DISSERTATION

Nociceptin/orphanin FQ opioid peptide receptor-related ligands as potential analgesics in the endometriosis-associated pain

Opioidpeptidrezeptor-verwandte Liganden von Nozizeptin/Ophanin FQ als potenzielle Analgetika bei Endometriose-bedingten Schmerzen

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Charité – Universitätsmedizin Berlin

von

Qihui Guan
aus Guangzhou, China

Erstbetreuung: Prof. Dr. med. Sylvia Mechsner
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List of abbreviations

CGRP, calcitonin gene-related peptide

COCs, combined oral contraceptives

DOR, delta opioid receptor

ELISA, enzyme-linked immunosorbent assay

EM, endometriosis

ESHRE, European Society of Human Reproduction and Embryology

GnRH, Gonadotropin Releasing Hormone

HIF-1α, hypoxia inducible factor 1 subunit alpha

IL, interleukins

KOR, kappa opioid receptor

MOR, mu opioid receptor

NFs, nerve fibres

NGF, nerve growth factor

NOP, nociceptin/orphanin FQ peptide

NSAIDs, nonsteroidal anti-inflammatory drugs

PGE_2, prostaglandins

PGP 9.5, protein gene product 9.5

RANTES, Regulated on Activation Normal T cell Expressed and Secreted

rASRM, American Society of Reproductive Medicine

ROS, reactive oxygen species

SERM, Selective estrogen receptor modulators

SPRM, Selective progesterone receptor modulators (SPRM)

SP, substance P

TH, tyrosine hydroxylase

TNFα, tumor necrosis factor α

VAS, visual analogue scale

VIP, vasoactive intestinal peptide

Abstract

Background: Endometriosis (EM) is a chronic inflammatory disease that mainly affects women of childbearing age. It occurs when endometrial tissue grows outside the uterine cavity. Pain is one of the most common symptoms of EM, however, there are currently no specific and ideal analgesic options available for EM-associated pain therapy. In this context, we aim to provide new insights and prospected analgesic options by summarizing the pathogenesis of EM-associated pain and discussing the expression of the nociception/ orphanin FQ peptide (NOP) receptor in EM-associated nerve fibres (NFs).

Methods: This prospective study spanned from May 2012 to May 2019 and included 94 female participants. Peritoneal samples were extracted via laparoscopy from 94 symptomatic women (73 EM and 21 non-EM patients). These samples underwent immunohistochemical staining for NOP, protein gene product 9.5 (PGP9.5), substance P (SP), calcitonin gene-related peptide (CGRP), tyrosine hydroxylase (TH), and vasoactive intestinal peptide (VIP). Furthermore, clear peritoneal fluids were collected during laparoscopy from patients with peritoneal EM (n = 17) and controls (n = 17). Enzyme-Linked Immunosorbent Assay was performed to compare the NOP concentration between EM and control group.

Results: Our results show that NFs density was significantly increased in the group of patients with EM compared to the control group. However, the EM group that received hormonal treatment had decreased innervation compared to the EM group without hormonal therapy. Additionally, more NOP-positive NFs and blood vessels were observed in the EM group than in the control group. The EM patients with hormonal treatment did not show any differences in NOP receptor compared to those without hormonal intake. Furthermore, we observed that NOP receptors co-localize with sympathetic, parasympathetic, and sensory fibres in the EM group. No changes in the NOP concentration in the peritoneal fluid of EM patients compared to healthy women could be found.

Conclusion: EM-associated pain have a significant impact on a patient's quality of life. Unfortunately, the current treatment strategies for this condition are not entirely satisfactory. Therefore, there is an urgent need for novel treatments that are more

effective and tolerable. Our study has shown a connection between the expression of NOP receptors, rASRM, and pain in patients with EM. This suggests that the NOP receptor and N/OFQ, as the endogenous ligand, may play a part in EM-associated pain. Further research is necessary to clarify these connections and determine whether the NOP receptor could be a target model for new therapeutic interventions.

Zusammenfassung

Hintergrund: Die Endometriose (EM) ist eine chronische entzündliche Erkrankung die hauptsächlichen Frauen im gebärfähigen Alter betrifft. Sie tritt auf, wenn Endometrium-artiges Gewebe außerhalb der Gebärmutterhöhle wächst. Schmerzen gehören zu den häufigsten Symptomen der EM, doch gibt es derzeit keine spezifischen und idealen analgetischen Optionen für die EM-assoziierte Schmerztherapie. In diesem Zusammenhang wollen wir neue Erkenntnisse und mögliche Analgetika evaluieren, indem wir die Pathogenese von EM-assoziierten Schmerzen zusammenfassen und die Expression des Nozizeptions-/Orphanin-FQ-Peptid-Rezeptors (NOP) in EM-assoziierten Nervenfasern (NFs) diskutieren.

Methoden: In diese prospektive Studie wurden 94 Frauen von Mai 2012 bis Mai 2019 eingeschlossen. Laparoskopisch entnommene Peritonealproben von 94 symptomatischen Frauen (73 mit EM und 21 Kontrollen) wurden immunhistochemisch auf NOP, Protein-Genprodukt 9,5 (PGP9,5), Substanz P (SP), Calcitonin-Gen-bezogenes Peptid (CGRP), Tyrosinhydroxylase (TH) und vasoaktives intestinales Peptid (VIP) angefärbt. Zusätzlich wurden während der Laparoskopie klare Peritonealflüssigkeiten von Patienten mit peritonealer EM (n = 17) und Kontrollen (n = 17) gewonnen. Zum Vergleich der NOP-Konzentration zwischen der EM- und der Kontrollgruppe wurde ein Enzyme-Linked Immunosorbent Assay durchgeführt.

Ergebnisse: Unsere Ergebnisse zeigen, dass die NFs-Dichte in der Gruppe der Patienten mit EM im Vergleich zur Kontrollgruppe deutlich erhöht war. In der EM-Gruppe, die eine Hormonbehandlung erhielt, war die Innervation jedoch geringer als in der EM-Gruppe ohne Hormonbehandlung. Außerdem wurden in der EM-Gruppe mehr NOP-positive NFs und Blutgefäße beobachtet als in der Kontrollgruppe. Die EM-Patienten mit Hormonbehandlung wiesen keine Unterschiede bei den NOP-Rezeptoren im Vergleich zu denen ohne Hormoneinnahme auf. Außerdem beobachteten wir, dass NOP-Rezeptoren in der EM-Gruppe mit sympathischen, parasympathischen und sensorischen Fasern ko-lokalisiert sind. Es konnten keine Veränderungen in der NOP-Konzentration in der Peritonealflüssigkeit von EM-Patientinnen im Vergleich zu gesunden Frauen festgestellt werden.

Schlussfolgerung: EM-assoziierte Schmerzen haben einen erheblichen Einfluss auf die Lebensqualität der Patientinnen. Leider sind die derzeitigen Behandlungsstrategien für diese Erkrankung nicht ganz zufriedenstellend. Es besteht daher ein dringender Bedarf an neuartigen Behandlungen, die wirksamer und verträglicher sind. Unsere Studie hat einen Zusammenhang zwischen der Expression von NOP-Rezeptoren, rASRM und Schmerzen bei Patienten mit EM gezeigt. Dies deutet darauf hin, dass der NOP-Rezeptor und N/OFQ als endogener Ligand bei EM-assoziierten Schmerzen eine Rolle spielen könnten. Weitere Forschungsarbeiten sind erforderlich, um diese Zusammenhänge zu klären und festzustellen, ob der NOP-Rezeptor ein Zielmodell für neue therapeutische Interventionen sein könnte.

1 Introduction

1.1 Current knowledge of Endometriosis

1.1.1 Definition and Pathogenesis

Endometriosis (EM) is a benign, chronic inflammatory and estrogen-dependent gynecological disease, which is characterized by the presence of endometrial-like tissue including epithelial, stromal, and smooth muscle cells, outside the uterine cavity (1). Although the first description of EM goes back to 1860, its pathogenesis is still unclear due to the complexity of this disease (2). Up to now, some hypotheses have been proposed to explain the potential origins of ectopic endometriotic lesions (Figure 1). In 1927, John Sampson, a well-known American gynecologist put forward the theory of **retrograde menstruation** explaining the etiology of EM (3). He believed sloughed endometrial cells that flow in a retrograde direction via the fallopian tubes into the pelvic cavity during menstruation result in EM. Studies suggest that retrograde menstruation transpires in 76-90% of women; however, not all of them suffer from EM (4). This hypothesis falls short in elucidating the occurrence of the condition in premenarcheal girls (5), female fetuses (6), and men (7, 8).

The embryonic rest theory offers insight into the rarer cases of EM observed in women devoid of endometrium or a menstrual cycle, as well as in men (9). Müllerian-origin embryonic cell rests are present in both sexes, possessing the ability to transform into ectopic endometrial tissue given specific circumstances. These cell remnants originate from müllerian ducts, which develop into female reproductive organs in women and undergo regression in men during early development (10). However, this theory proved overly restrictive upon further investigation of EM, particularly with the discovery of endometriotic lesions in diverse locations such as the appendix, small intestine, serosal surface of the colon, and primary umbilical EM (11).

Dr. Leyendecker put forward the **tissue injury and repair concept** to demonstrate the uterus as the origin of EM. Uterine hyperperistalsis results in microtraumatisation in the junctional zone and released mediators induce aromatase expression, the locally released estrogen promotes proliferation and angiogenesis (12). Locally released oxytocin subsequently enhances local peristaltic activity, initiating a cascade of escalating damage to the junctional zone. Another hypothesis, named **endometrial-myometrial**

interface disruption, was proposed to describe a potential ischemia and hypoxia process, which focuses on the up-regulation of HIF-1α is correlated with the establishment of EM lesions (13). Additionally, the development of lesions and associated fibrosis may be explained by increased collagen I deposition resulting from smooth muscle cells expressing oxytocin and vasopression receptors, as well as estrogen and progesterone receptors, and the process of fibroblast to myofibroblast transformation (14).

The **lymphovascular metastasis** concept aims to describe the dissemination of EM cells in the lymphovascular system. Prof. Mechsner et al. (15) showed that EM lesions are occasionally detected in randomly sampled lymph nodes during partial bowel resections with rectovaginal EM. A study with sentinel lymph node labeling in rectovaginal EM demonstrated the presence of EM lesions in labeled lymph nodes (16). As the presence of lesions was correlated with the size of the primary rectovaginal lesions and extensive lymphangiogenesis was also detectable, these data support the concept of a lymphatic distribution of EM.

While numerous hypotheses have been proposed in an attempt to elucidate the origins of EM, the etiology of this disease appears to be a complex amalgamation of various concepts previously delineated.
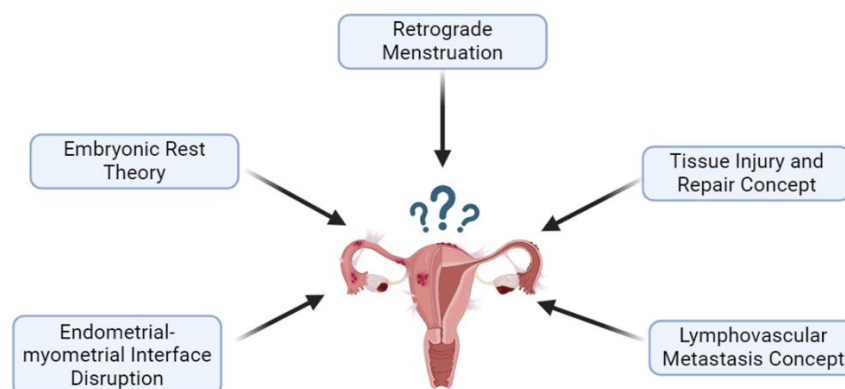
Figure 1. Summary hypotheses of Endometriosis. EM, a prevalent gynecological condition, manifests with tissue resembling the endometrium found outside the uterus. According to the Retrograde Menstruation theory, endometrial cells shed during menstruation can flow backward through the fallopian tubes into the pelvic cavity, leading to the development of EM. On the other hand, the Embryonic Rest Theory posits that müllerian-origin embryonic cell rests can transform into ectopic endometrial tissue under certain circumstances. The tissue injury and repair concept holds the opinion that uterine hyperperistalsis results in microtraumatisation in the junctional zone, locally released estrogen and oxytocin help to escalate

this damage. Endometrial-myometrial interface disruption thought the generation of EM lesions is corelated with the up-regulation of HIF-1α which results in a potential ischemia and hypoxia process. Lymphovascular metastasis concept aims to describe the dissemination of endometrial cells in the lymphovascular system, which may give an explanation of generation of EM lesions. (own representation: Guan)

1.1.2 Clinic

The range of symptoms associated with EM is diverse and is unrelated to the extent of the lesion, ranging from asymptomatic cases that are unintentionally discovered to serious diseases in patients (17). Typically, the onset of symptoms occurs before turning 20 years old (17), with common complaints including dyspareunia, dyschezia, dysmenorrhea, non-cyclic chronic pelvic pain, dysuria, and primary or secondary infertility. Of the infertile patients, 40–50% of them suffered from EM (18, 19). Various mechanisms of infertility caused by EM include pelvic structure inflammation, immune dysregulation, pelvic cavity anatomical distortions, impaired embryo implantation, adhesion development, fallopian tube fibrosis, and hormonal environment alterations within the uterus (17). Additionally, EM may elevate the risk of mental health issues such as anxiety and depression. The disease significantly diminishes patients' quality of life and social well-being due to pain, fatigue, heavy bleeding, and mood disturbances (20).

1.1.3 Diagnosis

It can be challenging to diagnose EM due to the spectrum of symptoms, and because the severity of the illness and the intensity of the symptoms often do not relate to one another. Some women may not experience any symptoms at all (21). Laparoscopic visualization of lesions with a histologic confirmation was the gold standard for diagnosis over many years (22-24), however, it is expensive and poses risks.

The advances in imaging technologies, such as transvaginal ultrasound and magnetic resonance imaging, raised the question of the need for laparoscopy for diagnosing EM. The European Society of Human Reproduction and Embryology (ESHRE) Endometriosis Guideline Core Group and other authors (25-27) believe that a combination of a thorough medical history, clinical examination, and imaging technology should be established as a gold standard diagnosis for EM.

1.1.4   Epidemiology

The Global Burden of Diseases, Injuries, and Risk Factors Study 2017 (GBD 2017) provides a thorough evaluation of the occurrence rates, prevalence, and years lived with disability attributed to 354 different causes across 195 countries and territories spanning from 1990 to 2017. According to this study, the global prevalence for EM counts in 44,656.0 per 1,000 (with a range of 37,289.1 to 52,852.4) (28). A comprehensive study undertaken in Australia sought to determine the precise prevalence of EM. Spanning nearly two decades, the research encompassed 13,508 women, integrating data from several sources such as survey responses and three health databases. The finds revealed that 6% of women had officially confirmed cases of EM, while 5.4% were suspected to have the condition. By aggregating both confirmed and suspected cases, the prevalence of EM was found to be 11.4% (95% CI 11.1-11.7%) (29).

