Endometriosis Is Associated With a Shift in MU Opioid and NMDA Receptor Expression in the Brain Periaqueductal Gray

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Abstract

Studies have examined how endometriosis interacts with the nervous system, but little attention has been paid to opioidergic systems, which are relevant to pain signaling. We used the autotransplantation rat model of endometriosis and allowed to progress for 60 days. The brain was collected and examined for changes in endogenous opioid peptides, mu opioid receptors (MORs), and the *N*-methyl-D-aspartate subunit receptor (NRI) in the periaqueductal gray (PAG), since both of these receptors can regulate PAG activity. No changes in endogenous opioid peptides in met- and leu-enkephalin or β -endorphin levels were observed within the PAG. However, MOR immunoreactivity was significantly decreased in the ventral PAG in the endometriosis group. Endometriosis reduced by 20% the number of neuronal profiles expressing MOR and reduced by 40% the NRI profiles. Our results suggest that endometriosis is associated with subtle variations in opioidergic and glutamatergic activity within the PAG, which may have implications for pain processing.

Keywords

endometriosis, rat, stress, opioid peptides, mu opioid receptor, periaqueductal gray

Introduction

Endometriosis is a gynecological disorder defined as the growth of endometrium-like tissue outside the uterine cavity, primarily on the pelvic peritoneum and organs.¹ Endometriosis is commonly characterized by chronic pelvic pain, pain during intercourse (dyspareunia), and painful periods (dysmenorrhea).^{2,3} Unfortunately, endometriosis negatively impacts the daily life activities of women who suffer from it, often more so than other chronic conditions.⁴⁻⁶ Other chronic conditions that produce pelvic pain have been shown to produce plastic changes in the brain, especially on structures functionally involved in pain and stress processing.⁷⁻⁹ However, little is known about the central changes that may occur in endometriosis.

In the rat model, endometriosis is surgically induced by autotransplantation of the uterine tissue. Similar to the clinical observations, hyperalgesic mechanisms develop at 4 weeks postendometriosis induction and stabilize by 6 to 8 weeks.¹⁰ We have recently found that in the same rat model used by Berkley's group,¹¹ there are alterations in the brain.¹² We specifically found that in the hippocampus, a structure highly impacted by physiological stress, there is a decrease in corticotropin-releasing factor within the CA3 region 60 days after endometriosis induction. This study lead us to

think that other brain structures, most likely the ones involved in analgesia, might also show adaptations due to endometriosis. Therefore, we focused our interest toward the periaqueductal gray (PAG).

The PAG is a cell dense structure located around the mid-brain aqueduct. Functions classically associated with PAG activity include analgesia and autonomic regulation and defensive behaviors.^{13,14} Although usually homogeneously classified, the PAG has 4 distinct anatomical modules: dorsomedial, dorsolateral, lateral, and ventrolateral, each with unique identifiable functions. In this study, we examined the ventrolateral

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compartment of the PAG because in the rat, this region has a dual role: expression of conditioned defensive responses and a role in descending projections that mediate analgesia.¹³ In other chronic conditions that cause pain, changes in PAG activity have been reported.^{15,16} Although changes in other brain regions such as the preoptic hypothalamic area have been reported in the endometriosis rat model,^{7,12,17} whether PAG activity is altered during endometriosis remains elusive.

