Amygdala Activation by Corticosterone Alters Visceral and Somatic Pain in Cycling Female Rats

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Running Title: Estrous cycle differences in pain perception

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ABSTRACT

Irritable bowel syndrome (IBS) is often seen in women and symptom severity is known to vary over the menstrual cycle. In addition, activation of the hypothalamic-pituitary-adrenal (HPA) axis enhances symptomology and patients with IBS have increased activation of the amygdala, a brain region known to facilitate HPA output. However, little is known about the effects of amygdala activation during different stages of the menstrual cycle. We therefore investigated the effects of amygdala activation on somatic and visceral pain perception over the rat estrous cycle. Female Wistar rats were implanted with either corticosterone (CORT) or cholesterol as a control, onto the dorsal margin of the central amygdala. Visceral sensitivity was quantified by recording the visceromotor response (VMR) to colorectal distension (CRD) and somatic sensitivity was assessed via the Von Frey test. In cholesterol controls, both visceral and somatic sensitivity varied over the estrous cycle. Rats in proestrus/estrus responded to CRD with an increased VMR as compared to rats in metestrus/diestrus. Somatic sensitivity followed a similar pattern with enhanced sensitivity during proestrus/estrus as compared to metestrus/diestrus. Elevated amygdala CORT induced visceral hypersensitivity during metestrus/diestrus, but had no effect during proestrus/estrus. In contrast, elevated amygdala CORT increased somatic sensitivity during both metestrus/diestrus and proestrus/estrus. These results suggest that amygdala activation by CORT eliminates spontaneously occurring differences in visceral and somatic pain perception, which could explain the lowered pain thresholds and higher incidence of somatic pain observed in women with IBS.

Key words: Irritable bowel syndrome, hypothalamic-pituitary-adrenal (HPA) axis, ovarian hormones, somatic sensitivity, visceral sensitivity.
INTRODUCTION

Irritable bowel syndrome (IBS) is one of the most common functional gastrointestinal (GI) disorders in the industrialized parts of the world and is characterized by a female predominance (8-9). The pathogenesis is still unknown, but a number of factors, including altered GI motility and secretory capacity, visceral hypersensitivity and psychosocial factors such as stress and anxiety are implicated in the development of the disorder (5). Due to the female predominance of the disorder, attention has been drawn to the role of ovarian hormones in the development of IBS and previous reports have shown that women with IBS often report exacerbation of symptoms during menses (11) (14). In addition, patients with IBS often report worsening of symptoms following periods of stress, involving activation of the hypothalamic-pituitary-adrenal (HPA) axis. Activation of the HPA axis occurs via series of events initiated by the hypothalamic paraventricular nucleus (PVN) resulting in synthesis and secretion of corticotrophin releasing factor (CRF). The released CRF then stimulates the anterior pituitary to secrete adrenocorticotropic hormone (ACTH) into the blood stream which in turn induces synthesis and secretion of glucocorticoids from the adrenal cortex. Dysregulation of the HPA axis in IBS patients has been related to blunted ACTH levels and enhanced cortisol response to visceral stimulation (3). Related to the enhanced response to visceral stimulation, a recent meta-analysis identified the amygdala as one of the most consistently activated brain regions following rectal stimulation in IBS patients as compared to controls (31). Increased activation of the amygdala is known to have a stimulatory effect on the HPA-axis thus resulting in increased glucocorticoid secretion. Although the amygdala is not in direct contact with the PVN, it can affect its activity by
activating sub cortical relay systems such as the bed nucleus of striata terminalis (BNST) which in turn are in direct contact with the PVN (10) (26). In rodents, activation of the amygdala by stereotaxic implants of corticosterone (CORT) onto the dorsal margin of the central amygdala (CeA) induces anxiety-like behavior and somatic and visceral hypersensitivity, which are common clinical features of IBS (20). The specificity of the model has been verified by specific targeting of adjacent areas to the CeA, such as the CA3 area of the hippocampus and the caudate putamen. In these areas stereotaxic delivery of CORT did not affect either somatic or visceral pain perception, supporting the theory that the CORT induced effects are specific to activation of the CeA (20) (19). In addition, we recently showed that the CORT effect on visceral sensitivity was CRF dependent, pointing to activation of the PVN (22). The physiological relevance of the models is supported by findings in animals models of stress induced activation of the HPA-axis which are characterized by similar features such as increased CRF expression, enhanced visceral sensitivity, altered bowel habits and anxiety like behavior (29) (35). Taken together, both ovarian hormones and HPA axis activation are implicated in the pathogenesis and regulation of symptom severity of IBS, however, the underlying mechanisms resulting in IBS are still unknown. We therefore studied visceral and somatic pain perception during control conditions e.g. in cycling females implanted with cholesterol onto the central amygdala (CeA) and after HPA axis activation, induced by CORT implants onto the CeA as described above. We hypothesize that remodeling of the amygdala alters the normally occurring differences in pain processing present during the estrous cycle and that these alterations play a role in the development of IBS.
MATERIALS AND METHODS

