.6 ± 0.1 pellets/h/ 100g bw in males $p < 0.01$ and $p < 0.001$; at 1 and 4 days of WAS, respectively) (Figs. 4A,B). Defecation was significantly higher in

females compared to males in response to one (Fig. 4A) or four (Fig. 4B) exposures to WAS ($p < 0.001$ each) whether FPO was expressed per animal (data not shown) or per 100g bw. The estrous cycle did not significantly affect WAS-induced defecation in female rats (7.1 ± 0.9 , 5.5 ± 1.0 , 8.6 ± 1.2 and 6.9 ± 0.9 pellets/h for proestrus, estrus, metestrus and diestrus, respectively [$F(5, 43) = 11.40$, $p > 0.05$]).

Modulatory influence of opioid pathway in the defecation response to rWAS in male and female rats—The defecation response in animals exposed to an acute session of WAS (1 day) and pretreated with naloxone, saline or non-injected did not significantly differ in either females (3.1 ± 0.4 and 3.6 ± 0.5 vs 4.6 ± 0.5 pellet/h respectively; [$F(2, 32) = 5.617$, $p > 0.05$]) or males (2.4 ± 0.3 and 2.2 ± 0.1 vs 2.1 ± 0.4 pellet/h/ 100g bw, respectively; [$F(2, 30) = 0.2086$, $p > 0.05$]) (Fig. 4A), despite a tendency for females to decrease in defecation with saline and naloxone sc injections. However, the sex difference observed in defecation in the non-injected animals ($p < 0.001$) was smaller in animals injected subcutaneously with saline ($p < 0.05$) and no longer observed in animals injected with naloxone (Fig. 4A).

Compared to non-injection conditions, pretreatment with naloxone or saline did not affect the average defecation response to 4 days of rWAS exposure in either males (1.7 ± 0.2 and 2.1 ± 0.2 vs 2.2 ± 0.1 pellet/h/100g bw for naloxone and saline vs no injection, respectively; [$F(2, 30) = 0.7250$, $p > 0.05$]) or females (3.6 ± 0.3 and 3.2 ± 0.2 vs 3.2 ± 0.3 pellet/h/100g bw for naloxone and saline vs no injection, respectively; [$F(2, 31) = 0.3109$, $p > 0.05$]) (Fig 4B).

Influence of repeated stress on body weight gain in male and female rats

Male rats had a significant higher body weight than females (263 ± 6 g vs 200 ± 4 g, $n = 14$ – 16 , $p < 0.0001$) at the beginning of the experiment. Both males and females exposed to rWAS showed a consistent increase in body weight gain over time ($5.9 \pm 0.7\%$, $n = 14$ and $2.7 \pm 0.8\%$, $n = 16$ at WAS day 4, respectively) that was significantly different between males and females at days 4 and 5 ($p < 0.05$, data not shown).

DISCUSSION

We showed that exposure to acute or repeated mild psychological stress in the form of 1-h WAS alters the visceral sensitivity to CRD in a time and sex-dependent manner. Immediately after exposure to 1 or 4 sessions of WAS, both male and female rats exhibited visceral analgesia that was partially naloxone-sensitive in females but naloxone-independent in males. Twenty-four hours after the last rWAS session, females, but not males, exhibited a naloxone-dependent visceral hyperalgesia, revealing sex differences in psychological stress-induced immediate and delayed alterations of visceral sensitivity, which appear to be mediated by endogenous opioidergic mechanisms.

Single and 4-day exposures to 1-h WAS reduced the VMR to CRD in both male and female non-treated rats when monitored after the stress session. These data extend the characterization of WAS-induced visceral analgesia to female rats and to different chronicity of rWAS. We previously reported using similar VMR non-invasive monitoring that 1 or 10 days of 1-h WAS results in an immediate analgesic response to CRD in male rats.¹³ In humans as well, increases of sensory thresholds in response to rectal balloon distension was shown in healthy controls exposed to acute mental stress.³⁷ However while both male and female rats display decreased VMR to CRD immediately after exposure to acute or rWAS, there were significant qualitative sex differences in the analgesic responses in function of the CRD pressure, the addition of a mild stressor and the influence of naloxone. The visceral analgesia induced immediately after an acute 1-h WAS occurred at both 40 mmHg and 60 mmHg of CRD in females and only at 40 mmHg in males. Moreover, the analgesic response