The PAG is rich in both opioid receptors and endogenous opioid peptides. Mu opioid receptors (MORs) are mostly activated by the release of endogenous peptides, mainly leu and met-enkephalin and β -endorphin.^{18–20} Enkephalin is distributed throughout the rostrocaudal span of the PAG, reflecting its relationship to components of analgesic systems.²¹ Similarly, β -endorphin is highly distributed within the PAG overlapping with some areas of the enkephalinergic system.^{22,23} In women with endometriosis, the concentration of β -endorphin within peripheral mononuclear cells has been found to be decreased compared to controls specifically during the luteal phase of the cycle,²⁴ suggesting that alterations in opioid systems play a role in the painful symptoms of endometriosis. On the other hand, activation of *N*-methyl-D-aspartate (NMDA) receptors within the PAG might antagonize the effects of endogenous opioid peptides and produce hyperalgesia.²⁵ Interestingly, under normal conditions, there is a high degree of colocalization of NMDA receptors and MORs within the PAG.²⁶ Molecular interaction of both of these receptors when activated within neuronal compartments has been mapped, showing that NMDA receptor activation along with MORs contribute to morphine tolerance.²⁷ In addition, blockade of NMDA receptors along with morphine produce greater analgesia.²⁷ Therefore, changes in the expression of these receptors within PAG profiles can impact analgesic signaling and thus pain responses in the individual.

It is still unknown whether endometriosis is associated with changes in endogenous opioid peptides or in opioid receptors in the central nervous system (CNS). Therefore, the current study was undertaken to answer the following questions: (1) is endometriosis associated with MOR levels or its colocalization with NMDA receptors within the PAG? and (2) is endometriosis associated with changes in endogenous opioid peptide levels within the PAG? Due to our reduced understanding of how endometriosis affects brain circuits, we have not yet developed effective therapies aimed at reducing disease presentation without having significant secondary effects on ovulation and reproductive abilities. The aim of this study was to shed light on endometriosis-associated brain changes that may hint to potential new therapeutic targets for pelvic pain.

Methods

Animal Model

Studies were performed in a well-established rat model of endometriosis using female Sprague Dawley rats weighing 200 to 250 g (Southern Veterinary Service, Ponce Health Sciences University, Puerto Rico), with 6 animals per treatment

group. Animals were part of a larger set of rats from our previous publication.²⁸ Rats were randomly assigned to either sham surgery or surgical induction of endometriosis (endo). All animals were maintained in a bio-bubble facility room on a 12-hour light–dark cycle and at constant temperature (23°C). Standard laboratory chow and tap water were provided ad libitum. The Institutional Animal Care and Use Committee at Ponce Health Sciences University approved all experimental procedures. Animals were handled for 7 days (5 min/d/rat) prior to beginning the experiment in order to reduce experimenter-induced stress on the animal, and daily vaginal cytological smears were carried out for all rats to check their reproductive cyclicity (Figure 1). Endometriosis was surgically induced following the method of Vernon and Wilson.^{12,29,30} Briefly, the distal 2 cm of the right uterine horn was removed and immersed in warm (37°C) sterile culture medium. The endometrium was exposed by opening the uterine horn lengthwise, and 4 pieces of uterine horn measuring 2 × 2 mm were cut. The implants were sutured with the serosal surface facing the mesenteric vessels of the small intestine and the endometrial surface exposed to the peritoneum. Sham-operated rats received 4 sutures to the mesentery of the intestine without uterine implants, and the right uterine horn was massaged with fingertips for 2 minutes to minimize any effects resulting from the mechanical handling of the uterine horn. Saline solution was continuously applied to the peritoneal cavity to keep it moist and reduce adhesions. Animals were killed at 60 days following the endometriosis induction surgery.^{12,29,30}

Tissue Collection

A laparotomy was performed to allow for the assessment of disease severity as described below and to collect tissues. A vaginal cytological smear was taken at the time of killing to allow for interpretation of any effects of the estrous cycle stage on the experimental results. The peritoneal cavity was systematically examined for the presence of the implants and the original sutures. The site of the implants was examined for the presence/development of vesicles or cysts, and their longest and shortest diameters were measured. Vesicle volume was calculated based on the following formula: $V = (1.33 \times \pi \times [a/2] \times [b/2]^2)$,³¹ where *a* corresponds to the longest diameter and *b* to the shortest diameter measured. For each individual rat, the total vesicle volume was calculated by adding up the individual volumes of the developed vesicles.