Animals

Experiments were performed on cycling and ovariectomized (OVX) female Wistar rats (n=54) (220-320g) purchased from Charles River Laboratories (Wilmington, MA). Upon arrival the rats were kept under standardized environmental conditions (21°C, 12 hour light/dark cycles) with full access to chow and water in the animal facility for at least 7 days before the experiments. To reduce stress associated with experimental settings rats were subjected to 5 days of acclimatization. The rats were then brought in to the laboratory between the hours of 10:00 am and 2:00 pm, were weighed and handled by the investigator. All experimental procedures were approved by the Animal Studies Subcommittee and Research and Development Committee at the Oklahoma City VA Medical Center.

Stereotaxic surgery

Rats were anesthetized with a combination of ketamine (100mg/kg ip.) and xylazine (10mg/kg ip.) and mounted in a stereotaxic surgical frame (Kopf, Tujunga, CA). Body temperature was maintained at 37°C by a homoeothermic heating blanket (Harvard, Ealing, UK). Following a midline incision, two small holes were made in the skull at coordinates 2.5 mm posterior to bregma and 4.2 mm to the right and the left of midline. A 25-gauge stainless steel canula containing a micropellet of CORT (30µg) or cholesterol (30µg) was lowered 7.0mm dorsally from the dura mater to the dorsal margin of the CeA. The micropellet was expelled and the canula was removed. Gel foam was used to fill the
holes in the skull and the incision was sutured. Antibiotic and analgesic cream was applied to the wound and animals were allowed a 5-day recovery period, during which their behavior was observed to ensure that they were not in distress or pain. Post mortem analysis of the brain was used to verify the exact location of the implants. Brains were dissected, frozen in isopentane 2-methylbutane (Fisher Scientific, Fair Lawn, NJ) and stored at -80°C until cryosectioned. Serial coronal sections were cryosectioned (Bright OTF, Fairfield, NJ) (40µm), mounted on glass slides and examined in visual light using the Paxinos and Watson rat brain atlas as a reference for correct placement of the micropellets e.g. within 750µm of the CeA which is the diffusion range of the CORT pellet (Fig. 1) (28).

Colonic sensitivity assessment

The level of colonic sensitivity was quantified by measuring the visceromotor response (VMR) to graded pressure colorectal distension (CRD). Rats were anesthetized with isoflurane (2%) for 30 min during which an incision was made to expose the left external oblique muscle. A strain gauge force transducer was sutured onto the muscle in parallel with the muscle fibers, followed by wound closure and application of antibiotic and analgesic cream. The led wires from the strain gauge was secured with one stitch to the back and taped to the base of the tail. To enable CRD a 4 cm latex balloon was inserted 9 cm into the distal colon and secured by taping the balloon catheter to the base of the tail. Following surgery the rats were allowed a 30 min recovery period ensuring that they were fully mobile upon initiation of the study and were eating and grooming as expected. The basal number of abdominal contractions was recorded for 10 min in unrestrained freely moving rats. The number of abdominal contractions was recorded by connecting
the strain gauge to a computer and analyzing the signal using the Polyview Data
Acquisition Software (Grass Technologies, West Warwick, RI). Following baseline
measurements, the colorectal balloon was distended for 10 min at a constant pressure of
20, 40 and 60 mmHg, separated by 10 min intervals without distension using a rigid
piston barostat (G&E Electronics, Toronto, ON, Canada). The number of abdominal
muscle contractions during each distension period was used as a measurement of colonic
sensitivity. All the measurements were performed in unrestrained freely moving rats.