In recent years, several authors have endeavored to elucidate the epidemiology of EM. However, the diversity in their chosen data sources, research designs, and definitions of an EM case has resulted in a persistent debate on the disease´s impact. A recent narrative review from our group(30) examined peer-reviewed data on EM prevalence, meticulously considering temporal references, data origin, and the studied population. This review aimed to reconcile the disparities between health insurance and clinical data. According to health insurance companies, approximately 1% of women are affected by EM. However, our narrative review, synthesizing information from clinical data studies (6.8%), population-based surveys/ self-reported studies (6.6%), and symptomatic patient data (21%), presented a contrasting perspective. Notably, the findings suggested that the prevalence of EM is higher than previously assumed by health insurance providers and other stakeholders. This nuanced exploration sheds light on the need for a comprehensive understanding of the disease's prevalence, challenging existing perceptions and encouraging a more inclusive dialogue on its impact.

1.1.5   Therapeutic strategies for Endometriosis and Endometriosis-associated pain

Patients commonly consult medical professionals for guidance on EM due to the presence of pain and infertility as its principal symptoms. In a recent review, six national and two international guidelines for EM were examined: the College National des

Gynecologues et Obstetriciens Francais, the National German Guideline (S2k), the Society of Obstetricians and Gynaecologists of Canada, the American College of Obstetricians and Gynecologists of Canada, the American Society for Reproductive Medicine (ASRM), and the National Institute for Health and Care (NICE) (31). With a high level of evidence, the majority of the included guidelines recommend progestins (dienogest or medroxyprogesterone acetate) in conjunction with combined oral contraceptives as initial treatment options for EM-associated pain. GNRH-agonists and the levonorgestrel intrauterine device are classified as second-line therapies. The effectiveness of alternative medical treatments such as danazol, gestrinone, aromatase inhibitors, Selective estrogen receptor modulators (SERM), and Selective progesterone receptor modulators (SPRM) is restricted, resulting in inconsistencies amongst guidelines. Surgery is a crucial method for treating pain associated with EM. The removal of endometriotic implants and endometriomas is the established standard procedure. Nonsteroidal anti-inflammatory medicines (NSAIDs) are commonly prescribed to alleviate symptoms of dysmenorrhoea and acyclic pelvic pain. The guidelines of these eight societies commonly define the usage of NSAIDs. As a first-line treatment for symptomatic conditions, nonsteroidal anti-inflammatory drugs (NSAIDs) are contraindicated for long-term use due to the potential for adverse effects. Pain management may involve the administration of metamizole, and in more severe instances, opioids (31, 32).

When considering infertility, the therapeutic choices offered are distinct from those for EM-associated pain. Surgery achieved the highest level of proof and use the standard procedure. Pharmacological treatments are generally not advised, with the exception of GnRH-agonists which might be utilized as a downregulation therapy prior to IVF or surgery (31).

However, these therapies often come with notable side effects. More than 30% of patients failed to relieve EM-associated pain via conventional medical and surgical therapies (33). Respectively, recurrence of chronic pelvic pain following laparoscopy was reported to be 21.5% within 2 years and 40-50% within 5 years (34-37). Managing the aftereffect reaction of hormonal therapy can pose challenges, even if EM symptoms improve. Reduced estrogen levels can result in problems such as decreased bone density, breast shrinkage, and mood instability. The occurrence of these side effects requires that the use of GnRH be limited to a maximum period of 6 months (38).

Furthermore, a risk of gastrointestinal hemorrhage is linked with the NSAIDs use. Regarding metamizole, the most prominent adverse event is agranulocytosis, along with hepatotoxicity related to metamizole (39). Opioids ought to be given beneath strict supervision due to the critical chance of enslavement (40). There is no proof to back the use of complementary treatments such as needle therapy, yoga, dietary supplements, and electrotherapy for EM-associated pain and fruitlessness (31, 32, 41).

Taken together, these facts underscore the urgent need for novel treatments that efficiently alleviate symptoms and prevent recurrence while preserving fertility. Looking for effective, fewer side effects and less traumatic strategies is an urgent call for EM treatment.

1.2   Physiopathology of pain development in Endometriosis

Most research on the pain mechanisms in EM has primarily centered around endometritotic lesions and adhesions as the main contributors to EM-related pain. While lesion-specific pain is evident and undoubtedly plays a crucial role in inducing EM-related pain, it's important to note that not all patients experience pain relief after lesion removal. As stated above, reports indicate that 20–28% of patients continue to experience pain even after surgery (34, 42). Pelvic diseases often result from the retrograde menstruation of hormone-sensitive endometrial cells, triggering inflammation, neuroangiogenesis, scarring, and fibrosis (19, 43, 44). Regarding the research progress of the mechanisms underlying the development of a chronic pain state in EM, recent studies mainly focus on inflammation (45-47), neurogenic inflammation (34, 48-50), peripheral sensitization (51, 52), central sensitization (53, 54), and cross-organ sensitization (55). In menstruation, due to the lowering levels of progesterone and estrogen, the endometrial tissue is naturally broken down and removed (56). In order to speed up the destruction of menstrual tissue, a sizable number of neutrophils, macrophages, and natural killer cells are recruited during this phase. Endometrial fragments that flow backward during retrograde menstruation still have endometrial features and undergo cyclical inflammation (57, 58). In EM, menstrual by-products originating from non-uterine lesions are released into the peritoneal cavity. This contributes to the accumulation of iron, reactive oxygen species (ROS), prostaglandins (PGE_2), and acidosis in the peritoneal fluid, triggering immune responses (59). The immune reaction spreads to lesion sites within the peritoneal

cavity, where increased concentrations of cytokines, chemokines, growth factors, and neutrophils create an inflammatory environment in patients (lesion inflammation). Notably, agents like PGE$_2$, tumor necrosis factor α (TNFα), nerve growth factor (NGF), Regulated on Activation Normal T cell Expressed and Secreted (RANTES), interleukins (IL) -8, and IL-1β directly stimulate sensory nerve endings, setting off a positive feedback loop (neurogenic inflammation). This increased activation of peripheral nerve endings (known as peripheral sensitization) intensifies the transmission of painful signals to the spinal cord, establishing and prolonging chronic pelvic pain (referred to as central sensitization). It is theorized that cross-organ sensitization occurs when sensitized nerve fibers from one organ trigger the sensitization of nerve fibers supplying another organ. Visceral nerve fibers converge in common areas of the spinal cord, creating the possibility of nearby cell sensitization due to their spatial proximity (55, 60). Due to the interconnected nerve pathways leading to the dorsal root ganglion and spinal cord, nerve fibers innervating the colon, bladder, uterus, and vagina have the capacity for cross-organ sensitization (60-63) (Figure 2).
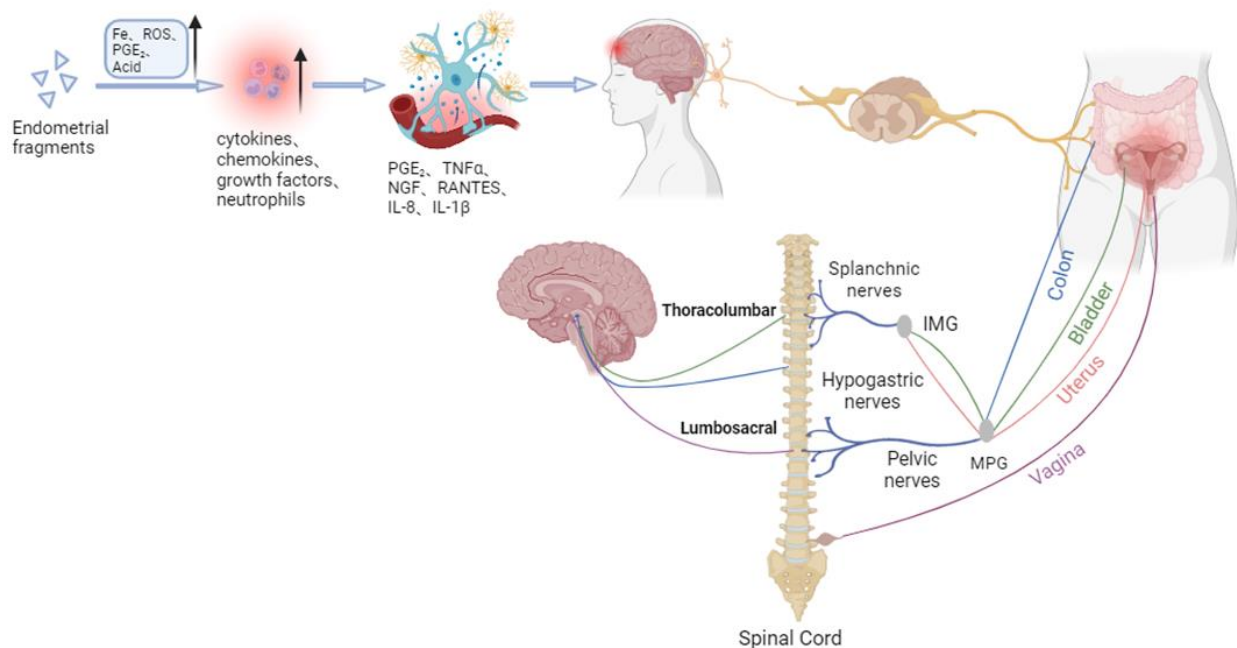
Figure 2. Schematic of development of pelvic pain in Endometriosis. Endometrial fragments deposited leads to the accumulation of iron, reactive oxygen species (ROS), prostaglandins (PGE$_2$), and acidosis in the peritoneal fluid. This triggers an immune response characterized by cytokines, chemokines, growth factors, and neutrophils. Inflammatory mediators like PGE$_2$, tumor necrosis factor α (TNFα), nerve growth factor (NGF), Regulated on Activation Normal T cell Expressed and Secreted (RANTES), interleukins (IL) -8, and IL-1β directly activate sensory nerve endings, initiating a positive feedback loop. Pain signals are then relayed to the spinal cord and transferred to nerves innervating the colon, bladder, uterus, and vagina,

resulting in cross-organ sensitization. Nociceptin signals are transmitted back to the spinal cord and eventually reach the brain cortex via spinal nerves. IMG, Inferior Mesenteric Ganglion; MPG, Major Pelvic Ganglion (38).

1.3 Endogenous opioid system and Endometriosis-associated pain

The endogenous opioid system is composed of endogenous opioid peptides and receptors (64). Belonging to the seven transmembrane-spanning superfamily of G-protein-coupled receptors, delta (DOR), mu (MOR), kappa (KOR) opioid receptor, and nociceptin/orphanin FQ peptide (NOP, initially called LC132, ORL-1) receptor are four main members in the endogenous opioid system (64, 65) (Figure 3). Involving in neurogenic inflammation modulation, the endogenous opioid system is a prospected therapy target for the treatment of EM-associated pain (66).
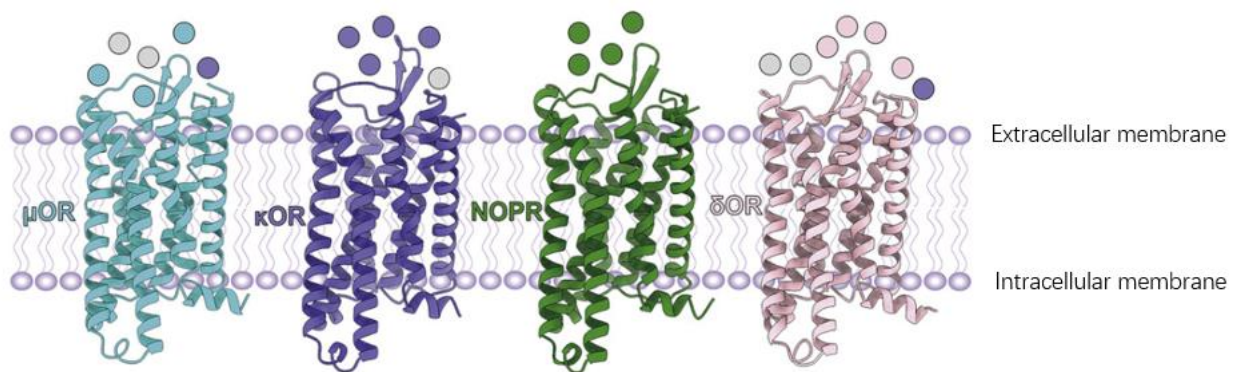
Figure 3. Schematic of main members in the endogenous opioid system. Opioid receptors are a group of G protein-coupled receptors (GPCRs), comprising MOR, DOR, KOR, and nociceptin receptor, all of which transduce signaling primarily through the inhibitory $G_{i/o}$ proteins (67).

KOR, one of the opioid receptors, is crucial for visceral and inflammatory pain (68-70), and it is expression has been shown in women's ectopic endometrial tissues (71). In female mice with EM, KOR could both prevent and reduce chronic pelvic mechanical sensitivity and discomfort (66).

MOR is expressed in the human endometrium with changing expression pattern throughout the menstrual cycle and endometrial compartments, suggesting that this opioid receptor may have various functions in the intricate remodeling process and, therefore, also in EM (70). In EM stromal cells, MOR receptor mRNA was upregulated, indicating potential involvement in the compromised immune system of this disease (72). Moreover, there was a significant elevation in MOR expression observed in ovarian EM

compared to eutopic endometrium (73). Stromal cells from patients with DIE treated with GnRH agonists exhibited a lack of MOR expression. In contrast, MOR levels were detectable in patients treated with progestin, albeit significantly lower compared to untreated patients (74).

While there is currently no evidence supporting the involvement of DOR in EM or suggesting that therapies targeting DOR could relieve EM-associated pain, reports indicate that DOR agonists exhibit activity in the periphery and may have the potential to alleviate chronic inflammatory visceral pain (75).

Nociceptin/orphanin FQ (N/OFQ) was identified in the 1990s (76), and because of its distinct structure, it does not bind to the classical opioid receptors, even though it shares a high degree of similarity with thosereceptors (77). The central and peripheral neurological systems, as well as peripheral organs like the heart and intestines, and the immune systems of rats and humans, all have high expression levels of the NOP receptor and the endogenous ligand N/OFQ (77). Additionally, N/OFQ has been implicated in pain modulation (78), with spinal NOP receptor activation leading to anti-hyperalgesic and anti-allodynic effects in chronic pain conditions (78). In contrast to the traditional opioid receptors, the expression of NOP receptors is unaffected by opioids and is decreased by combinations of LPS and PepG (79). Despite all of this knowledge, the possible connection between NOP or N/OFQ and pain associated with EM continues an uncharted territory.

1.4 Aims and hypotheses

To gain a better understanding of pain related to EM, we aimed to analyze the expression of NOP receptor in EM-associated nerve fibers for the first time. With the hypothesis that NOP receptors are present in both sensory and autonomic nerve fibers, our investigation aimed to delve into uncharted territory by exploring their expression in EM-associated nerve fibers. An additional facet of our hypothesis posited an augmentation in NOP receptor expression within these nerve fibers affected by EM. This innovative approach not only contributes to the evolving understanding of EM-related pain but also opens avenues for potential therapeutic interventions targeting NOP receptors in the intricate neural network associated with this challenging medical condition

2   Methods

2.1   Participants of the project

In this prospective study, a total of 94 women were enrolled between May 2012 and May 2019. Among them were 73 symptomatic EM patients who underwent laparoscopy with excision of endometriotic lesions. Twenty-one control samples were obtained from women without EM, who had undergone laparoscopy due to benign gynecological diseases such as peritonealised tissue, ovarian cysts, *hydrosalpinx*, uterine fibroids, pelvic pain, or infertility. Moreover, clear peritoneal fluids were collected during laparoscopy from peritoneal EM (n= 17) and non-EM patients (n= 17). The participants were chosen based on clinical intraoperative findings and histopathological results.   All patients underwent a comprehensive gynecological assessment, including palpation and transvaginal ultrasound.