Brain Section Preparation

After endometriotic vesicles and other peritoneal tissue samples were collected, rat brains were fixed by aortic arch perfusion with 3.75% glutaraldehyde and 4% paraformaldehyde in 0.1 mol/L phosphate buffer (PB; pH 7.6).³² The brains were removed from the skull and cut into 5 mm coronal blocks using a brain mold and postfixed for 4 hours in 4% paraformaldehyde in 0.1 mol/L PB. The brains were sectioned on a Leica Vibratome (40 μ m thick) and stored in

Figure 1. Timeline of experimental procedures. Animals were handled and vaginal smears collected 1 week prior to endometriosis induction. Endometriosis was allowed to progress. Between days 7 to 14 and 24 to 31, we checked for estrous cyclicity. All animals were killed 60 days after surgery.

cryoprotectant solution (30% sucrose and 30% ethylene glycol in 0.1 mol/L PB). Prior to immunocytochemistry, coronal sections of all treatment groups were rinsed in PB, and groups were coded with hole punches and pooled into single crucibles. Sections were treated with 1% sodium borohydride in PB for 30 minutes to neutralize free aldehydes.

Antisera

A guinea pig polyclonal antibody raised against C-terminal amino acids 384 to 398 of the MOR (AB5509; Millipore, Temecula, California) was used in single-label immunofluorescence studies. This antibody has been previously used in male Sprague Dawley rat spinal cord sections to evaluate colocalization of MOR with phosphorylated NMDA receptor 1 (NR1) receptors.³³ A rabbit polyclonal antiserum raised against amino acids 384 to 398 of the MOR was used in dual-labeling studies (24216; ImmunoStar, Hudson, Wisconsin). This antibody was raised against residues 384 to 398 from the carboxy terminus of MOR1.^{34,35} Specificity of the antibody has been demonstrated previously by Western blotting, adsorption, and omission controls.^{34,36} In our laboratory, comparison of both antibodies by Western blot using adult female rat brain PAG homogenates gave us similar results, yielding a band at approximately 45 kDa for the guinea pig antibody and 2 thin double bands between 45 and 50 kDa for the rabbit antibody (ImmunoStar; most widely used in other studies). The double-band migration pattern most likely corresponds to the glycosylated versus nonglycosylated form of the receptor, which might change the migration pattern.^{37,38} We also tested colabeling of both of the MOR antibodies in the same tissue sections from the endometriosis group. Figure 2 shows 4 different neurons from 2 different fields of the ventral PAG with an overlapping labeling pattern between both MOR antibodies (Millipore and ImmunoStar). The Millipore antibody was labeled using an anti-guinea pig Alexa488 secondary, whereas the ImmunoStar antibody was labeled using an antirabbit CY3 (both secondaries 1:400; Jackson ImmunoResearch Labs, Inc, West Grove, Pennsylvania). The membrane labeling pattern is very similar to what has been shown before in dorsal root ganglia neurons and lumbar spinal cord neurons of male adult rats.³⁹ A mouse

monoclonal antibody raised against amino acids 660 to 811 of the rat NR1 was used in dual-labeling immunofluorescence studies at a concentration of 1:100 (BD Biosciences, previously PharMingen, San Jose, CA). This antibody has been previously characterized.⁴⁰ A rabbit polyclonal antibody against methionine enkephalin (ImmunoStar) was used at a concentration of 1:5000. This antibody has been previously characterized.^{41,42} A rabbit polyclonal antibody against leucine enkephalin (Millipore) was used at a concentration of 1:2000. This antibody shows a cross-reactivity of 0.93% with met-enkephalin. A rabbit polyclonal against β -endorphin peptide (Bachem-Peninsula, Torrance, California) was used at a concentration of 1:8000. This antibody has been raised against the whole sequence of the rat β -endorphin peptide. Some cross-reactivity with met-enkephalin is expected due to the initial 5 amino acid immunogenic sequences used (Thy-Gly-Gly-Phe-Met) to develop the β -endorphin antibody (cross-reactivity not determined in the current study).