was no longer observed in males when a sc injection of saline was applied before the 1-h WAS while in females the decreased VMR to CRD at 60 mmHg was maintained under these conditions. As the basal VMR to CRD are similar in both male and female rats, the sex differential characteristics of the VMR to CRD are related to previous stressful environmental conditions which engage sex-modulated neurobiological systems subserving visceral analgesia.²² A common feature of many environmental changes that induces an antinociceptive response is aversion or fearfulness.³⁸ Therefore it can be speculated that females engage more antinociceptive circuits due to greater fearfulness/anxiety to WAS exposure either alone or with sc injection than males. In support of this possibility, defecation, which has been used early on as an index of fearfulness/anxiety in rodents exposed to unfamiliar environments^{39,40} is significantly increased in females than males in response to 1-h WAS alone or with sc injection of saline. We previously established that components of defecation induced by 1-hWAS involved corticotropin releasing factor (CRF) receptor dependent activation of the locus coeruleus and subcoeruleus.^{30,41} In addition, recent evidence established that the locus coeruleus arousal system is sexually dimorphic at the molecular and neuronal structural levels with females displaying increased dendritic structures allowing for increased receipt and processing of limbic information compared to males which is linked with the CRF signaling system.⁵ Therefore, it can be speculated that the environmental stress-related higher activation of the locus coeruleus in females versus males may enhance the recruitment of descending noradrenergic analgesic pathways which are well established to play a key role in spinal nociceptive processing.⁴² Regardless of mechanisms, our results indicate that acute exposure to WAS results in a more robust visceral analgesic response in female compared to male rats that may have a bearing with sex difference in the stress responsiveness to WAS. In clinical studies, a deficit in diffuse noxious inhibitory controls has been identified to be a risk factor for developing chronic pain syndromes such as IBS or fibromyalgia^{43–46} and may account for female predominance in these disorders.^{7,47}

Stress-induced analgesia has been extensively studied in somatic pain compared to the paucity of reports in the visceral pain field.¹⁴ Ovariectomy has been shown to significantly reduce stress-related analgesia suggesting that gonadal steroids appear to facilitate stress-related somatic analgesic responses in female rats.⁴⁸ However, results on sex-differences in the expression of somatic analgesia in rats showed variability across studies with females displaying a more pronounced,⁴⁹ lesser,⁵⁰ or equipotent⁵¹ analgesic response than males. Procedural variables that may account for these discrepancies have been related to the type of stressors, somatic nociceptive tests used to assess analgesia and genotypes influencing sex-related components of supraspinal descending inhibitory pain pathways and/or spinal gating processes.^{14,15,22,49,51}

Additional sexual dimorphism in the visceral analgesia induced by WAS relates to the mediation by distinct mechanisms that can be dissociated neurochemically. In male rats, the unchanged VMR to CRD by naloxone pretreatment before rWAS is indicative of opiate-independent mechanisms. By contrast, in females, the visceral analgesia arising from acute or rWAS exposure is partially opiate-dependent except at the highest CRD pressure under conditions of rWAS. This mixed opioid/non-opioid analgesia is in keeping with a distinct naloxone-insensitive antinociceptive response to somatic pain occurring under conditions of more intense or chronic stress and increased intensity of the noxious stimulus used in the pain test.¹⁵ While the present study provides the first report of sex-specific mechanisms underlying stress-induced visceral analgesia, a sexual dimorphism has been extensively studied in the somatic pain field.^{49,51,52} A number of neuromodulatory mechanisms have been associated with the mediation of non-opioid stress-induced somatic analgesia including serotonergic, adrenergic or cannabinoidergic systems, as well as peptide transmitters.^{15,53–58} A recent study indicates that naloxone-independent male-specific somatic analgesia is linked

with an interaction with vasopressin 1a receptor.⁵⁹ Regarding the stress-induced visceral analgesia, only one study has shown a non-opioid, neurotensin-mediated, analgesia in rodents in response to a combined forced swimming in cold water and WAS.⁶⁰ The scope of the present study did not include addressing the role of these above specific pathways in the observed sexually dimorphic WAS-induced visceral analgesic responses. However whether they are involved in the naloxone-insensitive WAS-induced visceral analgesia in male rats and the sexual dimorphism in the response will be further investigated.