2.2   Samples grouping

The localizations of peritoneal lesions were as follows: (a) 17 pelvic walls, (b) 4 bladders, (c) 14 pouch of Douglas, (d) 6 uterosacral ligaments, (e) 2 peritoneum, and (f) 30 fossa ovaricas. The stages of EM were classified according to the American Society of Reproductive Medicine (rASRM): (I) minimal, (II) mild, (III) moderate, and (IV) severe. In this study, rASRM I and II were considered as mild and rASRM III and IV were considered as severe. A visual analogue scale (VAS; no pain=0, strongest=10) was used as the standardized evaluation for the severity of pain (80). Based on the pain scale, patients were divided into two groups: moderate pain (scores 0-5) and severe pain (scores 6-10).

2.3   Immunofluorescence microscopy

2.3.1   Samples preparation

All peritoneal biopsies were fixed into 4% formalin for 18 - 24 h and then embedded in paraffin. After embedded, each biopsy section was cooled on the Tissue Cool Plate COP 20 (medite GmbH, Burgdorf W. Germany) for at least 40 min, thereafter was cut into 2 µm of thickness via microtome (pfm Slide 4004 M, pfm medical ag, Germany) and mounted on microscope slides (SuperFrost Plus, VWR International, LLC, Leuven

Belgium). The sections were then dried at 37°C for overnight and stored in the refrigerator at 4°C. Slides were de-waxed in the dry oven under 60°C for 45 min and cooled down for 20 min before deparaffinization and rehydration.

2.3.2   Immunofluorescence double staining

After preparing the samples, the sections underwent deparaffinization in the Xylene for 10 min, followed by a rehydration process in a descending series of ethanol (100%, 100%, 96%, 80%, 70%; 3 min per step) and finally in distilled water for 5 min. The antigen retrieval was then performed in the preheated Target 9 buffer (10 mM Tris Base, 1 mM EDTA sol., 0.05% Tween) for 20 min, and cooled down in room temperature for 20 min. After washing with Tris-Buffered Saline (TBS) buffer (twice, 5 min per round), the sections were permeabilized in 0.1% TBS-T (triton X-100) for 20 min at room temperature. Protein blocking was carried out in 5% Bovine Serum Albumin (BSA, Sigma, St. Louis, MO, USA) in TBS and 0.1% Tween for 30 min in a humid box in room temperature. Sections were incubated under 4°C overnight with the primary antibodies listed in Table 1. After washing the sections three times in TBS for 5 min each, they were incubated with the secondary antibody (Table 1) for 1 hour at room temperature. The sections were washed with TBS buffer twice for 5 min each and then incubated with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI, 100ng/ml, 32670-5MG-F – Sigma, USA) for 10 min. Finally, the sections were mounted with Kaisers Glyceringelatine mounting medium (1092420100, Merck K GaA, Germany) and left to dry in a dark hood for 24 hours.

The negative control sections were prepared by excluding the primary antibody during the processing. The positive control consisted of a skin incision and a tissue segment of peritoneal EM with significant nerve incisions.

Table 1. Primary and secondary antibodies used in the Immunofluorescence double staining

Antibodies	Code	Company	Dilution
Primary antibodies			
Human Orphanin FQ/Nociceptin	sc-398073	Santa Cruz, DEU	1:50
Human Orphanin FQ/Nociceptin	ab66219	Abcam, UK	1:380
Protein gene product 9.5 (PGP 9.5)	NB110-58872	Novus, DEU	1:300
Substance P (SP)	sc-21715	Santa Cruz, DEU	1:500
Calcitonin gene-related peptide (CGRP)	sc-8857	Santa Cruz, DEU	1:100

Tyrosine hydroxylase (TH)	T2928	Sigma, USA	1:100
Vasoactive intestinal peptide (VIP)	sc-25347	Santa Cruz, DEU	1:100
Secondary antibodies			
Alexa Fluor 594 donkey anti-rabbit IgG (H+L)	A21207	Invitrogen, USA	1:300
Alexa Fluor Plus 594 donkey anti-mouse IgG (H+L)	A32744	Invitrogen, USA	1:300
Alexa Fluor 488 donkey anti-rat IgG (H+L)	A21208	Invitrogen, USA	1:300
Alexa Fluor 488 donkey anti-mouse IgG (H+L)	A21202	Invitrogen, USA	1:300
Alexa Fluor 594 donkey anti-goat IgG (H+L)	A11058	Invitrogen, USA	1:300
Alexa Fluor 488 goat anti-chicken IgG (H+L)	A11039	Invitrogen, USA	1:300

(own representation: Guan)

2.3.3 Images capture

An axiophot microscope (Carl Zeiss, Göttingen, Germany) was used for detecting the staining outcome. Photomicrographs were taken at two different magnifications of 100X and 200X. The photos were then processed using Adobe Photoshop (2022 Full Version, cs6, Adobe Systems, Unterschleissheim, Germany). The density of PGP 9.5 NFs was accessed by quantifying the number of immunostained nerves adjacent to the endometriotic lesions (including epithelial, stromal, and smooth muscle cells) within the distal area of 1 mm^2. In the control group, the nerve fiber density was determined using the "hotspot" method as previously described (81).

Two experienced observers performed a double-blind investigation throughout the assessment of nerve fiber density. The code of each patient was not disclosed until the study analysis was completed. If there were any discrepant results, the two observers repeated the evaluation together until they reached a consensus.

2.4 Measurement of NOP ligand concentration

After the trocars were inserted, peritoneal fluids were extracted from the Douglas' pouch, centrifuged at 3000 rpm for 5 minutes, and the supernatants stored at -80°C. Using the commercially available Human Orphanin FQ/Nociceptin enzyme-linked immunosorbent assay (ELISA) kit (plate number 1—Nordic BioSite AB, Täby, Sweden), the concentration of the endogenous Orphanin FQ/Nociceptin ligand was determined in duplicate. The sensitivity of this kit is 2.813 pg/mL, and its detection range is 4.688–300 pg/mL. The entire process was performed in compliance with the manufacturer's guidelines. Thermo Scientific Multiskan FC (Waltham, MA, USA, Unity Lab Services) was used to

automatically quantify the optical density (absorbance at 450 nm) after the substrate reaction.

2.5 Statistical Analysis

The statistical analysis was conducted using IBM SPSS for Windows (version 29.0.0.0). Parametric t-test or nonparametric Mann-Whitney U, Kruskal-Wallis, and Spearman correlation tests were used to evaluate the data. Chi-square and Fisher's exact tests were employed to assess the qualitative variable. For $p < 0.05$, statistical significance was defined.

2.6 Ethical Considerations

Ethics Institutional Review Board of the Charité University Medicine approved the study (Ethic vote EA4/036/12). All participants gave their consent prior to enrolment.

3   Results

3.1   Population characteristics of participants

Table 2. Characteristics of the study participants

	EM patients (N= 73)	Controls (N= 21)
Age (years)		
Mean	31.2	35.6
SD	6.93	10.65
Stages (rASRM)		
I-II	49 (67.1%)	-
III-IV	24 (32.9%)	-
Hormone treatment		
Yes	22 (30.1%)	2 (9.5%)
Missing data	5 (6.8%)	8 (38.1%)
Pain (EM-associated pain)		
Number of patients	70 (95.9%)	8 (38.1%)
Missing data	3 (4.1%)	11 (52.4%)
Patients with EM-associated pain		
Pelvic pain		
Number of patients	66	8
Pain intensity (mean, SD)	5.27 ± 1.62	N.A.
Missing data	50	8
Dysmenorrhea		
Number of patients	64	6
Pain intensity (mean, SD)	5.59 ± 2.33	N.A.
Missing data	41	6
Dyspareunia		
Number of patients	43	4
Pain intensity (mean, SD)	4.64 ± 2.29	N.A.
Missing data	27	4
Dyschezia		
Number of patients	25	
Pain intensity (mean, SD)	4.55 ± 2.70	N.A.
Missing data	15	
Dysuria		
Number of patients	11	
Pain intensity (mean, SD)	3.25 ± 1.50	N.A.
Missing data	7	
Menstrual cycle		
Menses	5	0
Proliferative	14	1
Secretory	11	4
Hormone intake	23	2
Menopause	0	0
Missing data	20	14

N.A. No answer.(82)

In this study, 94 women which is comprised of 73 EM patients and 21 women without EM were recruited. The population characteristics including different kinds of painful

symptoms are summarized in Table 2. The average age of the EM group was 31.2 (18 –
50) years, while women in the control group were on average 35.6 (18–52) years old. No
significant difference in age between EM and non-EM patients was observed ($p = 0.131$).
Twenty-two (30.1%) EM patients were under hormonal treatment during the period of
surgery, while 2 (9.5%) of non-EM patients received hormonal therapy. In the EM group,
50 out of 73 women (68.49%) had minimal to mild EM (rASRM I and II). The remaining
23 women (31.51%) had moderate to severe EM (rASRM III and IV).

3.2   Nerve Fibre Density in Peritoneal Endometriotic Lesions

In both the EM and control group, nerve fibers density was observed using anti-PGP 9.5
(Figure 4A). Notably, compared with the control group (mean ± SD: 0.28 ± 0.70 NF/mm^2),
PGP9.5 nerve fibre density was significantly increased in endometriotic lesions (mean ±
SD: 1.76 ± 1.57 NF/mm^2; $p < 0.001$; Figure 4B). Hormonal therapy in the EM group
resulted in a substantial decrease in nerve fibre density (mean ± SD: 1.05 ± 0.79 NF/mm^2)
compared to the EM group without hormonal treatment (mean ± SD: 2.15 ± 1.76 NF/mm^2;
$p = 0.011$; Figure 4C).

Furthermore, the density of PGP9.5-positive nerves exhibited a correlation with the
rASRM stages (Table 3, r = 0.403; $p < 0.001$).
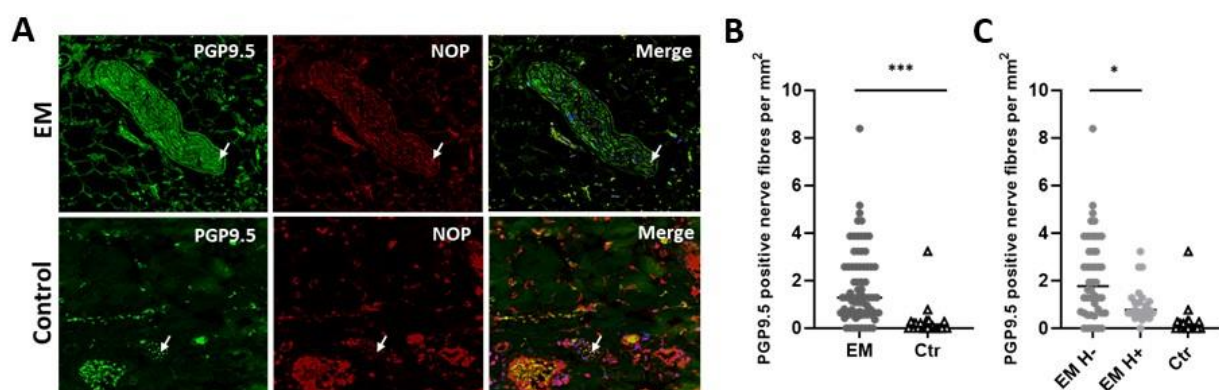
Figure 4. Endometriosis patients showed increased nerve fibers density. (a) EM and control samples
stained with PGP9.5 (green) and NOP (red) antibody. Merge images shown the colocalization of pan
marker (PGP9.5) and the NOP receptor. All pictures are in 200x magnification. (b - c) PGP9.5 positive
nerve fibers per mm^2 in EM patients and controls. Arrows indicate colocalisation points. EM: endometriosis
patients; Crt: control; H+: under hormonal treatment; H-: without hormonal treatment; All the results are
presented as median, 25% - 75% Percentile. Mann-Whitney test and Kruskal-Wallis with Dunn's multiple
comparison tests. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.(82)

Table 3. Correlation analysis

			P value
Hormonal therapy in EM[a]	Pelvic pain		0.545
	Dysmenorrhea		0.599
	Dyspareunia		1.000
	Dyschezia		0.579
	Dysuria		1.000
Pain level and hormonal therapy in EM[a]	Pelvic pain		0.674
	Dysmenorrhea		1.000
	Dyspareunia		0.388
	Dyschezia		0.543
	Dysuria		1.000
Pain level and rASRM[a]	Pelvic pain		0.611
	Dysmenorrhea		1.000
	Dyspareunia		1.000
	Dyschezia		0.560
	Dysuria		0.405
Pelvic pain and nerve fibres density/ pain receptor[b]	PGP9.5	Nerve fibres/ mm^2	0.108
	NOP	Nerve fibres/ mm^2	0.253
		Blood vessels/ mm^2	0.806
Dysmenorrhea and nerve fibres density/ pain receptor[b]	PGP9.5	Nerve fibres/ mm	0.480
	NOP	Nerve fibres/ mm^2	0.481
		Blood vessels/ mm^2	0.270
Dyspareunia and nerve fibres density/ pain receptor[b]	PGP9.5	Nerve fibres/ mm^2	0.475
	NOP	Nerve fibres/ mm^2	0.958
		Blood vessels/ mm^2	0.729
Dyschezia and nerve fibres density/ pain receptor[b]	PGP9.5	Nerve fibres/ mm^2	0,740
	NOP	Nerve fibres/ mm^2	0.742
		Blood vessels/ mm^2	0.863
Dysuria and nerve fibres density/ nerve fiber receptor[b]	PGP9.5	Nerve fibres/ mm^2	0.674
	NOP	Nerve fibres/ mm^2	0.288
		Blood vessels/ mm^2	0.801
rARSM and nerve fibres density/ nerve fiber receptor[b]	PGP9.5	Nerve fibres/ mm^2	**<0.001****
	NOP	Nerve fibres/ mm^2	**<0.001****
		Blood vessels/ mm^2	**0.024***

Analyses made with [a]Chi-square or Fisher and [b]Spearman correlation; *p* < 0.05; ** *p* < 0.005.(82)

3.3   Increased Expression of NOP could be seen in the EM group

Compared with the control group (mean ± SD: 0.11 ± 0.17 NOP-positive NF/mm^2), a significantly increased of NOP-positive nerve fibers could be seen in the EM group (mean ± SD: 1.22 ± 1.62 NOP-positive NF/mm^2; *p* <0.001; Figure 5A). EM patients with hormonal treatment did not show any difference in the expression of NOP receptor (mean ± SD: 0.83 ± 0.99 NOP-positive NF/mm^2) compared to those without hormonal treatment (mean ± SD: 1.49 ± 1.86 NOP-positive NF/mm^2; *p*= 0.381; Figure 5B).

Furthermore, the EM group showed more blood vessels (mean ± SD: 6.0 ± 4.7 blood vessels/mm²) than the controls (mean ± SD: 2.7 ± 4.3 blood vessels/mm²; *p* = 0.007; Figure 5C). A significant increase of NOP-positive stained vessels in the EM group (mean

± SD: 1.1 ± 1.8 blood vessels NOP-positive/mm²) could be seen compared with women without EM (mean ± SD: 0.2 ± 0.7 blood vessels NOP-positive/mm²; p = 0.013; Figure 5D). The number of blood vessels and their NOP-positivity in EM patients was not affected by the hormonal treatment.