Light Microscopy Immunohistochemistry and Analysis

For single-labeling immunohistochemistry of neuropeptides, the tissue was processed according to the avidin-biotin complex (ABC) method.⁴³ For this, tissue sections were rinsed in PB followed by 0.1 mol/L Tris saline (TS) and incubated in (1) 0.5% bovine serum albumin (BSA) in TS, 30 minutes; (2) opioid receptor antibodies in 0.025% Triton (TX) X100 and 0.1% BSA/TS for 18 to 24 hours at room temperature and 24 hours at 4°C; (3) a 1:400 dilution of biotinylated secondary immunoglobulin (IgG), 30 minutes; (4) ABC (at twice the recommended dilution; Vector, Laboratories, Burlingame, CA), 30 minutes; and (5) 3,3'-diaminobenzidine (Sigma, St Louis, Missouri) and H₂O₂ in TS for 6 minutes. All incubations were separated by washes in TS. Sections were dehydrated in ascending concentrations of alcohols and mounted using DPX mounting media (Sigma). For quantitative densitometry, images of regions of interest (ROIs), dorsal and ventral PAG, were captured on a Nikon 200 (Armonk, NY) using an Infinity 3.0 CCD camera (Lumenera Corp. Ontario, Canada) under equal lighting and background-corrected conditions. The mean gray value (of 256 gray levels) for each

Figure 2. Both types of mu opioid receptor (MOR) antibodies used have a high degree of overlapping in their immunofluorescence. A1 and A2, Sample MOR immunoreactivity of 2 different fields within the ventral periaqueductal gray (PAG) of an endometriosis rat. Neurons were labeled using the rabbit anti-MOR (1:1000) from ImmunoStar followed by CY3 secondary antibody. This antibody has been widely characterized. B1 and B2, Sample immunoreactivity of the same fields shown in (A), using the guinea pig anti-MOR (1:1000) from Millipore followed by Alexa488 secondary antibody. C1 and C2, Image merge of the immunoreactive fields from both antibodies showing a high degree of colocalization (yellow; arrows). (The color version of this figure is available in the online version at <http://rs.sagepub.com/>.)

selected ROI was determined as previously described.^{44,45} To compensate for background staining and control for any possible variations in illumination level between images, the average pixel density for 3 regions lacking labeling was subtracted. A single PAG from each animal with the best morphology and consistent immunoperoxidase labeling was included in the analysis. Optical density values were measured using ImageJ software (<http://imagej.nih.gov/ij/>), and net optical density values obtained after subtracting background values.

Fluorescent Microscopy Immunohistochemistry and Analysis

Sections of each group were rinsed in 0.05 mol/L phosphate-buffered saline (PBS; pH 7.4) and incubated in (1) 0.1% TX/0.3% normal goat serum (NGS) in PBS for 1 hour; (2) a combination of guinea pig polyclonal MOR (1:1000) antisera in 0.1% TX/0.3% NGS in PBS for 48 hours at 4°C; and (3) a cocktail of goat anti-guinea pig Cy3 IgG (1:400; Jackson

ImmunoResearch Labs, Inc) in 0.1% NGS in PBS for 1 hour at room temperature. All incubations were separated by washes in PBS. Sections were mounted on gelatin-coated slides, dehydrated in ascending concentrations of alcohol and xylene, and coverslipped with DPX mounting media (Sigma). For dual-labeling experiments, primary antisera cocktails also contained mouse monoclonal NR1 (1:100) and secondary antisera cocktails included goat antimouse Alexa 488 IgG (1:400; Jackson ImmunoResearch Labs, Inc). As controls, these immunocytochemical procedures were utilized on sections with the omission of the primary or secondary antisera. Immunofluorescence images were acquired sequentially using an Olympus (Corporate Parkway, NJ) BX-60 equipped with X-cite 120Q lamp (Excelitas Tech. Waltham, MA) and photographed with a Nikon DS-F11 camera. Two different excitation filters from Semrock Inc (Rochester, New York) were used for detecting the fluorescent stains: FITC-3540B-OMF-ZERO for Alexa Fluor 488-conjugated secondary antibodies and TxRed-4040C-OMF-ZERO for Cy3. Areas of interest were photographed sequentially under each filter set.