Sex difference in VMR to CRD was also observed when assessed 1 day after exposure to 1-h WAS with females developing hyperalgesia to CRD. By contrast, males exhibited values similar to those of basal levels 24 h after a single or 4 days of WAS without or with sc injection. A similar normalization of the VMR to CRD occurred also 24 h after WAS 1-h/day applied for 10 days in male rats showing the lack of delayed visceral hyperalgesia.¹³ This contrasts with the previously reported development of visceral hyperalgesia 24 h after an acute (1 day) or repeated (10 days) exposure to WAS in male rats.^{9,61} However, the VMR in these studies was tested using the mainstay method of electromyographic recording. We have recently shown that male mice equipped with electromyographic electrodes and single housed thereafter develop visceral hyperalgesia in response to rWAS while those tested non-invasively and group housed exhibit a strong visceral analgesia.¹⁰ Our previous work suggests that the combination of surgery and post-surgical social isolation contribute to a higher sensitization of VMR by stress in electrode-equipped male rodents. In fact, postsurgical hyperalgesia has been associated with long-lasting visceral hypersensitivity in male rats.²¹ By contrast female rats, 24 h after rWAS and CRD at 60 mmHg or sc saline injection + acute WAS and CRD at 40 or 60 mmHg, display enhanced VMR to CRD. In addition the hyperalgesia observed 24 h after acute WAS combined with sc injection of saline was partially prevented by naloxone. Because repeated CRD under the same conditions (basal, days 4 and 5) without WAS lead to a reproducible similar VMR in cyclic female rats, the switch from WAS-induced analgesia immediately after WAS to hyperalgesia 24 h later in females is driven by the previous exposure to the environmental stressor and not to an hyperalgesic response to repeated CRD. These data further underlie the sex difference in stress-related circuitries that impact on pain modulation.²² The naloxone-sensitive analgesia and hyperalgesia in female rats may be consistent with the dual actions of opioids that cause paradoxical pain amplifications upon their withdrawal,⁶² while the opiate-independent stress-induced visceral analgesia in males is not subjected to this dual effect. However, further experiments are needed to ascertain these mechanisms.

In summary, our results provide evidence that single or rWAS reduced the VMR to CRD in both males and females. However, sex differences in the modality of the stress-induced visceral analgesia are present, with females showing a more robust analgesia after an acute exposure that was partially naloxone-sensitive, while in male rats the analgesic response arising from rWAS is opiate-independent. In addition, only females developed a delayed hypersensitivity in response to a previous exposure to an acute sc injection + WAS or rWAS which is largely naloxone-sensitive. The present findings provide an experimental model to assess sex-related opiate-dependent and independent mechanisms underpinning stress-related activation of endogenous analgesic system that impacts on visceral pain. In addition the different neurochemical substrates of stress-induced visceral analgesia in males and female rats highlight the importance of sex-specific interventions to modulate visceral pain based on differential underlying analgesic mechanisms. This opens new venues to delineate the dual anti-and pro- nociceptive role of endogenous opiate in stress-related modulation of visceral pain in female rats.

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Abbreviations

CRD	colorectal distension
CRF	corticotropin releasing factor
EMG	electromyography
IBS	irritable bowel syndrome
ICP	intracolonic pressure
rWAS	repeated water avoidance stress
VMR	visceromotor response
WAS	water avoidance stress.

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Figure 1.

Visceral analgesic responses induced by an acute or repeated exposure to water avoidance stress (WAS) in female and male rats: **A:** Representative raw traces of baseline intracolonic pressure recording in response to 10, 20, 40 and 60 mmHg CRD in a female and a male rat. Black bars represent the distension (20 s in duration). **B,C:** An acute session of WAS induces an immediate visceral analgesia to colorectal distension (CRD) at 40 mmHg in both male and female and only females exhibit analgesia at 60 mmHg (day 1); 24 h later (day 2), both females and males are back to normal (baseline values), * $p<0.05$, *** $p<0.001$ vs respective CRD baseline. **D,E:** Repeated water avoidance (rWAS) for 4 days induces a strong visceral analgesic response at 40 mmHg in both female and male rats immediately after the last session of stress (day 4). Twenty-four hours later (day 5), females exhibit a visceral hyperalgesia at 60 mmHg while males VMR is back to normal (baseline values), * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs respective CRD baseline. Data are mean $\pm$ SEM of VMR expressed as % of 60 mmHg response to the 1st CRD; $n=10-15$ as indicated in parenthesis for each group. P: proestrus, E: estrus, M: metestrus, D: diestrus.

Figure 2.

Influence of saline or naloxone injection on visceral analgesic response to an acute exposure to WAS in female and male rats. In female rats injected subcutaneously with saline before the 1-h WAS session, the immediate visceral analgesia to CRD occurred at 60 mmHg when tested 45 min after the last session of stress (day 1; **A**), while males do not exhibit changes in their visceromotor response (VMR) (**B**); 24-h later at day 2, males VMR is still similar to baseline values (**B**) while females developed a visceral hyperalgesia to CRD that is significant at 40 and 60 mmHg (**A**), ** $p < 0.01$ vs CRD baseline. When compared to saline-injected groups (**A,B**), naloxone injected subcutaneously once before WAS partially prevents the visceral analgesia exhibited by female rats at 60 mmHg immediately after the 1st session of WAS (**C**) but does not affect the VMR of male rats which is similar to baseline (**D**); 24 h later, naloxone prevents the development of delayed visceral hyperalgesia in females (**C**), while still not affecting males VMR (**D**). Data are mean $\pm$ SEM of VMR expressed as % of 60 mmHg response to the 1st CRD; $n = 9-12$ as indicated in parenthesis for each group. P: proestrus, E: estrus, M: metestrus, D: diestrus.