The density of NOP-positive nerve fibers (r = 0.410; p< 0.001) and blood vessels (r = 0.307; p = 0.024) correlated with the rASRM stages but not with the pain levels (Table 3).
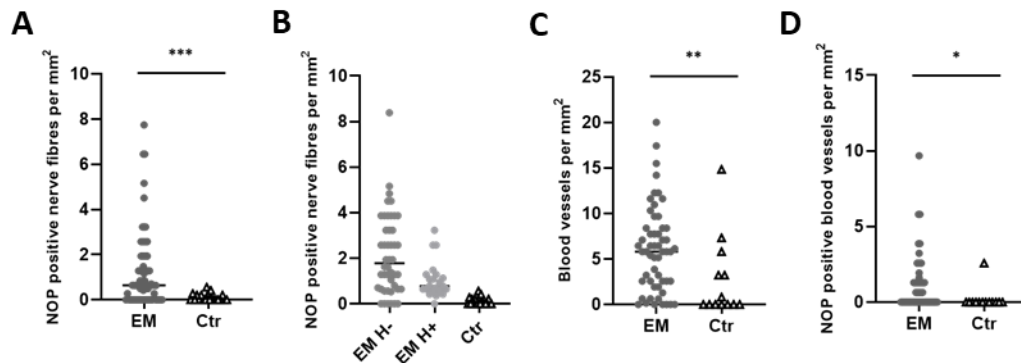

Figure 5. Endometriosis patients showed increased blood vessels density and NOP-receptor expression. (a-b) NOP positive nerve fibers per mm² in EM patients and controls. (c) Blood vessels per mm² in EM patients and controls. (d) NOP positive blood vessels mm² in EM patients and controls. EM: endometriosis patients; Crt: control; H+: under hormonal treatment; H-: without hormonal treatment; All the results are presented as median, 25% - 75% Percentile. Mann-Whitney test and Kruskal-Wallis with Dunn's multiple comparison tests. * = p < 0.05; ** = p < 0.01; *** = p < 0.001. (Guan et al. 2023)

3.4 NOP Expression across Sympathetic, Parasympathetic, and Sensory Fibers surrounding the endometriotic lesions

The result of double-staining immunofluorescence indicated that NOP receptors were co-labeled with most (>75%) sympathetic fibers which are labelled with TH-positive, many (50%-75%) parasympathetic fibers which are labelled VIP-positive and many (50%-75%) sensory fibers which are labelled with SP and CGRP positive both in the EM and the control group (Figure 6).
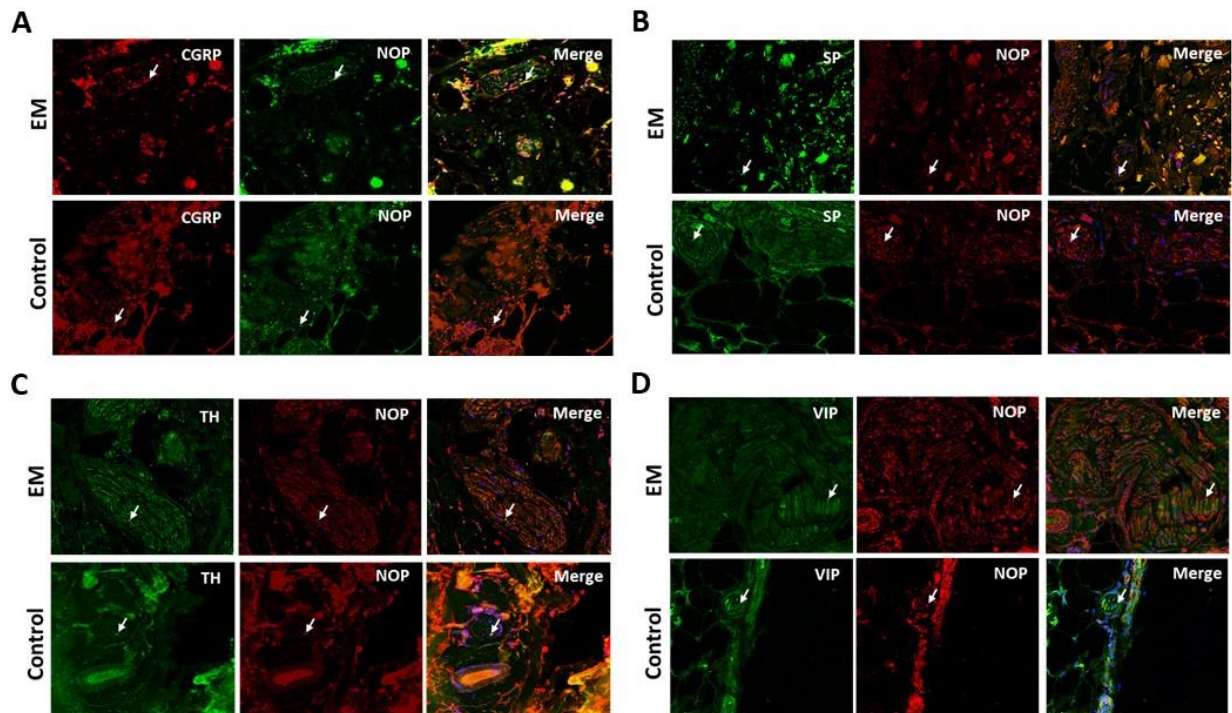
Figure 6. The presence of NOP receptors has been observed in sympathetic, parasympathetic, and sensory fibers that innervate endometriotic lesions. (a) EM and non-EM samples were stained with CGRP (red) and NOP (green) antibodies to visualize sensory fibers.(b) EM and non-EM samples were stained with SP antibody for sensory fibers (green) and NOP antibody (red). (c) The EM and non-EMsamples were stained with TH antibody to visualize sympathetic fibers in green, and NOP antibody in red.(d) Samples were stained with VIP (parasympathetic fibers - green) and NOP (red) antibody for analysis. Arrows demonstrate the points of colocalisation between the nerve marker and the NOP receptor. All pictures are in 200x magnification.(82)

3.5 Comparable Expression of Endogenous Orphanin FQ/Nociceptin Ligand Concentration in Endometriosis and Control Groups

The endogenous Orphanin FQ/Nociceptin ligand concentration expression in the peritoneal fluid did not show any statistically difference between EM (mean ± SD: 2.81 ± 11.59) and the control group (7.88 ± 22.07, p= 0.586; Figure 7). All women with EM (rASRM I= 29.41%, II= 35.29%, III= 00.00%, IV= 35.29%) were premenopausal, with a mean age of 31.4 ± 4.4 years (ranging from 26 to 40 years), and had a regular menstrual cycle (41% in the secretory phase and 59% in the proliferative phase). The control group comprised premenopausal individuals with a mean age of 32.6 ± 5 years (ranging from 20 to 50 years). They exhibited regular menstrual cycles, with 29.4% in the secretory phase, 41.2% in the proliferative phase, and 5.9% during menses. Additionally, one

woman in the control group was using an oral contraceptive.

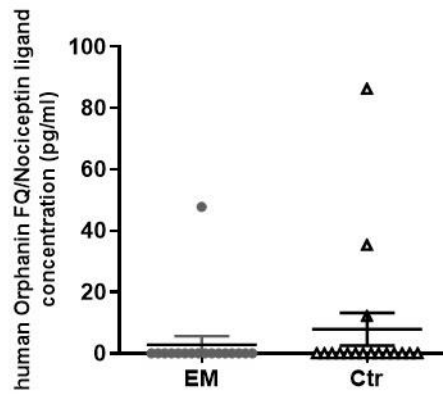**Figure 7. Measurement of Orphanin FQ/Nociceptin ligand concentration (pg/ml) in the peritoneal fluid of women diagnosed with peritoneal endometriosis and controls.** Endogenous Orphanin FQ/Nociceptin ligand concentration expression in the peritoneal fluid of women with peritoneal EM and controls was determined using an ELISA kit. EM: Endometriosis; Ctr: Control; Mann-Whitney test. *p* = 0.586.(82)

4   Discussion

Current treatments for EM are often deemed inadequate due to their limited efficacy and the prevalence of undesirable side effects. Both medical professionals and patients have recognized the urgent need for better and more effective interventions to address this chronic condition. Specifically, there is a high demand for advancements in pain management strategies tailored to the unique challenges posed by EM. A few NOP-R agonists have exhibited notable anti-nociceptive and anti-allodynic impacts in experimental torment models (78). In rodent models of neuropathic pain (83, 84) and non-human primate models of acute and inflammatory pain (85-87), enactment of the NOP-R via its endogenous peptide ligand (N/OFQ) or non-peptide agonists produces strong anti-hypersensitive and anti-nociceptive effects. The perplexing localization and useful versatility of the N/OFQ-NOP-R framework torment pathway, counting the DRG, shallow dorsal horn of the spinal line, tangible trigeminal complex, and periaqueductal dim, adjust with its set up part in tweaking nociceptive flagging in assorted settings, depending on the location, torment state, and species included (87). Nevertheless, the characterization of the N/OFQ-NOP-R system in the periphery remains relatively underexplored, with limited investigation conducted in human tissue. In the current investigation, our hypothesis were confirmed: (1) NOP receptors are present in both sensory and autonomic nerve fibers, and (2) their expression is increased in nerve fibers from EM patients. Furthermore, our findings have shown that the expression of NOP receptors is correlated with EM stage (rARSM) and pain levels. This represents the inaugural exploration of the relationship between NOP localization and EM-associated pain, suggesting a potential role for NOP in the management of this pain condition.

The physiological and pathological mechanism of pain associated with EM is not yet fully understood. However, the abnormal innervation of EM lesions is considered crucial in the role of chronic pelvic pain among EM patients. The innervation associated with the lesion of EM was initially observed by Anaf et al. (88) in deep infiltrating EM in the rectovaginal septum, where perineural and interneural invasion occurred. Similarly, in this study, which enrolled 73 EM patients, hyper-innervation in endometriotic lesions was confirmed, as described in previous studies (89-94).

Nerve presence within endometriotic lesions has been confirmed across human, murine, and rat models. When compared to the normal peritoneum, ectopic endometrial implants

in these models exhibit high levels of sympathetic, parasympathetic, and sensory nerve fibers in the peritoneal lesion (93, 95, 96). EM is a painful chronic inflammatory disease with a possible disturbance of the opioid system. This disturbance may contribute to the inflammatory condition, pain origination, and development (70, 71, 74, 97, 98). As a member of the opioid family, NOP, which is widely expressed both in the nervous system and immune system (77), has become the focus of attention among scientific researchers due to its correlation with pain. Our study revealed a notable increase in the expression of the NOP receptor in nerve fibers and blood vessels of patients with EM compared to non-EM. This corroborates the idea that peripheral sensitization and EM-associated nerve fibers play a significant role in the generation of pain. A striking increment in NOP receptor immunoreactive nerve fibers was observed in bladder samples from patients with overactive bladder and bladder pain syndrome - another chronic pelvic pain condition (99). Additionally, we found that the NOP receptor was co-localized in the sensory nerve marked as SP and CGRP positive, and in the autonomic nerve marked as TH and VIP positive, as previously reported by Schroder et al. (100).

Regarding hormonal therapy, we conducted a comparison between the nerve fibre density of EM patients who received hormonal treatment and those who did not. Our findings indicate that there is a significant decrease in nerve fiber density in EM patients who did receive hormonal treatment when compared to those who did not. Tokushige et al. reported that conventional hormone treatments, as progestogens and oral contraceptives, that reduce EM-associated pain, decreased nerve fiber density in ectopic endometrium (101). Another study associated the use of hormonal therapy with over 65% reduction of nerve fibers in rectovaginal EM specimens (102), a suggestion previously noted in superficial peritoneal EM (103). A report from Lancet performed a Randomized Controlled Trial, indicating that GnRHa helps to alleviated chronic pelvic pain induced by EM (104). These findings suggest that hormonal therapy can reduce nerve fiber density and ultimately alleviate the chronic pelvic pain caused by EM. However, the administration of hormonal treatment did not yield discernible differences either in the expression of NOP receptors or in the density of blood vessels among the EM patients. In a study reported by Constantin et al. (105), Nociceptin/orphanin-FQ exerted a potent inhibition on GnRH neurons. In vivo, double-labeled OFQ/POMC fibers were found in the vicinity of GnRH neurons, and OFQ fibers apposed GnRH neurons. Common anti-angiogenic drugs used in EM mainly includes anti-VEGF, Cox-2 inhibitors, angiotensin II

receptor blockers, immunomodulators, antioxidants, NF-κB inhibitors (106). Khan et al. (107) demonstrated that GnRH agonist significantly decreased the infiltration of micro-vessel density in the endometrium of women with EM. Levonorgestrel-releasing intra-uterine system, a commonly used intrauterine device, has been reported to decreased VEGF expression both in the endometrial glands and stroma (108).

Our study unveiled a positive correlation between the rASRM stage and nerve fiber density, suggesting that EM women in rASRM stages III and IV exhibit more pronounced hyper-innervation compared to those in stages I and II. To date, there have been no reports addressing the relationship between rASRM scores and nerve fiber density. Additionally, a noteworthy positive correlation between NOP receptor density and rASRM stages was revealed, indicating a higher abundance of NOP receptors in EM patients with moderate to severe endometriosis (rASRM stages III and IV) compared to those with minimal to mild endometriosis (stages I and II). Up to date, although there is a strong correlation between the plasmatic N/OFQ levels and the severity of sepsis (109-111), arthritis (112), and inflammatory bowel diseases (113-115), relationship between EM and NOP or N/OFQ remains unknown. Furthermore, our correlation analysis indicated a positive association between NOP-positive stained nerve fibers and blood vessel density with the rASRM stage, but not with the pain levels or symptoms.

4.1   Strengths and weaknesses of the study(s)

Our study has strengths. We are pleased to report that our study is the first to observe NOP in EM. This initial exploration provides useful context and identifies potential areas for further investigation. It also aids in developing hypotheses, leading researchers towards more focused inquiries and experimental designs. Also, the good sample size for the patient groups is a positive point. However, we acknowledge that this study has some limitations, such as a small sample size for controls and the lack of detailed functional assessments. Nevertheless, the unbiased sampling approach compensates for these limitations, and a larger sample size would provide a more conclusive outcome. To minimize subjectivity, two experienced observers conducted a double-blind investigation during the assessment of NF density.