Figure 3. Endometriosis reduces mu opioid receptor (MOR)-like immunoreactivity within the ventral periaqueductal gray (PAG) neurons. A, Sample MOR immunoreactivity in the ventral PAG from sham animal and (B) from an endometriosis animal. C, Average cell fluorescence (corrected for area) was decreased in the endo group. $n = 4$ to $5 \pm$ standard error of the mean (SEM). * $P < .05$ versus sham on a Mann-Whitney U test. Bar = $40 \mu\text{m}$.

Alexa Fluor 488 (NR1) was pseudocolored green, and Cy3 (MOR) was pseudocolored red. To verify dual labeling, MOR and NR1, red and green pictures of the same field, were merged using ImageJ.

For single-labeling studies, all images were taken the same day they were stained and using the same excitation parameters and illumination. Mu opioid receptor fluorescence within individually labeled neurons was quantified in ImageJ. Images from the ROI were converted to 16-bit photos. The average optical density and area from 4 randomly selected neurons within each PAG brain section per animal were outlined and quantified. The mean optical density of nonlabeled neuropil background within each brain section was also quantified. Corrected optical density (COD) was calculated by the following formula: $\text{COD} = \text{neuron integrated optical density} - (\text{area of neuron} \times \text{mean background density})$. For dual-label studies, the number of MOR- and NR1-labeled neurons in $80\,000 \mu\text{m}^2$ areas of the ventral PAG and dorsal raphe nucleus (anteroposterior: -6.30 to -7.80 caudal to bregma)⁴⁶ was counted on a computer screen using the ImageJ cell counting tool. Cell counts were summed per animal and presented as mean $\pm$ standard error of the mean (SEM) of MOR- and NR1-labeled neurons per group. Since the purpose of counting cells was not to obtain absolute numbers but to provide a relative estimate between groups, no correction factors were applied to compensate for error of overestimation.⁴⁷ Preplanned comparison for the average percentage of MOR-immunoreactive neurons showing colocalization with NR1 was obtained for each animal group. A minimum of 2 different ventral PAG fields from 2 different brain sections from the same animal was analyzed.

Statistical Analysis

Data graphs were prepared using GraphPad Prism version 6.0 (GraphPad Software, San Diego, California). Data were

analyzed using SPSS version 21.0 (IBM, Armonk, NY). A $P < .05$ was considered to represent a statistically significant difference. The mean difference $\pm$ SEM was used to assess the differences among treatment groups. Normal distribution of the data was corroborated by a Shapiro-Wilk test. When only 2 variables were present, a nonparametric Mann-Whitney U test was used for variables not normally distributed (Figure 3), whereas an unpaired Student t test was used for variables normally distributed (Figure 5). For changes in MOR and NR1 in the dual-immunoreactivity experiments, the data were analyzed using a 2-way analysis of variance with group (endo or sham) and type of immunoreactivity (MOR, NR1, or dual labeling) as the variables (Figure 4). This test was followed by a multiple comparisons Tukey test.

Results

Endometriotic Vesicle Volume

No difference in the body weight between the groups was found at the time of killing. Vesicles were identified by laparotomy and measured as described.³⁰ As expected, none of the sham animals developed vesicles at the suture sites. All of the implanted rats developed vesicles in at least 2 of the implant sites. The endo group developed a vesicle in 91% of their sutures. The average vesicle volume for the endo group was $35.58 \pm 9.12 \text{ mm}^3$.