**Somatic pain assessment**

Somatic pain sensitivity was recorded using the Von Frey test as a measurement of
cutaneous sensitivity. Rats were placed on an elevated mesh floor (12 x 12mm grid),
enclosed and unstrained in a clear plastic enclosure (21 x 27 x 15 cm). An automatic IITC
2390 series Electronic Von Frey Anesthesiometer (IITC Life 164 Science Inc, Woodland
Hills, CA), was used to record the force required to induce a withdraw reflex. The tip of
the probe was pushed against the hind paw until a positive response was obtained e.g.
withdrawal of the hindlimb. The process was repeated three times with 5 minutes
intervals and the average force was calculated as a measurement of cutaneous sensitivity.

**Experimental design**

In cycling females vaginal cytology was used to determine the phase of the estrous cycle
(6). Vaginal samples were taken at the same time of the day for 8 contiguous days prior
to experiment to ensure regular cycling. The vaginal sample was put on a glass slide and
examined using light microscopy. The phase of the estrous cycle was determined by two independent researchers. On day 0 rats were implanted with either CORT or cholesterol as a control. Cholesterol was chosen as an inactive control since it is a precursor in the biosynthesis of CORT but is not metabolized by the surrounding tissue. On day 5, somatic sensitivity was assessed using the Von Frey assay and on day 7 visceral sensitivity was measured by recording the VMR to CRD. Vaginal samples were collected directly after the respective experiment and the slides were read after the last experimental point to blind the investigator to the phase of the estrous cycle. During the initial data analysis we observed that neither somatic nor visceral sensitivity varied between metestrus and diestrus or between proestrus and estrus. The cycling females were therefore divided into two groups, metestrus/diestrus and proestrus/estrus. The number of animals in each phase was evenly distributed in the proestrus/estrus group, however in the metestrus/diestrus group two thirds of the animals were in diestrus, depending on the short duration of metestrus as compared to diestrus (6).

**Drugs and chemicals**

CORT and Cholesterol was obtained from Sigma-Aldrich (St. Louis, MO). Ketamine was purchased from Phoenix Pharmaceuticals (St. Josephs, MO), Xylazine was obtained from Hospira (Lake Forest, IL) and Isoflurane was obtained from Vedco (St. Josephs, MO).

**Data analysis**

Data is presented as mean ± standard error of the mean (SEM). To analyze the data from the Von Frey experiments we used a one-way ANOVA with a Bonferroni post-test. A
two-way repeated measurements ANOVA followed by the Bonferroni post-test was used to analyze colonic sensitivity data with treatment and distension pressure as factors. A p-value < 0.05 was considered statistically significant.
RESULTS

Effects of estrous cycle on colonic sensitivity

In cholesterol implanted control rats, graded colonic distension induced a linear increase in the VMR in the metestrus/diestrus, proestrus/estrus and OVX groups. By comparing the magnitude of the responses in the different groups we observed a significantly greater VMR to distension pressures of 40 and 60 mmHg during proestrus/estrus as compared to metestrus/diestrus and the OVX group for the respective pressure. No significant difference was observed between rats in metestrus/diestrus and the OVX group (Fig. 2A).

Effects of CORT on colonic sensitivity

Exposure of the amygdala to elevated levels of CORT has previously been shown to increase colonic sensitivity in male rats (7) (19). Here we studied the CORT-induced effects on visceral sensitivity during the rat estrous cycle. CORT implanted cycling and OVX females responded to CRD with a pressure-dependent increase in the number of abdominal contractions. Elevated amygdala CORT induced visceral hypersensitivity at 60 mmHg in cycling females in metestrus/diestrus (cholesterol: 15.6±1.9; CORT: 27.4±3.9, p<0.01) and in OVX rats (cholesterol: 13.3±5.4; CORT: 29.0±6.8, p<0.05). However, CORT did not affect visceral sensitivity during proestrus/estrus and the cycle dependent differences observed in cholesterol implanted rats was no longer seen (Fig. 2B). Thus, these findings suggest that elevated amygdala CORT attenuates cycle-dependent differences in visceral sensitivity and the effect was not dependent on the presence of ovarian hormones.
Effects of estrous cycle and elevated CORT on somatic sensitivity