4.2   Implications for practice and/or future research

The main therapies currently used in the clinical treatment of EM have several adverse effects. Among those are alterations in lipid profile, vaginal dryness, depression, mood changes, exhaustion, and bone mineral loss. As a result, approximately 10% of patients are incapable of proceeding with the treatment (33, 116, 117). Endogenous opioid peptides are secreted by the CNS and immune system cells (65). These peptides bind to opioid receptors located on sensory nerve terminals producing analgesic effects by either reducing the nerve's ability to transmit signals or by suppressing the pro-inflammatory neuropeptides release. However, the widespread problems of drug abuse and addiction to opioids are particularly prominent in modern society. The widespread availability and extensive use of opioids for medical reasons have contributed to the alarming rise of opioid use disorder (OUD) and overdose-related fatalities. Both MOR and KOR activities play pivotal roles in the initiation, progression, and perpetuation of addiction, whereas the involvement of DOR presents a more nuanced picture (118). N/OFQ applies a wide inhibitory impact on different neurotransmitter systems implicated in drug reward and has been demonstrated to reduce the dopamine levels induced by drug intake within the core accumbens (119, 120). Moreover, evidence indicating that the N/OFQ-NOP complex acts as an anti-opioid for specific reactions, as prove by the barricade of the supraspinal analgesia induced by morphine and conditioned side preference by N/OFQ. Targeting the NOP-N/OFQ complex offers a promising approach to reduce the pleasurable effects of many substances commonly misused and to develop effective medication for addiction treatment. Increasingly, studies advocate for the viability of multi-mechanistic opioids as a more secure choice to the classical opioids. A novel pain relieving NOP agonist, cebranopadol, developed by Grünenthal that has gained increasing attention in recent years. It demonstrates strong and effective pain-relieving and hypersensitivity-reducing effects in various rat models of both acute and chronic pain when given by intravenous and oral routes (83). In addition, cebranopadol was tried in rodent models of visceral, incendiary, and painful bone cancer conditions. The data shows that the inhibition of referred visceral pain coming from pancreatitis is depending on the dosage (121). In osteoarthritis pain rodent model, the anti-hypersensitive efficacy of cebranopadol was observed to be influenced by NOP and MOP receptor antagonists (122). Cebranopadol demonstrated significant efficacy in acute pain mice models, with a strength comparable to the solid opioid fentanyl (123). Overall, this novel pain-relieving NOP agonist shows to

have a wide pain-relieving profile counting hindrance of reactions to warm, mechanical, or chemical incitement in several pain aetiologies, counting intense nociceptive as well as (sub)chronic fiery, visceral, and persistent neuropathic pain. In 2018, Kleideiter et al. (124) described the clinical pharmacokinetic characteristics of cebranopadol, detailing its suitability for once-daily dosing, even without the need for an extended-release formulation. In 2018, Scholz et al. (125) assessed for the primary time, the pain-relieving adequacy, security, and tolerability of this drug in patients enduring direct to extreme intense pain taking after bunionectomy. The administration of 400 and 600 μg of cebranopadol (single doses) resulted in more efficient postoperative pain relief compared to the classical opioid morphine. Furthermore, cebranopadol exhibited superior tolerability and obtained a higher overall rating from the patients. A randomized, double-blind, no inferiority trial also demonstrated positive outcomes in individuals with chronic moderate-to-severe cancer-related pain (126). Cebranopadol was successful, secure and well endured within the studied dosage range of 200-1,000 μg. Additionally, it outperformed morphine PR in terms of the major outcome measure among the patients. Taking together, our research offers potential novel insights into the effective treatment of EM- associated pain, with fewer side effects compared to current therapeutic modalities.

5   Conclusions

Our research has shown that the NOP receptor is highly expressed in the nerve fibers and blood vessels surrounding endometriotic lesions. Additionally, we have found a correlation between NOP receptor expression and rASRM and pain in patients with EM. This indicates that the NOP receptor as the N/OFQ endogenous ligand, may play a role in EM-associated pain. Our findings suggest that the NOP receptor could be a potential target for new therapeutic interventions, provided that further investigations and detailed research are conducted.

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Statutory Declaration

"I, Qihui Guan, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic *Nociceptin/orphanin FQ opioid peptide receptor-related ligands as potential analgesics in the endometriosis-associated pain/ Opioidpeptidrezeptor-verwandte Liganden von Nozizeptin/Ophanin FQ als potenzielle Analgetika bei Endometriose-bedingten Schmerzen*, independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me."

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Date   Signature

Declaration of your own contribution to the publications

<u>Qihui Guan</u> contributed the following share to the below listed publications:

<u>Publication 1:</u> **Qihui Guan**, Renata Voltolini Velho, Alice Jordan, Sabrina Pommer, Irene Radde, Jalid Sehouli, Sylvia Mechsner. Nociceptin/Orphanin FQ Opioid Peptide-Receptor Expression in the Endometriosis-Associated Nerve Fibers—Possible Treatment Option? *Cells*, 2023 May 15;12(10):1395.

Contribution in detail: Q.G. contributed to sample collection, group division, sample pre-processing, optimization of the dilution of primary antibodies, immunofluorescence double staining, image capture, image optimization, nerve fiber density calculation and assessment. **A.J., S.P., I.R.** helped **Q.G.** with the immunofluorescence double staining and analysis. **R.V.V.** prepared the first version of the manuscript. All the authors critically reviewed and corrected the manuscript. **J.S.** discussed and gave important suggestions regarding this project. **S.M.** supervised the project and, together with **Q.G**. and **R.V.V.**, edited the final version of the manuscript.

<u>Publication 2</u>: **Qihui Guan**, Renata Voltolini Velho, Jalid Sehouli and Sylvia Mechsner, Endometriosis and Opioid Receptors: Are Opioids a Possible/Promising Treatment for Endometriosis? *International Journal of Molecular Sciences*, 2023 Jan 13;24(2):1633.

Contribution in detail: Q.G. performed the literature search, gave a critical evaluation of the source of literatures, identified themes, debates, and gaps, outlined the structure, and drafted the first version of the manuscript. **Q.G.** created all the figures and legends. **S.M.** supervised the project. **R.V.V.** corrected and edited the final version of the manuscript. All the authors corrected and approved the final version of the paper.

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Signature, date and stamp of first supervising university professor/ lecturer

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Signature of doctoral candidate

Excerpt from Journal Summary List

Journal Data Filtered By: **Selected JCR Year: 2022** Selected Editions: SCIE,SSCI
Selected Categories: **"CELL BIOLOGY"** Selected Category Scheme: WoS
Gesamtanzahl: 191 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfaktor
1	NATURE REVIEWS MOLECULAR CELL BIOLOGY	65,845	112.7	0.08361
2	NATURE MEDICINE	139,574	82.9	0.23615
3	CELL	338,069	64.5	0.47782
4	CANCER CELL	55,579	50.3	0.07603
5	CELL RESEARCH	29,723	44.1	0.04001
6	Signal Transduction and Targeted Therapy	19,678	39.3	0.03255
7	Cell Discovery	6,697	33.5	0.01481
8	Cell Metabolism	58,914	29.0	0.07653
9	Cell Stem Cell	33,385	23.9	0.05575
10	NATURE CELL BIOLOGY	50,849	21.3	0.05678
11	Protein & Cell	7,357	21.1	0.01099
12	TRENDS IN CELL BIOLOGY	19,549	19.0	0.02252
13	Science Translational Medicine	52,013	17.1	0.08619
14	NATURE STRUCTURAL & MOLECULAR BIOLOGY	30,445	16.8	0.04040
15	Journal of Extracellular Vesicles	12,477	16.0	0.01241
16	Molecular Cell	86,313	16.0	0.12609
17	Cell Reports Medicine	4,157	14.3	0.01354
18	TRENDS IN MOLECULAR MEDICINE	13,691	13.6	0.01241
19	Autophagy	27,639	13.3	0.02571

Selected JCR Year: 2022; Selected Categories: "CELL BIOLOGY"

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfaktor
60	Cells	60,246	6.0	0.09176
61	Cancer & Metabolism	1,436	5.9	0.00158
62	Stem Cell Reports	11,404	5.9	0.02244
63	MOLECULAR MEDICINE	7,013	5.7	0.00438
64	STRUCTURE	15,623	5.7	0.01469
65	JOURNAL OF CELLULAR PHYSIOLOGY	42,434	5.6	0.04186
66	AMERICAN JOURNAL OF PHYSIOLOGY-CELL PHYSIOLOGY	18,242	5.5	0.00883
67	Frontiers in Cell and Developmental Biology	39,252	5.5	0.06380
68	JOURNAL OF LEUKOCYTE BIOLOGY	20,982	5.5	0.01321
69	Journal of Molecular Cell Biology	3,711	5.5	0.00483
70	JOURNAL OF CELLULAR AND MOLECULAR MEDICINE	30,560	5.3	0.03284
71	MECHANISMS OF AGEING AND DEVELOPMENT	7,649	5.3	0.00560
72	MOLECULAR AND CELLULAR BIOLOGY	48,740	5.3	0.00673
73	Aging-US	28,970	5.2	0.04014
74	MOLECULAR CANCER RESEARCH	11,324	5.2	0.01099
75	STEM CELLS	18,981	5.2	0.00879
76	BIOCHIMICA ET BIOPHYSICA ACTA-MOLECULAR CELL RESEARCH	19,579	5.1	0.01024
77	INFLAMMATION	9,132	5.1	0.00730

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Article

Nociceptin/Orphanin FQ Opioid Peptide-Receptor Expression in the Endometriosis-Associated Nerve Fibers—Possible Treatment Option?

Qihui Guan [†], Renata Voltolini Velho [†], Alice Jordan, Sabrina Pommer, Irene Radde, Jalid Sehouli and Sylvia Mechsner *

Endometriosis Research Center, Department of Gynecology Charité with Center of Oncological Surgery, Charité, Campus Virchow-Klinikum, Augustenburger Platz 1, 13353 Berlin, Germany; qi-hui.guan@charite.de (Q.G.); renata.voltolini-velho@charite.de (R.V.V.)
* Correspondence: sylvia.mechsner@charite.de; Tel.: +49-030-450664866
† These authors contributed equally to this work.

Abstract: Endometriosis (EM) is a chronic inflammatory disease affecting millions of women worldwide. Chronic pelvic pain is one of the main problems of this condition, leading to quality-of-life impairment. Currently, available treatment options are not able to treat these women accurately. A better understanding of the pain mechanisms would be beneficial to integrate additional therapeutic management strategies, especially specific analgesic options. To understand pain in more detail, nociceptin/orphanin FQ peptide (NOP) receptor expression was analyzed in EM-associated nerve fibers (NFs) for the first time. Laparoscopically excised peritoneal samples from 94 symptomatic women (73 with EM and 21 controls) were immunohistochemically stained for NOP, protein gene product 9.5 (PGP9.5), substance P (SP), calcitonin gene-related peptide (CGRP), tyrosine hydroxylase (TH), and vasoactive intestinal peptide (VIP). Peritoneal NFs of EM patients and healthy controls were positive for NOP and often colocalized with SP-, CGRP-, TH-, and VIP-positive nerve fibers, suggesting that NOP is expressed in sensory and autonomic nerve fibers. In addition, NOP expression was increased in EM associate NF. Our findings highlight the potential of NOP agonists, particularly in chronic EM-associated pain syndromes and deserve further study, as the efficacy of NOP-selective agonists in clinical trials.

Keywords: chronic inflammation; endometriosis; nerve fibers; NOP; opioid receptors; pelvic pain

1. Introduction

Endometriosis (EM) is an unrecognized, chronic inflammatory gynecological disease that affects approximately 10% of women of reproductive ages, i.e., two million women in Germany and 270 million worldwide [1,2]. Characterized by the presence of epithelial, stromal, and muscle cells outside the uterine cavity, EM affects the uterus itself (adenomyosis uteri) and the peritoneum of the pelvic cavity. Several factors interplay in the genesis of EM-associated pain such as the lesions themselves, pain-mediating substances, nerve fibers, and immune cells [2]. Due to the dissemination of endometriotic lesions, the associated symptoms show a wide variation including chronic pelvic pain, dysmenorrhea, dyspareunia, dyschezia, dysuria, and sub- or infertility [1–4]. Chronic pelvic pain is one of the main problems of this condition, affecting patients' psychological and social wellbeing and imposing a substantial economic burden on society [5–9].

Currently, the standard treatment options are not able to solve the daily problems of these women accurately [2]. Hormonal administration and surgical intervention are the most applied treatment options; however, countless side-effects, as well as high recurrence rates and ongoing pain after intervention, are frequent [2,3]. As pain is an important factor in EM, analgesia should be applied. To date, nonsteroidal anti-inflammatory drugs

(NSAD), metamizole, or, in extreme cases, opioids are the drugs used for EM-associated pain treatment [2]. Clinical observations often show NSAD and metamizole failure in the case of ongoing symptoms over many years and chronic pelvic pain syndrome, implying that central sensitization seems to be part of the pain chronification leading to a decrease in the pain threshold [2,4,10]. Therefore, the development of more effective therapeutic strategies is still an unmet clinical need, and it is hindered by the lack of knowledge of the mechanisms underlying the generation of EM pain and its associated comorbidities.

Opioid receptors are membrane-bound receptors belonging to the family of G-protein-coupled receptors (GPCRs). There are four opioid receptor subtypes, including the three classical opioid receptors, μ (MOR), δ (DOR), and κ (KOR), and the more recently discovered nociceptin/orphanin FQ peptide (NOP) receptor [11,12]. Since NOP receptors are distributed in various regions (dorsal root ganglia—DRG, spinal dorsal horn—SDH, and brain) that are involved in pain transmission, they are under investigation primarily as alternatives for MOP receptor opioid analgesics, in addition to their anxiolytic and antidepressant-like effect [13]. However, in the earlier phases of the discovery of nociception, the NOP receptor was considered a controversial drug target for analgesics because of its unique pharmacological effects on pain modulation (antinociceptive vs. nociceptive effects) [14–20]. Currently, the NOP receptor has become the main focus as a promising target for analgesics as NOP receptor ligands have been reported to show antinociceptive effects in nonhuman primates regardless of their administered doses and administration routes. Moreover, NOP/opioid receptor agonists have recently displayed potent antinociceptive activity with favorable side-effect profiles [13].

To understand pain generation in EM patients in more detail, NOP expression was analyzed for the first time in EM-associated nerve fibers (NF).

2. Materials and Methods

2.1. Patients

This prospective study enrolled 94 women from May 2012 until May 2019. Seventy-three EM patients, who underwent laparoscopy due to symptomatic EM with excision of endometriotic lesions, were included. The peritoneal lesions were localized in the lateral pelvic wall ($n = 17$), bladder ($n = 4$), pouch of Douglas ($n = 14$), uterosacral ligament ($n = 6$), peritoneum ($n = 2$), and fossa ovarica ($n = 30$). The diagnosed EM was staged according to the revised classification of the American Society of Reproductive Medicine (rASRM) as (I) minimal, (II) mild, (III) moderate, and (IV0 severe. In the analysis, two stages were considered: mild (rASRM I and II) and severe (rASRM III and IV). Twenty-one control samples were collected from women without EM, who had undergone laparoscopy for benign gynecological presentations such as non-EM associated with ovarian cysts, uterine fibroids, *Hydrosalpingx*, pelvic pain, peritonealized tissue, or the unfulfilled wish to have children. Additionally, clear peritoneal fluids were obtained during laparoscopy from patients with peritoneal EM ($n = 17$) and controls ($n = 17$).

Patients were selected on the basis of clinical intraoperative and subsequent histopathologic findings. All patients were given a complete gynecological examination including palpation and transvaginal ultrasound. The severity of pain was documented using a standardized questionnaire with a visual analogue scale (VAS; 0 = no pain, 10 = strongest imaginable pain) [21–23]. The women were divided into two groups according to the pain scale: moderate pain (0–5 on the scale) and severe pain (6–10 on the scale).

The study was approved by the Institutional Review Board of the Charité University Medical Centre (Ethic vote EA4/036/12). All patients gave their consent.

2.2. Immunofluorescence Double Staining and Determination of Nerve Fiber Density

All peritoneal biopsies were immediately fixed in buffered formalin (4%) for at least 12 h and thereafter embedded in paraffin. Sections of 2 μm thickness were cut and used for immunofluorescence double staining using antibodies against NOP receptor (Santa Cruz, Heidelberg, Germany, sc-398073, 1:50 and Abcam, Cambridge, UK, ab66219, 1:380),

protein gene product 9.5 (PGP 9.5—Novus, Wiesbaden Nordenstadt, Germany, NB110-58872, 1:300), substance P (SP, Santa Cruz, sc-21715, 1:500), calcitonin gene-related peptide (CGRP—Santa Cruz, sc-8857, 1:100), tyrosine hydroxylase (TH—Sigma, St. Louis, MO, USA, T2928, 1:100), and vasoactive intestinal peptide (VIP—Santa Cruz, sc-25347, 1:100).