Mu Opioid Receptor Immunoreactivity

Mu opioid receptor immunoreactivity was abundant in the ventrolateral areas of the PAG and the dorsal raphe. Consistent with a previous report,²⁶ MOR was mostly observed labeling somata and some dendritic processes (Figures 2 and 3A and B). To be able to quantify group differences in immunoreactivity, pictures were all taken under the same

Figure 4. Endometriosis is associated with a shift in N-methyl-D-aspartate (NMDA) receptor 1 (NR1) more than mu opioid receptor (MOR). Sample MOR (A) and NR1 (B) immunofluorescence in the periaqueductal gray (PAG). C, The merged image of dually labeled immunoreactive profiles (neuronal and or glial). D, MOR⁻NR1⁺ profiles and dually labeled profiles were significantly higher than MOR⁺NR1⁻ profiles. In the endo animals, we observed a nonsignificant decrease in all types of profiles. E, Percentage change in the mean number of labeled profiles in the endo group compared to the sham group. There was double the decrease in MOR⁻NR1⁺ profiles as in the other 2 categories. $n = 4$ to $6 \pm$ standard error of the mean (SEM). * $P < .05$ on a 2-way analysis of variance (ANOVA) followed by Tukey multiple comparison test. Bar = $60 \mu\text{m}$.

illumination parameters and at the same time. The MOR immunoreactivity was significantly decreased in the endo group as compared to the sham group. Immunoreactivity in sham neurons showed an average optical density of 34.79 ± 3.05 , whereas the immunoreactivity in neurons from the endo group was 26.99 ± 2.63 (Mann-Whitney $U = 140$, $P < .05$). Based on previous reports showing differences during the estrous cycle in MOR distribution on hippocampal inhibitory neurons,⁴⁸ we analyzed the data by grouping the animals based on the stage of the estrous cycle. Estrous cycle distribution was very similar between treatment groups. No difference in MOR immunoreactivity was observed between groups when analyzed by the stage of the estrous cycle.

Mu Opioid Receptor-NR1 Immunoreactivity

Consistent with a previous report,²⁶ we found a high number of neurons that colocalized MOR and NR1 (Figure 4D). N-methyl-D-aspartate receptor 1 can be found in both glial and neuronal compartments; thus, we refer to them as immunoreactive positive “profiles.” In the sham animals, the number of

profiles expressing only NR1 (MOR⁻NR1⁺) was significantly higher than the number of profiles showing only MOR (MOR⁺NR1⁻). The number of MOR⁻NR1⁺ was 5.01 ± 1.05 , whereas the number of MOR⁺NR1⁻ was 1.1 ± 0.28 per $80\,000 \mu\text{m}^2$ of tissue quantified. Interestingly, this difference was no longer evident in the endo group. In the endo group, the number of MOR⁻NR1⁺ was 2.95 ± 1.34 , whereas the number of MOR⁺NR1⁻ was 0.80 ± 0.41 per $80\,000 \mu\text{m}^2$ of tissue quantified. There was a main statistical effect in the type of immunoreactivity observed ($F_{(2,21)} = 8.57$, $P < 0.01$) but no statistical effect between the treatment groups ($F_{(1,21)} = 1.807$, $P = .19$). However, we quantified the percentage change in the number of labeled profiles for all types. We observed that in the endo group, both single-labeled profiles (MOR⁺NR1⁻ and MOR⁻NR1⁺) showed a reduction compared to sham, with the larger decrease of 41% in single-labeled NR1⁺ profiles. Despite the lack of significance, we calculated the effect size of our data showing a large effect as described by Cohen⁴⁹: $D = 1.71$, $r = .65$. Dual-labeled profiles (MOR⁺NR1⁺) also showed a decline (17%) in the endo rats (sham: 5.38 ± 0.98 ; endo: 4.44 ± 1.46). This suggests that the inflammatory processes of endometriosis could be associated with a bigger variation in

Figure 5. Endometriosis did not alter opioid peptide immunoreactivity in the periaqueductal gray (PAG). A, Sample met-enkephalin immunoreactivity in the dorsal part of the PAG was diffuse and some somata-like structures were evident. B, Semiquantitative measures of met-enkephalin immunoreactivity revealed no differences between groups. C, A cluster of leu-enkephalin immunoreactive fibers was evident in the ventrolateral part of the PAG. D, There were no differences in immunoreactivity between groups. E, β -Endorphin immunoreactivity was abundant and most evident in the ventrolateral compartments. Punctae immunoreactivity was very evident. F, Similar to enkephalin, we found no differences in immunoreactivity between groups on a Student *t* test. Aq represents cerebral aqueduct (A, C, and E all from endo rats. Bar = 50 μ m).

the number of profiles expressing NR1 than the variation observed in MOR immunoreactivity.