Somatic hypersensitivity has been reported in patients with IBS (36) (37), however there are other reports showing both normal sensitivity as well as somatic hyposensitivity (2) (4). Although there is evidence for hormonal regulation of somatic sensitivity in humans, little is known about the interplay between the HPA axis and ovarian hormones in regulation of somatic sensitivity. In cholesterol treated rats somatic sensitivity varied over the estrous cycle, with rats in proestrus/estrus being significantly more sensitive as compared to rats in metestrus/diestrus. However, no significant differences were observed between cycling and OVX rats. Elevated amygdala CORT induced an increase in somatic sensitivity in both the metestrus/diestrus and proestrus/estrus group however, the cycle dependent differences observed between the cholesterol treated rats were not present in the CORT implanted groups. Furthermore, CORT implants did not affect somatic sensitivity in OVX rats (Fig. 3).
DISCUSSION

Recent studies have identified enhanced amygdala activation as a possible regulator of disease activity in patients with IBS (24, 31). The mechanisms by which the amygdala can regulate disease activity have been related to the stimulatory effects on the HPA axis which is known to worsen symptomology. In addition to the amygdala, ovarian hormones are also known to affect IBS symptomatology, however, little is known about the effect of increased amygdala activation during various levels of ovarian hormones. In the present study we found that both visceral and somatic sensitivity vary over the rat estrous cycle and that high levels of ovarian hormones were associated with enhanced sensitivity as rats in proestrus/estrus were significantly more sensitive as compared to rats in metestrus/diestrus. We also discovered that amygdala activation by CORT attenuated estrous cycle-dependent differences mainly by enhancing sensitivity during metestrus/diestrus.

The VMR to CRD is a behavioral response that is usually monitored in rodents via EMG electrodes or strain gauge force transducers. In the current study, the technique employed to assess visceral sensitivity involves a brief period of anesthesia when a skin incision is made to attach a strain gauge force transducer to the external oblique muscle. The technique has the advantage of allowing measurement of colonic sensitivity in freely moving rats, which avoids possible influence of restrained stress associated with keeping the animals in Bollman cages. However, one disadvantage with the method is the possibility that post-surgical somatic pain could influence the data and affect its interpretation. The effects of somatic injury on visceral sensitivity is not very well
understood however, it was recently shown that a hind paw incision induced colonic hypersensitivity that was first observed one day post surgery and sustained for 8 days. In contrast the hind paw incision did not affect visceral sensitivity when measured 6h post surgery (1). In addition, hind paw injections of low pH saline induced colonic hypersensitivity that was observed 72-h post surgery and sustained for up to 2 weeks (18). Although a hind paw incision and an abdominal incision might have different effects on visceral sensitivity, the results from these studies suggest that the sensitization does not occur in the acute phase of the injury rather in the recovery phase. Combined with the fact that all animals in the present study underwent the same experimental procedure and that local anti inflammatory and analgesic treatment was used to block sensitization of visceral input we consider it unlikely that our findings are the result of pain and stress from the surgical procedure.

The rat estrous cycle can be divided into 4 phases, metestrus, diestrus, proestrus and estrus. Plasma hormone levels peak during proestrus and reach their lowest level during estrus (17). Although plasma hormone levels drop during estrus we observed increased somatic and visceral pain sensitivity during both proestrus and estrus. This finding suggests that the observed increase in sensitivity is not directly linked to plasma hormones levels, more likely due to sustained genomic effects, which persists long after the plasma hormone peak. For example estrogen induced expression of CRF in the PVN and estrogen induced increase in glucocorticoid receptor expression in the amygdala (25, 32). Our finding of enhanced visceral sensitivity during proestrus/estrus, which includes the plasma hormone peak correlate with previous findings from Ji et al. which showed
that estradiol treatment of OVX females increase the VMR response to CRD and the 
same group also recently showed that cycling female rats are more sensitive to CRD 
during proestrus as compared to metestrus and diestrus (12-13). Similarly to the effects 
on visceral pain, our measurements of somatic sensitivity also varied over the estrous 
cycle, with rats in proestrus/estrus being more sensitive as compared to rats in 
metestrus/diestrus. The role of ovarian hormones in regulation of somatic sensitivity is 
less studied than the effects on visceral pain, however, it has been shown that rats in 
proestrus and estrus have a lower threshold of pressure induced vocalization as compared 
to rats in metestrus and diestrus (15). This observation as well as our results also correlate 
with findings in humans where it has been shown that women are more sensitive to 
painful stimuli during the luteal phase as compared to the follicular phase (30).