Negative control sections were processed by omitting the specific primary antibody. A skin incision and a tissue section of peritoneal EM with large nerve incisions were used as the positive control. Staining was detected using an axiophot (Carl Zeiss, Göttingen, Germany) microscope. Photomicrographs were taken at different magnifications ($100\times$ and $200\times$) and were further processed using Adobe Photoshop (2022 Full Version, cs6, Adobe Systems, Unterschleissheim, Germany).

The density of PGP9.5 nerve fibers was assessed by counting the number of immunostained nerves proximal to the endometriotic lesions (epithelial, stromal, and smooth muscle cells) and in the distal area at 1 mm^2. The "hotspot" method [24] was used to determine the nerve fiber density of the control tissue as already described.

The density was measured by sequential assessment of two blinded investigators. Each patient had a code, which was unbroken until after the analysis at the end of the study. In cases of discrepant results, both the first and the second observers repeated the analysis together and reached a consensus.

2.3. Enzyme-Linked Immunosorbent Assay

Peritoneal fluids were aspirated from the pouch of Douglas immediately after the insertion of trocars to minimize contamination with blood. Grossly hemorrhagic specimens were excluded. Peritoneal fluids were centrifuged for 5 min at 3000 rpm, and the supernatants were aliquoted and stored at $-80\,^\circ$C until used. The endogenous Orphanin FQ/Nociceptin ligand concentration was measured in duplicate using the commercially available Human Orphanin FQ/Nociceptin enzyme-linked immunosorbent assay (ELISA) kit (EKH6946—Nordic BioSite AB, Täby, Sweden). This kit presents a detection range of 4.688–300 pg/mL and a sensitivity of 2.813 pg/mL. The analysis was conducted according to the manufacturer's protocol. After the substrate reaction, the optical density was measured (absorbance at 450 nm) automatically by the ELISA-READER Thermo Scientific Multiskan FC (Waltham, MA, USA, Unity Lab Services).

2.4. Statistical Analysis

Statistical analysis was performed using IBM SPSS for Windows (version 29.0.0.0). The data were evaluated using t-test (parametric) or Mann–Whitney U, Kruskal–Wallis, and Spearman correlation tests (nonparametric). Chi-square and Fisher's exact tests were used for the qualitative variable. Statistical significance was defined for $p < 0.05$.

3. Results

3.1. Population Characteristics

The population characteristics including pain aspects from the 94 women recruited for this study are summarized in Table 1. The present study group comprised 73 patients: 50 (68.49%) presented with minimal to mild endometriosis (rASRM I and II) and 23 (31.51%) presented with moderate to severe endometriosis (rASRM III and IV). Twenty-two (22/73) of them were under hormonal therapy at the time of the surgery. The mean age of the EM patients was 31.2 (18–50) years. The control group was a composite of 21 patients, two of which received hormonal therapy. Women in the control group were on average 35.6 (18–52) years old. No significant difference in age between EM and non-EM patients was observed ($p = 0.131$).

Table 1. Characteristics of the study participants.

	EM Patients (N = 73)	Controls (N = 21)
Age (years)		
Mean	31.2	35.6
SD	6.93	10.65
Stages (rASRM)		
I–II	49 (67.1%)	-
III–IV	24 (32.9%)	-
Hormone treatment		
Yes	22 (30.1%)	2 (9.5%)
Missing data	5 (6.8%)	8 (38.1%)
Pain (EM-associated pain)		
Number of patients	70 (95.9%)	9 (42.8%)
Missing data	3 (4.1%)	11 (52.4%)
Pelvic pain		
Number of patients	66 (90.4%)	8 (38.1%)
Pain intensity (mean, SD)	5.27 ± 1.62	N.A.
Missing data	45 (61.6%)	8 (38.1%)
Dysmenorrhea		
Number of patients	64 (87.7%)	6 (28.6%)
Pain intensity (mean, SD)	5.59 ± 2.33	N.A.
Missing data	41 (56.2%)	6 (28.6%)
Dyspareunia		
Number of patients	47 (64.4%)	4 (19.0%)
Pain intensity (mean, SD)	4.64 ± 2.29	N.A.
Missing data	16 (%)	4 (19.0%)
Dyschezia		
Number of patients	25 (34.2%)	2 (9.5%)
Pain intensity (mean, SD)	4.55 ± 2.70	N.A.
Missing data	15 (20.5%)	2 (9.5%)
Dysuria		
Number of patients	11 (15.1%)	2 (9.5%)
Pain intensity (mean, SD)	3.25 ± 1.50	N.A.
Missing data	6 (8.2%)	2 (9.5%)
Menstrual cycle		
Menses	5 (6.8%)	0 (0.0%)
Proliferative	14 (19.2%)	1 (4.8%)
Secretory	11 (15.1%)	4 (19.0%)
Hormone intake	23 (31.5%)	2 (9.5%)
Menopause	0 (0.0%)	0 (0.0%)
Missing data	20 (27.4%)	14 (66.7%)

N.A.: no answer.

3.2. Characterization of Nerve Fibers in Peritoneal Endometriotic Lesions

Using anti-PGP9.5, nerve fibers could be detected in both EM and healthy peritoneal specimens. PGP9.5 nerve fiber density was significantly increased in endometriotic lesions (mean ± SD: 1.76 ± 1.57 NF/mm²) compared to the healthy peritoneum (mean ± SD: 0.28 ± 0.70 NF/mm²; $p < 0.001$). When the hormonal therapy was taken into consideration, a decreased innervation could be observed in the EM group with hormonal intake (mean ± SD: 1.05 ± 0.79 NF/mm²) compared with the EM group without hormonal treatment (mean ± SD: 2.15 ± 1.76 NF/mm²; $p = 0.011$) (Figure 1A–C).
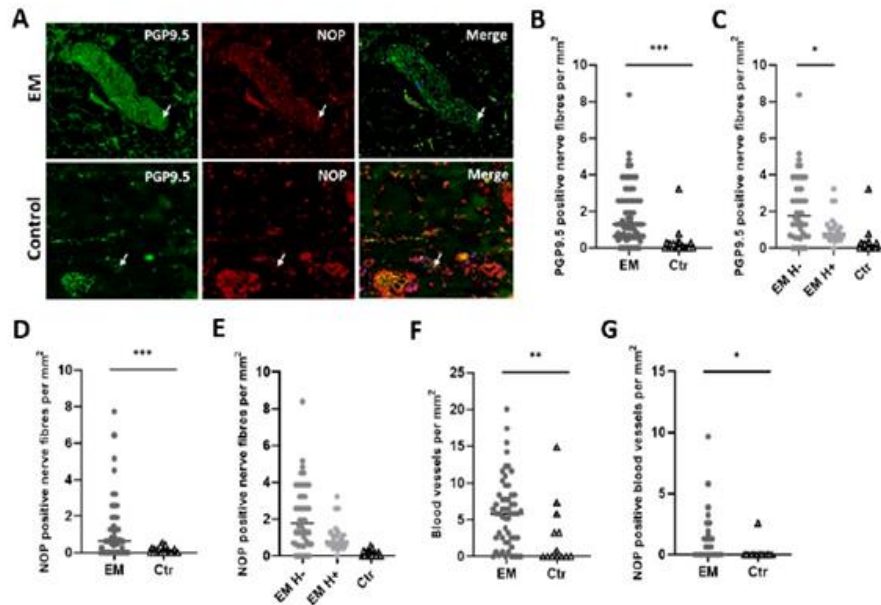
Figure 1. Endometriosis patients showed increased nerve fiber and blood vessel density, as well as NOP−receptor expression. (**A**) EM and control samples stained with PGP9.5 (green) and NOP (red) antibody. Merged images showi the colocalization of pan marker (PGP9.5) and the NOP receptor. All pictures are at 200× magnification. (**B,C**) PGP9.5−positive nerve fibers per mm^2 in EM patients and controls. (**D,E**) NOP−positive nerve fibers per mm^2 in EM patients and controls. (**F**) Blood vessels per mm^2 in EM patients and controls. (**G**) NOP−positive blood vessels mm^2 in EM patients and controls. Arrows indicate colocalization points. EM: endometriosis patients; Ctr: control; H+: under hormonal treatment; H−: without hormonal treatment. All results are presented as the median, and 25th–75th percentile. Mann–Whitney and Kruskal–Wallis with Dunn's multiple comparison tests. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

A correlation between the nerve density (PGP9.5—positive NF/mm^2) and the rASRM stages could be seen ($r = 0.403$; $p < 0.001$) (Table 2).

Table 2. Correlation analysis.

		Value
Hormonal therapy in EM	Pelvic pain	$p = 0.263$ [b]
	Dysmenorrhea	$p = 0.599$ [b]
	Dyspareunia	$p = 1.000$ [b]
	Dyschezia	$x^2 (1) = 0.512$; $p = 0.579$ [a]
	Dysuria	$x^2 (1) = 0.046$; $p = 1.000$ [a]
Pain level and hormonal therapy in EM	Pelvic pain	$p = 0.674$ [b]
	Dysmenorrhea	$x^2 (1) = 0.022$; $p = 1.000$ [a]
	Dyspareunia	$p = 0.388$ [b]
	Dyschezia	$p = 0.543$ [b]
	Dysuria	$p = 1.000$ [b]

Table 2. *Cont.*

			Value
Pain level and rASRM	Pelvic pain		$p = 0.611$ [b]
	Dysmenorrhea		$p = 1.000$ [b]
	Dyspareunia		$p = 1.000$ [b]
	Dyschezia		$p = 0.560$ [b]
	Dysuria		$p = 0.405$ [b]
Pelvic pain and nerve fiber density/pain receptor	PGP9.5	Nerve fibers/mm^2	$r = 0.344; p = 0.108$ [c]
	NOP	Nerve fibers/mm^2	$r = -0.248; p = 0.253$ [c]
		Blood vessels/mm^2	$r = 0.067; p = 0.806$ [c]
Dysmenorrhea and nerve fiber density/pain receptor	PGP9.5	Nerve fibers/mm^2	$r = 0.142; p = 0.480$ [c]
	NOP	Nerve fibers/mm^2	$r = -0.142; p = 0.481$ [c]
		Blood vessels/mm^2	$r = -0.275; p = 0.270$ [c]
Dyspareunia and nerve fiber density/pain receptor	PGP9.5	Nerve fibers/mm^2	$r = 0.119; p = 0.475$ [c]
	NOP	Nerve fibers/mm^2	$r = 0.009; p = 0.958$ [c]
		Blood vessels/mm^2	$r = 0.69; p = 0.729$ [c]
Dyschezia and nerve fiber density/pain receptor	PGP9.5	Nerve fibers/mm^2	$r = -0.050; p = 0.740$ [c]
	NOP	Nerve fibers/mm^2	$r = 0.049; p = 0.742$ [c]
		Blood vessels/mm^2	$r = -0.032; p = 0.863$ [c]
Dysuria and nerve fiber density/nerve fiber receptor	PGP9.5	Nerve fibers/mm^2	$r = -0.060; p = 0.674$ [c]
	NOP	Nerve fibers/mm^2	$r = -0.152; p = 0.288$ [c]
		Blood vessels/mm^2	$r = -0.044; p = 0.801$ [c]
rARSM and nerve fiber density/nerve fiber receptor	PGP9.5	Nerve fibers/mm^2	$r = 0.403; p < 0.001$ **,[c]
	NOP	Nerve fibers/mm^2	$r = 0.410; p < 0.001$ **,[c]
		Blood vessels/mm^2	$r = 0.307; p = 0.024$ *,[c]

Analyses using [a] chi-square or [b] Fisher and [c] Spearman correlation; * $p < 0.05$, ** $p < 0.001$.

3.3. EM Patients Showed Increased Expression of NOPReceptor

EM patients presented more NOP-positive nerve fibers (mean $\pm$ SD: 1.22 ± 1.62 NOP-positive NF/mm^2) when compared with controls (mean $\pm$ SD: 0.11 ± 0.17 NOP-positive NF/mm^2; $p < 0.001$). Interestingly, EM patients that received hormonal therapy did not differ in the expression of NOP receptor (mean $\pm$ SD: 0.83 ± 0.99 NOP-positive NF/mm^2) from the patients that did not receive this treatment (mean $\pm$ SD: 1.49 ± 1.86 NOP-positive NF/mm^2; $p = 0.381$) (Figure 1D,E).

When looking at blood vessels, EM patients presented more blood vessels (mean $\pm$ SD: 6.0 ± 4.7 blood vessels/mm^2) than women without EM (mean $\pm$ SD: 2.7 ± 4.3 blood vessels/mm^2; $p = 0.007$). In addition, EM patients showed more NOP-positive stained vessels (mean $\pm$ SD: 1.1 ± 1.8 blood vessels NOP-positive/mm^2) than the control group (mean $\pm$ SD: 0.2 ± 0.7 blood vessels NOP-positive/mm^2; $p = 0.013$) (Figure 1F,G). The hormonal intake did not affect the number of blood vessels or their NOP-positivity.

The NOP-positive stained nerve fibers ($r = 0.410; p < 0.001$) and blood vessels ($r = 0.307; p = 0.024$) correlated with the rASRM stages but not with the pain levels (Table 2).

3.4. NOP Receptors Are Located on Sympathetic, Parasympathetic, and Sensory Fibers That Innervate the Lesions

If the NOP receptor is involved in EM and its associated pain, this receptor should be located on the axonal fibers innervating the lesions. With double-labeling fluorescence immunohistochemistry, most (>75%) sympathetic fibers (TH-positive), many (50–75%) parasympathetic fibers (VIP-positive), and many (50–75%) sensory fibers (SP- and CGRP-positive) in the EM and control samples were co-labeled with an antibody for NOP receptor (Figure 2).
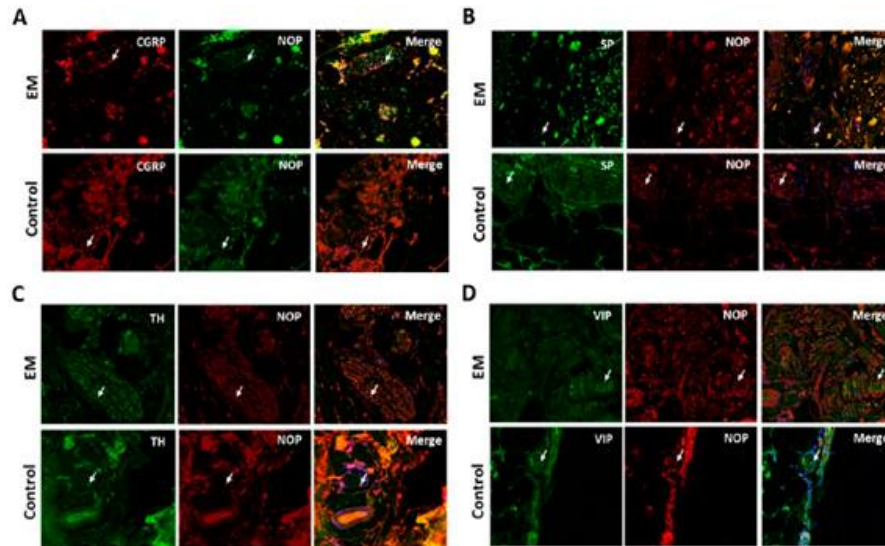
Figure 2. NOP receptors are present in sympathetic, parasympathetic, and sensory fibers innervating endometriotic lesions. (**A**) EM and control samples stained with CGRP (sensory fibers—red) and NOP (green) antibody. (**B**) EM and control samples stained with SP (sensory fibers—green) and NOP (red) antibody. (**C**) EM and control samples stained with TH (sympathetic fibers—green) and NOP (red) antibody. (**D**) EM and control samples stained with VIP (parasympathetic fibers—green) and NOP (red) antibody. Arrows indicate colocalization points between nerve marker and the NOP receptor. All pictures are 200× magnification.