Figure 3.

Influence of saline or naloxone injection on visceral analgesic response to repeated exposure to WAS (rWAS) in female and male rats. rWAS induced a visceral analgesia to CRD at 40 and 60 mmHg when tested immediately after the last session of stress (day 4) in female (A) in male (B) rats injected subcutaneously with saline before each stress session compared to baseline. Twenty-four hours later (day 5), both female (A) and male (B) VMR are back to baseline values, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs respective CRD baseline. Compared to saline-injected groups (C,D), naloxone injected subcutaneously before WAS prevents the development of visceral analgesia in female rats at 40 mmHg (C) but has no influence on the visceral analgesic response of males immediately after the 4th session of WAS nor the return to basal 24 h later (D). Furthermore, 24 h later, there was a visceral analgesia at 60 mmHg in female rats (C), ** $p<0.01$, *** $p<0.001$ vs respective CRD baseline. Data are mean $\pm$ SEM of VMR expressed as % of 60 mmHg response to the 1st CRD, $n=8-9$ as indicated in parenthesis for each group. P: proestrus, E: estrus, M: metestrus, D: diestrus.

Figure 4.

Mean defecation of male and female rats subjected to no stress or WAS for 1 (**A**) or 4 (**B**) consecutive days pretreated with either saline or naloxone or non-injected. Mean defecation was significantly increased over the course of WAS (1 or 4 days) compared to basal (home cage) defecation (**A**, **B**). Females had a significantly higher defecation compared to males on the first and fourth exposure to WAS (**A**, **B**). **A**: Saline or naloxone pretreatment did not significantly influence the defecation response to acute (A) or rWAS (B) in either male or female rats, $p < 0.05$ and *** $p < 0.001$ vs female same WAS day, + $p < 0.05$ and ++ $p < 0.01$ vs respective basal. **B**: None of the pretreatment significantly affected the defecation response to 4 days rWAS in either males or females, *** $p < 0.001$ vs female same WAS day, + $p < 0.05$, ++ $p < 0.01$ and +++ $p < 0.001$ vs respective basal. Each bar represents the mean $\pm$ SEM of number of rats indicated at the bottom.

Visceromotor response to repeated colorectal distension in naïve female rats at different stages of the estrous cycle

Table 1

Estrous stage	VMR (%) ^a				
	n	CRD (mmHg)			
		10	20	40	60
Proestrus	6	2.0 ± 1.7	13.2 ± 7.6	67.0 ± 10.7	85.0 ± 12.2
Estrus	9	1.4 ± 1.2	10.6 ± 5.1	51.5 ± 7.4	70.0 ± 3.6
Metestrus	17	2.6 ± 1.9	15.5 ± 7.2	69.6 ± 11.6	72.0 ± 12.9
Diestrus	22	1.2 ± 1.0	23.2 ± 4.3	70.9 ± 11.7	75.2 ± 8.0

^aData are means ± SEM of number of rats indicated in the “n” column.

Table 2

Visceromotor response to repeated colorectal distension in naïve male and female rats.

		VMR (%) ^a					
	CRD (mmHg)	Baseline	Day 1	Day 2	Baseline	Day 4	Day 5
Male	10	-5.2 ± 3.5	-3.2 ± 1.9	0.4 ± 2.7	0.6 ± 1.5	1.7 ± 1.7	-1.5 ± 1.0
	20	4.4 ± 1.0	6.0 ± 2.9	13.0 ± 7.5	6.0 ± 2.1	10.4 ± 4.8	11.6 ± 9.1
	40	32.2 ± 15.9	43.5 ± 14.0	82.7 ± 29.3*	48.6 ± 2.9	53.6 ± 15.6	47.6 ± 5.4
	60	100.0 ± 0.0	140.2 ± 23.8	112.3 ± 13.3	100.0 ± 0.0	80.3 ± 16.1	119.9 ± 12.0
Female	10	0.1 ± 1.1	-3.2 ± 0.6	-1.5 ± 2.9	1.0 ± 2.2	-1.2 ± 1.1	-1.6 ± 3.6
	20	3.0 ± 2.8	4.2 ± 3.8	2.3 ± 3.7	24.0 ± 12.4	33.7 ± 16.7	20.3 ± 6.3
	40	65.1 ± 22.0	45.9 ± 15.4	61.1 ± 19.3	72.2 ± 15.8	93.9 ± 40.1	87.7 ± 38.6
	60	100.0 ± 0.0	105.1 ± 8.0	129.2 ± 15.1	100.0 ± 0.0	129.1 ± 36.0	135.2 ± 30.7

^aValues are means ± SEM, n=3-5/group. There were no statistical differences between days in each sex or between sexes for each day.