Opioid Peptides

Met-enkephalin immunoreactivity was most prominent in the dorsal regions of the PAG as compared to the ventrolateral compartments (Figure 5A). Ventrolateral immunolabeling was similar to a previous report.⁵⁰ In some brains, well-defined somata-like structures were easily identified with punctae staining around them. However, semiquantitative assessment of immunostaining revealed no difference between the groups (Figure 5B; sham: 19.59 ± 1.91 ; endo: 20.20 ± 2.38). On the other hand, the immunoreactivity of leu-enkephalin and

β -endorphin was most prominently observed in the ventral and ventrolateral regions of the PAG, consistent with previous reports.²¹ Leu-enkephalin exhibited a fiber-like arrangement that was most evident in the rostralateral portions of the PAG (Figure 5C). β -Endorphin showed a punctae distribution, with very few distinguishable fiber-like staining or cell bodies (Figure 5E). Similar to met-enkephalin, the immunoreactivity of leu-enkephalin and β -endorphin was no different between groups (Figure 5D and F). Leu-enkephalin immunoreactivity in the sham group was 17.21 ± 1.66 and endo group: 21.33 ± 2.18 . β -Endorphin immunoreactivity in the sham group was 29.05 ± 2.08 and endo group: 27.23 ± 4.9 .

Discussion

In the current study, we demonstrate that in the rat model, endometriosis can be associated with a decrease in MOR immunoreactivity within neuronal compartments in addition to a shift in MOR and NMDA—NR1 receptor expression within the ventral PAG. However, opioid peptide immunoreactivity remains invariable suggesting adaptive mechanisms in the endometriosis group. Our data show that there was a nonsignificant variation toward decreased immunoreactivity of both MOR and NR1 in the endometriosis rats, but the effects were more prominent for the latter. The impact of endometriosis on the PAG-opioid system is associated with changes in MOR and NR1 receptor expression, suggesting a possible alteration in PAG excitability produced by the course of endometriosis.

Cross Talk Between MOR and NMDA Functional Implications

In the ventrolateral PAG, MOR exerts a potentiatory effect on NMDA responses, even at low concentrations of endogenous opioid peptides.⁵¹ We observed a 41% decrease in NMDA-NR1-expressing neurons in the endometriosis animals compared to the sham animals. Although the difference in NR1 receptor expression between the sham and endo groups was not significant, a shift toward decreased number of profiles expressing the receptor might result in a depotentiation of the PAG activity. Since inflammatory parameters are exacerbated in endometriosis rats,¹² at the functional level, the changes produced by endometriosis might be an effort to homeostatically regulate the PAG circuit in response to a physiological stressor. We hypothesize that the shift in NR1 in endometriosis rats is an attempt of the system to homeostatically regulate pain perception. However, the shift in the number of neurons expressing MOR was 22%, smaller than the decrease in NMDA receptors; but on those neurons expressing MOR, there was a significant decrease in receptor immunoreactivity. A decrease in MOR receptor immunoreactivity in endometriosis animals implies that opioid signaling is altered within the PAG by the disease, strongly suggesting a decreased modulatory activity of MORs. Previous studies have shown that endometriosis produces hyperalgesia.^{10,52-54}

Due to the known role of PAG in pain perception, our study indirectly suggests that reduced MOR expression is one of the possible contributing factors leading to endometriosis-induced hyperalgesia. A decrease in MOR expression has been shown to be associated with substantial changes in biological functions in neurons, in particular.^{55,56} It is important to remember that PAG projects to the rostral ventrolateral medulla and from there to spinal cord compartments that receive somatosensory inputs from visceral organs.⁵⁷ It remains to be elucidated whether opioidergic synaptic activity at different levels of the spinal cord is affected due to either stress or endometriosis. Further work to confirm our hypothesis is deemed necessary both in animal models and in the clinical scenario.