3.5. Orphanin FQ/Nociceptin Ligand Is Not Overexpressed in the Peritoneal Fluid of Women with Peritoneal Endometriosis

The endogenous Orphanin FQ/Nociceptin ligand concentration expression in the peritoneal fluid of women with peritoneal EM (mean $\pm$ SD: 2.81 $\pm$ 11.59) and controls (7.88 $\pm$ 22.07) was not statistically different ($p = 0.586$; Figure 3). All EM women were premenopausal with a mean age of 31.4 $\pm$ 4.4 years (range 26–40 years) and had a regular menstrual cycle (secretory phase: 41%, proliferative phase: 59%). EM was classified according to the rASRM at stages I to IV (I = 29.41%, II = 35.29%, III = 00.00%, IV = 35.29%). Controls included women who were premenopausal with a mean age of 32.6 $\pm$.5 years (range 20–50 years) and had a regular menstrual cycle (secretory phase: 29.4%, proliferative phase: 41.2%, menses: 5.9%), while one woman was taking an oral contraceptive.
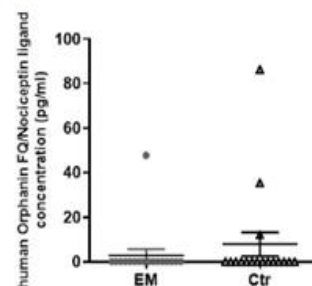

Figure 3. Orphanin FQ/Nociceptin ligand concentration (pg/mL) in the peritoneal fluid of women with peritoneal endometriosis and controls. Using an ELISA kit, the endogenous Orphanin FQ/Nociceptin ligand concentration expression in the peritoneal fluid of women with peritoneal EM and controls was measured. EM: endometriosis; Ctr: control. Mann–Whitney test; $p = 0.586$.

4. Discussion

There are many symptoms connected with EM. However, the main symptom is cyclic and noncyclic chronic pelvic pain [2,25]. The pain pathology is still largely unexplained; however, since the discovery of the NOP receptor and N/OFQ as the endogenous ligand, evidence has appeared demonstrating the involvement of this receptor system in pain. This is not surprising for members of the opioid receptor and peptide families, particularly since both the receptor and N/OFQ are highly expressed in brain regions involved in pain, as well as in the spinal cord and dorsal root ganglia [26,27]. Moreover, most of the data on NOP receptor expression are derived from rodents, and using mRNA expression analysis which may not translate in the human scenario [28]. NOP is expressed both in the central nervous system and in peripheral tissues. Nevertheless, little is known about the localization of the NOP receptor in human tissues, and information about any changes in expression levels in human disease is absent [29]. Whole-body images of a healthy 22 year old man showed radioactivity of ^{11}C-NOP-1A in the brain and peripheral organs expressing NOP receptors, such as the heart, lungs, liver, pancreas, small bowel, and urinary bladder [30]. In human visceral disease, we could only find data about NOP expression in bladder pain syndrome [30]. Accordingly, we analyzed, for the first time, the nociceptin/orphanin FQ peptide receptor expression in EM-aNF. Our goal was to understand pain generation in EM patients in more detail, relating this pain to the localization and expression of NOP receptors in nerve fibers from the female reproductive system and visceral organs.

The hyper-innervation already described in endometriotic lesions [31–36] was confirmed in the 73 EM women enrolled in this study. Interestingly, the NOP receptor was significantly more expressed in nerve fibers and blood vessels from EM patients than in controls. This is substantial evidence for peripheral sensitization and involvement of EM-aNF in pain generation. A marked and significant increase in NOP receptor immunoreactive nerve fibers was observed in bladder specimens from patients with overactive bladder and with bladder pain syndrome—another chronic pelvic pain condition [29]. Subclassification of the EM-aNF quality showed colocalization of the NOP receptor in sensory (SP- and CGRP-positive NF) and autonomic NF (TH- and VIP-positive NF), which have been seen for other groups [37], demonstrating the complexity of the EM-associated pain.

The Involvement of the NOP receptor system in pain modulation has been carefully investigated [37,38]. Indeed, depending on route, concentration, and pain model, NOP receptor activation could lead to either pronociceptive or antinociceptive effects [38,39]. The effects of NOP receptor agonist activation appear to be considerably clearer for chronic than acute pain [37,38]. Early studies examining the effects of N/OFQ on pain induced by inflammation or sciatic nerve injury suggested potential neuroplasticity, as the peptide was very effective in inducing anti-allodynic and anti-hyperalgesic activity in these chronic pain models [40–43].

EM-associated pain has a strong impact on the patient's quality of life [44,45]. Unfortunately, current treatment strategies are not fully satisfactory [2]; thus, novel treatments with better effectiveness and tolerability are urgently needed. Our study demonstrated a link among NOP receptor expression, rASRM, and pain in EM patients, suggesting that the NOP receptor and N/OFQ as the endogenous ligand may be involved in EM-associated pain. Further investigation will be needed to elucidate these links and to evaluate whether the NOP receptor could provide a target model for new therapeutic intervention.

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Review

Endometriosis and Opioid Receptors: Are Opioids a Possible/Promising Treatment for Endometriosis?

Qihui Guan [†], Renata Voltolini Velho [†], Jalid Sehouli and Sylvia Mechsner *

Department of Gynecology Charité with Center of Oncological Surgery, Endometriosis Research Center Charité, Campus Virchow-Klinikum, Augustenburger Platz 1, 13353 Berlin, Germany
* Correspondence: sylvia.mechsner@charite.de; Tel.: +49-030-450664866
† These authors contributed equally to this work.

Abstract: Endometriosis (EM), defined as the presence of endometrial-like tissue with surrounding smooth muscle cells outside the uterus, is a disregarded gynecological disease reported to affect 6–10% of women of reproductive age, with 30–50% of them suffering from chronic pelvic pain and infertility. Since the exact pathogenic mechanisms of EM are still unclear, no curative therapy is available. As pain is an important factor in EM, optimal analgesia should be sought, which to date has been treated primarily with non-steroidal anti-inflammatory drugs (NSAIDs), metamizole or, in extreme cases, opioids. Here, we review the pain therapy options, the mechanisms of pain development in EM, the endogenous opioid system and pain, as well as the opioid receptors and EM-associated pain. We also explore the drug abuse and addiction to opioids and the possible use of NOP receptors in terms of analgesia and improved tolerability as a target for EM-associated pain treatment. Emerging evidence has shown a promising functional profile of bifunctional NOP/MOP partial agonists as safe and nonaddictive analgesics. However, until now, the role of NOP receptors in EM has not been investigated. This review offers a thought which still needs further investigation but may provide potential options for relieving EM-associated pain.

Keywords: chronic pelvic pain; drug abuse; NOP receptor; opioid tolerance; opium; therapeutic option

1. Introduction

Endometriosis (EM), defined as the presence of endometrial-like tissue with surrounding smooth muscle cells outside the uterus, is a disregarded gynecological disease reported to affect 6–10% of women of reproductive age and 30–50% of them suffering from chronic pelvic pain and infertility [1–3]. Patients complain that pain appears within the reproductive and urinary system, or lower part of the digestive system, which is related to the location of EM focus [4–6]. Furthermore, typical EM symptoms include dyspareunia, dysmenorrhea, dysuria, dyschezia, and noncyclic chronic pelvic pain [7]. Some patients also complain of atypical symptoms, such as pain in the lumbar-sacral region of the spine, diarrhea, constipation, fatigue, flatulence, nausea, anxiety, depression, and headaches, which seem to be stress-induced [8]. All these symptoms and a long course of the disease lead to a significant decrease in patient quality of life [9].

Since the exact pathogenetic mechanisms of EM are still unclear, no curative therapy is available. The treatment goal is to alleviate the symptoms and prevent the further spread of EM while preserving fertility [3,10]. The first line of therapy includes combined oral contraceptives or progestins. The second line includes gonadotropin-releasing hormone analogues (GnRHa) and aromatase inhibitors [11]. Since these therapies interfere with ovulation, the desire for an active or future pregnancy must be considered when prescribing them [12]. Moreover, about 30–60% of EM patients do not respond to conventional treatment [13,14]. In addition, about 10% of patients cannot continue the treatment because of the adverse effects (vaginal dryness, decreased libido, depression, irritability, fatigue, bone

mineral loss, changes in lipid profile, weight gain, oedema, acne, hot flashes, liver toxicity, breast atrophy, voice alteration, hirsutism, and oily skin) inherent to the therapy [15–17]. Moreover, a systematic review was able to show that the various drugs do not differ significantly in terms of their pain-relieving effect [18]. Although the drugs can prevent the further progression of the disease, existing endometriotic structures remain, regardless of the specific drug used, the dosage, or the duration of use [10]. Therefore, EM-related symptoms can reappear when drug use is terminated or interrupted, which is why drug therapy is regarded as a long-term treatment.

As the third line of treatment, the macroscopically visible EM foci can be removed by a surgical procedure (laparoscopy). Patients experiencing symptoms including severe involvement of the ovaries or ovarian cysts, stenosis in the bladder or gastrointestinal tract caused by EM, pain resistant to therapy, or a unfulfilled desire to have children are recommended for this procedure [19]. Our group has showed that 23.8% of patients had no pain after laparoscopy, while 52% had an improvement in symptoms [20]. Although this intervention seems to be a successful treatment option, pain remains in 25% of patients and EM recurrence is between 40 and 45% [14]. However, independent of the treatment chosen, the most important point is the early intervention to avoid pain chronification. Given that most adult patients report symptoms of EM as well as CPP during adolescence, and that approximately 80% of adolescent girls with CPP not responding to conventional medical therapy have EM, this would be a critical time to intervene [21].

As pain is an important factor in EM, optimal analgesia should be sought, which to date has been treated primarily with non-steroidal anti-inflammatory drugs (NSAIDs), metamizole or, in extreme cases, opioids [3]. However, the response to NSAIDs is often ineffective [22]. In addition, NSAIDs are associated with a higher risk of gastrointestinal bleeding. As for metamizole, agranulocytosis is the most notorious adverse event, as well as metamizole-associated hepatotoxicity [23]. Opioids should be prescribed but strongly supervised, as there is a risk of addiction [24].

Here, we review the mechanisms of pain development in EM, the endogenous opioid system and pain, as well as the opioid receptors and EM-associated pain. We also explore the drug abuse and addiction to opioids and the possible use of NOP receptors in terms of analgesia and improved tolerability as a target for EM-associated pain treatment. This review offers a thought which still needs further investigation, but may provide potential options for relieving EM-associated pain.

2. Mechanisms of Pain Development in Endometriosis

Mechanisms that lead to the development of pain in EM are very complex and multi-layered. Peripheral pain generation mechanisms that take place at the local level can be distinguished from those that take place in the central nervous system. Regionally, nociceptors are activated in and around the endometriotic lesions, which convert the stimulus into action potentials (Figure 1). These are in turn transmitted via the spinal cord to the brain, where signal processing takes place. This results in the subjective perception of pain [25]. Nociceptors are sensory receptors activated by noxious stimuli, which in EM represent pain mediator release and nerve infiltration or compression, but that is not usual. A pain receptor involved in this mechanism is the transient receptor potential vanilloid channel 1 (TRPV1), which acts as a cation channel and is increased in EM patients [26,27]. When the pain originates in the parietal peritoneum, it is described as a well-localized somatic pain. Visceral pain results from the activation of nociceptors in organs such as the uterus, bladder, or rectum. Compared to somatic pain, visceral pain tends to be dull and difficult to localize [7].
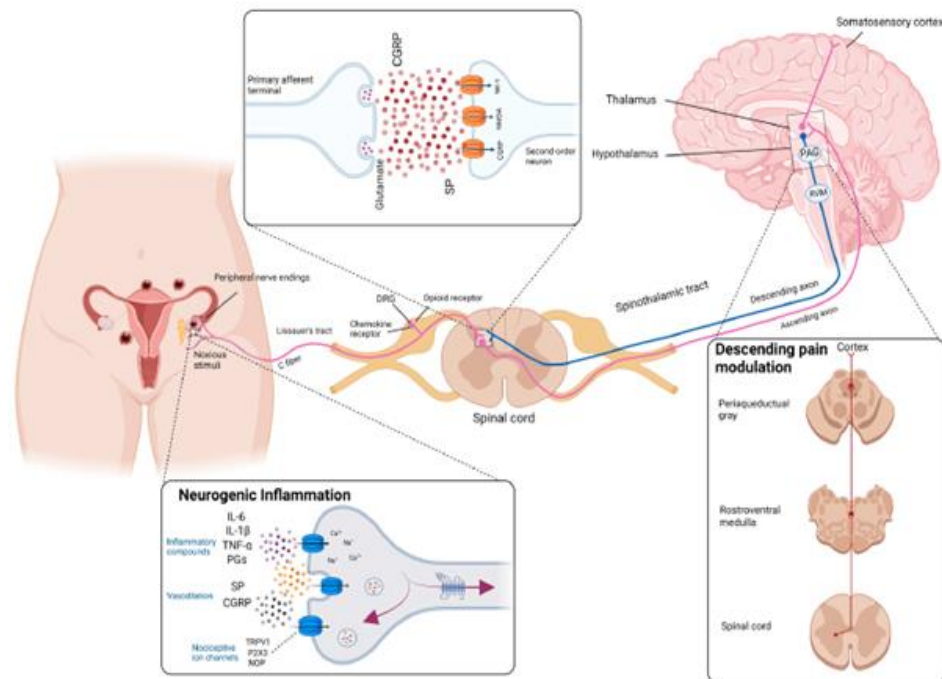
Figure 1. Pain and neurogenic inflammation in endometriosis. Endometriotic cytokines in the peritoneum, prostaglandins, interleukins (ILs), calcitonin gene-related peptide (CGRP), substance P (SP), nociceptive opioid peptide (NOP), and transient receptor potential vanilloid 1 (TRPV1) stimulate sensory peripheral nerve endings. The nociceptive signal conduct in the myelinated Aδ fibers or unmyelinated C fibers to the spinal cord. Subsequently, opioid peptides, which are synthesized as chemokine receptors, promote an antinociceptive effect in the dorsal root ganglia (DRG). In the DRG, chemokine receptors and opioid receptors receive the nociceptive message and transfer it to the spinal cord. Within the spinal cord, neurotransmitters such as CGRP, SP, and glutamate are released to activate the second-order neurons. Then, CGRP will combine with the CGRP receptor complex and SP will act on the neurokine-1 receptor (NK-1). The noxious message travels up to the thalamus via ascending axons and then finally reaches the somatosensory cortex. The nociceptive input can be attenuated or facilitated through endogenous opioid release via a descending pain-modulated system, which includes two crucial structures, periaqueductal gray (PAG) and rostroventral medulla (RVM).