Regulation of pain sensitivity by ovarian hormones can directly be linked to activation of 
the HPA axis via estrogen induced expression of CRF in the PVN and increased 
glucocorticoid receptor expression in the amygdala as mentioned above. Both these 
systems have a stimulatory effect on the HPA axis and CRF is known to mediate visceral 
sensitivity via the CRF₁ receptor (23). Direct effects of estrogen on CRF expression has 
been related to binding of estrogen receptors to estrogen response elements (ERE) and 
cAMP response elements in the CRF promoter, thus resulting in a rapid onset of gene 
expression (16) (32). In addition, administration of estrogen increase the endogenous 
diurnal CORT peak and block negative feedback, resulting in prolonged CORT effects 
(33) (34).
With the observation that somatic and visceral sensitivity vary over the rat estrous cycle and that ovarian hormones have the ability to act via activation of the HPA axis we also studied the effects of amygdala activation on somatic and visceral sensitivity in cycling females during proestrus/estrus, metestrus/diestrus and in OVX rats. Amygdala activation was induced by stereotaxic implantation of micropellets of CORT onto the dorsal margin of the CeA, which previously have been shown to induce visceral and somatic hypersensitivity in male Fischer 344 rats (6, 17-18). Activation of the amygdala by CORT resulted in enhanced somatic sensitivity during both metestrus/diestrus and proestrus/estrus but did not affect somatic sensitivity in the OVX rats. The lack of a CORT effects in the OVX groups could possibly be explained by somatic hyperalgesia induced by the ovariectomy. This phenomenon is known to occur in mice 4-5 weeks post surgery, which is within the time frame of our experiments (27). Another explanation could be that the CORT effect requires the presence of ovarian hormones. In contrast to the effects on somatic sensitivity, CORT implants induced visceral hypersensitivity during metestrus/diestrus and in OVX rats, but failed to enhance sensitivity during proestrus/estrus. The discrepancy between the CORT induced effects on somatic and visceral sensitivity during proestrus/estrus as well as in OVX rats show that somatic and visceral sensitivity are regulated by different mechanisms, which correlate with findings in male rats where we found that visceral sensitivity was mediated via both mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) receptors whereas somatic sensitivity was specifically mediated via GR receptors (21). The relative abundance of MR and GR receptors in the amygdala during the experiment and the effect
of ovarian hormones on the respective receptor expression will likely affect the outcome of the experiments.

In summary, we here show that both somatic and visceral sensitivity varied over the rat estrous cycle, with proestrus/estrus being associated with enhanced sensitivity. Amygdala activation by CORT attenuated cycle dependent differences mainly by enhancing sensitivity during metestrus/diestrus. Taken together, these results could explain the lowered pain threshold and higher incidence of somatic pain observed in women with IBS.
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DISCLOSURE

The authors have no conflicts of interest to disclose.
REFERENCES


Figure 1: Stereotaxic localization of Cholesterol (white circle) and CORT (black circle) micro pellets. Figures are adapted from the Paxinos and Watson rat brain atlas (1998). The distance marked in the picture represents the distance posterior to bregma.

Figure 2: Colonic sensitivity measured as the VMR to graded pressure CRD in cholesterol and CORT implanted female rats. A: Cholesterol implanted rats in metestrus/diestrus (n=14), proestrus/estrus (n=8) and OVX (n=6). B: CORT implanted rats in metestrus/diestrus (n=12), proestrus/estrus (n=10) and OVX (n=4). Data is presented as mean ± SEM, a p-value < 0.05 was regarded as statistically significant. *Represents comparison between metestrus/diestrus and proestrus/estrus, ¤represents comparison between OVX and proestrus/estrus.

Figure 3: Somatic sensitivity in cholesterol and CORT implanted rats in metestrus/diestrus (cholesterol n=12, CORT n=15), proestrus/estrus (cholesterol n=10, CORT n=7) and OVX (cholesterol n=6, CORT n=4). Data is presented as mean ± SEM. A p-value < 0.05 was regarded as statistically significant. *Represents comparison between CORT and cholesterol treated rats in the same phase and ¤represents comparison between cholesterol implanted rats in metestrus/diestrus and proestrus/estrus.