In contrast to our original hypothesis, we did not observe changes in endogenous opioid peptides (leu-enkephalin, met-enkephalin, and β -endorphin) within PAG compartments related to endometriosis. We focused on rostral and medial compartments of PAG, but even on immunohistochemical runs where a few caudal sections were included, minimal qualitative changes due to endometriosis were observed. Our data imply that after 60 days of endometriosis, in the rat model, endogenous opioid peptides within the PAG have adapted to the pathological condition with similar levels to those of sham-operated animals. We also examined a subset of animals at 22 days postendometriosis induction, and similar to the current results, no changes in opioid peptides within the PAG were observed (unpublished results). In vivo hydrolysis of leu-enkephalin has been reported to be decreased by 4 weeks after trauma⁵⁸; thus, changes in opioid peptides after endometriosis are probably short-lived and are likely associated with time points closer to the onset. However, even small alterations in the receptors expression, as observed here, can lead to changes in structure activity and output signaling, regardless of equal production in opioid peptides.

Study Limitations

The design of the current study did not intend to collect physiological outcomes related to pain sensitivity. Berkeley's group has previously examined hyperalgesic mechanisms of endometriosis in the same rat model demonstrating that hyperalgesia develops at 4 weeks postendometriosis induction and stabilizes by 6 to 8 weeks.¹⁰ Therefore, here, we take advantage of their previously published findings to draw indirect relations between our findings in the PAG and their results. In addition, our study design does not allow for determining whether the changes in expression of opioid peptides and their receptors occur gradually or have an acute onset after a certain time point, which might have implications in the presentation of PAG-related behaviors. As in any animal disease model, it is difficult to completely mimic the type of conditions that patients with endometriosis might go through (eg, work/study time lost, being less productive at work, concerns of infertility, and deteriorated sexual lives), which

result from them being aware/conscious of the multifaceted impact of this disease.

Clinical Considerations

Chronic conditions, such as irritable bowel syndrome, fibromyalgia, and chronic fatigue syndrome, share the common characteristic of facilitated pain signaling and abnormal pain inhibition.⁵⁹ Communication between the prefrontal cortex, the PAG, and the rostral ventrolateral medulla seems to be crucial in these chronic conditions. Studies have shown that PAG activity is increased in animal models of irritable bowel syndrome (IBS), which might be indirectly linked to visceral hypersensitivity in this chronic painful condition.⁶⁰ Despite the similarities in visceral hypersensitivity between endometriosis and IBS, current endometriosis treatments do not take into account possible abnormalities in systems that modulate stress and pain perception. Currently, multiple approaches for the treatment of endometriosis-associated symptoms are being used, which span psychotherapy to drug treatments and surgical interventions.¹¹ Despite this, we still don't have a clear understanding of whether CNS signaling circuits are altered due to endometriosis. In light of this, treatments such as exercise, relaxation techniques, cognitive psychotherapy to modulate/teach appropriate coping skills and other types of complementary therapies might prove useful for alleviating endometriosis-related symptoms. Future studies might be aimed at taking advantage of the rat model of endometriosis to empirically study the beneficial effects that stress-reducing techniques might have on the endometriosis pathophysiology and symptoms, taking also into consideration the neurofunctional changes that can occur such as those presented here.

In conclusion, our study is one of the first to demonstrate that endometriosis is associated with changes in MOR and NMDA-NR1 receptors at the level of the PAG, suggesting a dysregulation in ventrolateral PAG-mediated activity. Our results are the first steps to further explore how endometriosis might influence opioidergic and glutamatergic activity in the PAG, with the hope of eventually developing better and more specific stress management therapeutics for the treatment of this debilitating disease.

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Declaration of Conflicting Interests

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