Inflammatory pain is also a subtype of nociceptive pain [28,29]. EM is associated with a local inflammatory reaction in which regional inflammatory mediators such as cytokines, tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-6, and also immune cells are present in high concentrations [10,30]. The inflammatory mediators are secreted both by the endometriotic lesions themselves and by surrounding cells and nerve tissue [25]. The resulting neurogenic inflammation leads to hypersensitivity and activation of pain receptors [31], which is often associated with regional hyperalgesia (increased local pain perception) [12,30]. This is the result of longstanding chronic inflammation in which pain loses its function as a barrier to noxious stimuli [32]. In addition, neuropathic pain is described in up to 75% of EM patients. Neuropathic pain is defined as pain caused by a lesion or disease of the somatosensory nervous system. The peripheral mechanisms of EM-associated pain are mainly focused on the interplay among the immune system, peripheral nerve system, and endometriotic lesions [33]. In the case of EM, the nerves in the damaged area are activated without an active stimulus and patients often perceive it as a sharp pain [25].

After the pain is perceived via the peripheral anatomical area, the consciousness of pain emerges from the central nervous system (CNS) [34]. The modulation of chronic pelvic pain in women with EM and EM-associated pain correlates with the CNS [35,36]. Neural mechanisms similar to the generation of memory may be why central sensitization can cause pain without a peripheral noxious input [37]. Factors such as mood, feelings, and previous pain experiences significantly influence pain processing and can lead to both reduced and increased pain perception (centralized pain). Therefore, there is no linear relationship between the perceived pain intensity and the strength of the stimulus [25]. For example, women who suffer from dysmenorrhea react more sensitively to painful noxae, such as heat, than women without menstrual pain. In addition to the subjectively perceived stronger pain, an increased pain response in the brain was also shown in dysmenorrhea patients. Dysmenorrhea is a risk factor for chronic pelvic pain [38].

Additionally, one widely accepted view is that nociceptive pathways change in an activity-dependent manner, i.e., show plasticity [39–43]. The concept of functional plasticity or neuroplasticity provides mechanistic links between specific changes in molecules, synapses, microcircuits, and systems and thereby links a variety of modulatory factors to a change in perception and behavior. Over the last three to four decades, studies in animal models of chronic pain have established that peripheral afferents sensitize in response to a variety of molecules secreted by nonneuronal cells (immune cells and blood vessels, including inflammatory cytokines and growth factors) [41].

Increasing evidence has led to EM being considered as a neurogenic inflammatory disease [27,30]. Elevated expression and activation of nociceptors and elevated levels of neuropeptides, other proinflammatory chemicals, and cytokines imply that neuroinflammatory processes are present in the CNS in EM [30]. Although the precise mechanism of how pain in EM generates is unknown, it is clear that adequate analgesia is an integral and important part of EM treatment.

3. Endogenous Opioid System and Pain

The endogenous opioid system is integrated with endogenous opioid peptides and receptors [44]. The opioid receptors are transmembrane proteins belonging to the seven transmembrane-spanning superfamily of G-protein-coupled receptors (GPCR). GPCRs are of fundamental physiological importance, mediating actions of the majority of known neurotransmitters and hormones. Binding studies and bioassays defined four main types of opioid receptors: delta (DOR), mu (MOR), kappa (KOR) opioid receptor, and nociceptin/orphanin FQ peptide (NOP, initially called LC132, ORL-1) receptor [44,45].

The endogenous opioid system plays a fundamental role in modulating neurogenic inflammation and the ensuing pain and is implicated in the physiological control of emotional and cognitive responses [46]. Corticotropin-releasing factors, cytokines, catecholamines, and environmental stimuli such as stress can liberate endogenously occurring opioid peptides (e.g., β-endorphin) [47]. These activate the neuronal opioid receptors leading to the inhibition of the excitability of these nerves or the release of proinflammatory neuropeptides that results in analgesia [48]. Analgesic effects of locally administered opioids are particularly prominent under inflammatory conditions. Studies have shown that there may be an inflammation-dependent upregulation of opioid receptors in the periphery. After induction of peripheral inflammation, axonal transport of opioid receptors to the nerve endings will enhance, which leads to an increase in their density on peripheral nerve terminals [49,50]. In inflamed tissue, mRNAs for β-endorphin and enkephalins were found in lymphocytes, monocytes, and macrophages. Immune cells express the required machinery to process proopiomelanocortin into β-endorphin and release it from secretory granules [51].

4. Opioid Receptors and EM-Associated Pain

Since EM is a chronic inflammatory disease with disturbing pain mediation and analgesia, opioid system disruption (opioid peptides and/or receptors) might be involved

in the inflammatory condition and pain pathogenesis. Indeed, some studies have focused on opioid receptor expression in the endometrium (Figure 2) [52–56].
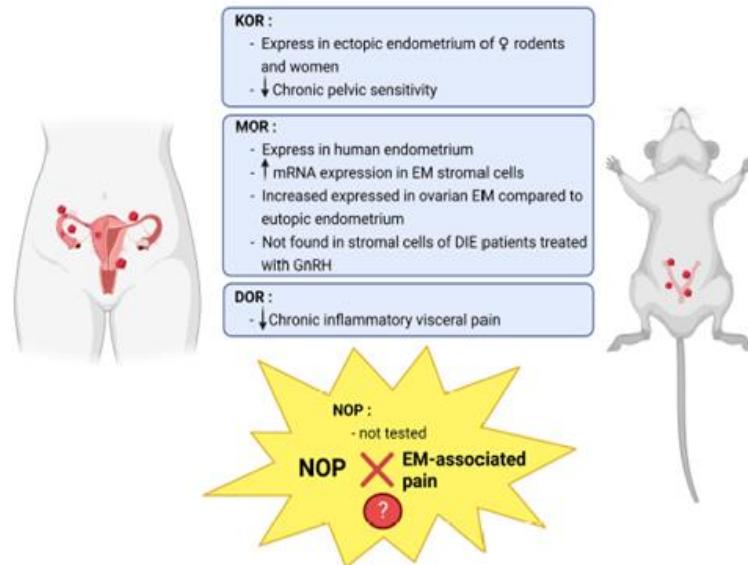

Figure 2. Opioid receptors and EM-associated pain. The endogenous opioid system is involved in modulating neurogenic inflammation, while endometriosis is a chronic inflammatory disease. In the studies regarding opioid receptor expression in the endometrium, both MOR and KOR are more or less related to EM-associated pain. Although to date, no evidence has shown that DOR antagonists can alleviate EM-associated pain, DOR agonists were reported to be active in the periphery and probably alleviate the chronic inflammatory visceral pain. Regarding NOP, what we know is its wide expression both in the central and peripheral nervous systems. Meanwhile, NOP receptors also have a high distribution within the immune system. To date, the relationship between EM-associated pain and NOP or N/OFQ remains unknown. KOR: kappa opioid receptors; MOR: mu-opioid receptors; DOR: delta opioid receptors; NOP: nociceptin/orphanin FQ peptide receptor; EM: endometriosis; GnRH: gonadotropin-releasing-hormone; DIE: deep infiltrating endometriosis.

Among the opioid receptors, KOR plays an important role in visceral and inflammatory pain [57,58] and its stimulation produces greater analgesia in women than in men due to sex differences associated with κ-opioid agonism [59]. Moreover, kappa-opioid receptor expression has been described in ectopic endometrial tissues of female rodents and women [53,60,61]. A recent study has shown that KOR stimulation can alleviate and prevent chronic pelvic mechanical sensitivity and discomfort in female mice subjected to EM. This KOR-mediated pain relief was void of antiallodynic tolerance and was highly effective during estrus, the phase of the estrous cycle in which mice become more sensitive. Such an increased sensitivity resembles the intense perimenstrual pain observed in EM patients. Interestingly, KOR-mediated pain relief did not modify the anxiety-like behavior or the memory impairment of mice with ectopic endometrial growths [46]. Kappa-opioid receptor agonists showed analgesia in patients with non-ulcer dyspepsia [62] and irritable bowel syndrome [63,64].

MOR is expressed in the human endometrium and its expression pattern changes during the menstrual cycle differently in all endometrial compartments. These findings suggest that MOR could have several functions in the complex remodeling process that the endometrium undergoes every month and, therefore, in EM [56]. The mu-opioid receptor mRNA was upregulated in EM stromal cells, indicating that it may be involved

in a defective immune system in this disease [65]. Additionally, MOR expression was significantly higher in ovarian EM than in eutopic endometrium [66]. Mu-opioid receptor expression was not detected in stromal cells from GnRH agonist-treated patients with deep infiltrating EM (DIE). Although MOR expression in stromal cells was detectable in progestin-treated patients, the expression levels were significantly lower than those in untreated patients [55]. These findings suggest that neuroimmune interactions may play a crucial role in the pathogenesis of EM-associated pain. Mediators including MOR in the neuroimmune pathways may be new targets for non-hormonal treatments.

In an EM rat model, a decrease in MOR immunoreactivity within neuronal compartments was reported [67]. This study demonstrated that the EM impact on the periaqueductal grey (PAG) opioid system is related to changes in MOR and N-methyl-D-aspartate receptor (NR1) subunit expressions. It indicated that EM might influence opioidergic and glutamatergic activities in the PAG. In addition, studies showed that there may be an interaction between psychological stress and EM development and progression [68]. EM-associated pain may be alleviated after physical interventions and aggravated under psychological stress [69,70].

The discovery of *OPRD1*, which is the DOR encoding gene, began an era of molecular and genetic investigations of the opioid system. *OPRD1*-knockout mice revealed that DORs have anxiolytic and antidepressant functions [71], decidedly distinguishing this receptor from MORs. Many pharmacological studies have shown DORs in mood disorders and chronic pain [72–74]. Other researchers have reported that DOR has great potential for the treatment of chronic pain with ancillary anxiolytic- and antidepressant-like effects [73,75,76]. DOR agonists were reported specifically active in the periphery, and may significantly improve the treatment of chronic inflammatory visceral pain [77]. The DOR is widely spread in the brain, synthesized in primary afferents and transported to the spinal cord [78]. The precise distribution of DOR in dorsal root (DRG) neurons remains controversial. Some studies infer that DOR is mainly expressed in large myelinated DRG neurons and shows a low level of co-expression with MOP [79,80]. Others have indicated that DOR is also distributed in small DRG neurons and that DOR and MOR can inhibit the noxious heat and mechanical-induced release of SP in the spinal cord [81–83]. However, to date, there is no evidence showing that EM-associated pain can be relieved by DOR antagonists.

In the mid-1990s, nociceptin/orphanin FQ (N/OFQ) was identified as a multifunctional ligand for the opioid receptor-like 1 (ORL1), with ORL1 renamed to N/OFQ peptide (NOP) receptor [84]. Despite the high homology of the NOP receptor and other classical opioid receptors (MOP, DOP, and KOP), N/OFQ does not bind to classical opioid receptors owing to its unique structure [85]. Both the NOP receptor and the endogenous ligand N/OFQ are widely expressed both in the central and peripheral nervous systems, as well as in peripheral organs, such as the heart and intestines, and the immune system of rodents and humans [85]. Given their distribution, N/OFQ and NOP receptors contribute to the regulation of different functions such as memory, emotion, reward, motor function, and sensory processing. Moreover, they are related to the regulation of renal and cardiovascular functions, respiratory functions, cough reflexes, urinary bladder function, and micturition reflexes, with special regard to sympathetic and parasympathetic regulation [86–88]. Moreover, its involvement in pain modulation has been reported [89]. Spinal NOP receptor activation produces anti-hyperalgesic and anti-allodynic effects in chronic pain [89].

In animal models, intrathecal N/OFQ administration inhibited thermal hyperalgesia of chronic inflammatory pain [90,91], and similar effects were observed in neuropathic pain models caused by chronic constriction injury (CCI) or spinal nerve ligation [90]. Compared with the other classical opioid receptors (MOR, KOR, and DOR), NOP receptors can be detected and their expression was unaffected by opioids and reduced by LPS/PepG combinations, while the aforementioned three classical opioid receptors could not be detected [92]. This study highly indicated that classical opioid receptors are not expressed in circulating immune cells. Indeed, NOP receptors have a high distribution within the immune system, including in lymphocytes, monocytes, B/T cells, and mononuclear cells [93–95].

There is a strong correlation between the plasmatic N/OFQ levels and the severity of sepsis [96–98], Parkinson's disease [99–101], arthritis [102], and inflammatory bowel diseases [103–105]. Other researchers have reported NOP receptors as a novel potential target in the treatment of gastrointestinal diseases, including inflammatory bowel diseases and irritable bowel syndrome [106,107]. In addition, its therapeutic potential in the treatment of traumatic brain injuries, traumatic stress, and their co-morbidities has also been reported [108].

Everything considered, it makes sense to believe that NOP may play a crucial role in alleviating EM-associated pain via the anti-neuroinflammatory process. However, to date, the relationship between EM-associated pain and NOP or N/OFQ remains unknown.

5. Opioids and Female Reproduction

The presence of the opioid system in peripheral reproductive tissues in both women and men is a recent revelation [109]. Specifically, researchers have reported the expression of opioid receptors in the granulosa cells of the ovarian follicle, in the oocyte, and in the human endometrium [56,110,111]. Opioid receptors are concurrently found in the somatic and germ cells of the testis. The clinical correlations of these findings include effects on follicular maturation, embryo implantation, spermatogenesis, and sexual function [109].

In the female central nervous system, they are involved in controlling the release of GnRH and thus the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). This affects ovarian hormonal production, follicular growth, and ovulation. Peripherally, endogenous opioids act directly as important neuromodulators as well as signaling peptides within several reproductive organs and tissues. These include the endocrine pancreas, the different compartments of the ovary, including the ovarian follicles and the oocyte, as well as the endometrium. The endogenous opioid system also has an important role during pregnancy and parturition. In addition to its direct roles, opioids also affect the prolactin and oxytocin systems, exerting additional indirect effects [109].

An interaction between opioid tone, GnRH secretion and subsequent release of LH and FSH has been proposed regarding to the menstrual cycle [112]. In the early follicular phase, the opioid tone is low, resulting in an uninhibited pulsatile GnRH secretion with a high frequency. The rise in estradiol levels towards the midfollicular phase is accompanied by a rise in the opioid tone. In turn, the rise in opioids promotes slower GnRH pulses leading to an increase in LH pulse amplitude. The frequency of GnRH and LH pulses is reduced in the luteal phase, caused by a high opioid tone induced through high progesterone levels [109]. A study with 131 healthy volunteers corroborated the postulated interaction, in which it was shown that β-endorphin levels, one of the endogenous opioid peptides which mainly act on μ-opioid receptors, increase during the follicular phase, reaching a maximum approximately 4 days before ovulation. The β-endorphin levels further increase in the luteal phase, before decreasing again before the next menstrual bleed. A positive linear correlation between endogenous opioids and progesterone levels during the luteal phase has been found [113].

The effect of opioids on the hypothalamic–pituitary axis (HPA) depends on the phase of the menstrual cycle. In ovariectomized rats, a high dose (10 mg/kg) of morphine increased LH but decreased it in low doses (1 mg/kg), which provides evidence that the effect of a single injection of morphine on LH is dose-dependent [114]. The primary mechanism by which opioids affect gonadotropin secretion is through their effects on GnRH. By in situ hybridization, GnRH mRNA levels are downregulated by opioids, suggesting that the suppression of the biosynthesis of GnRH may be stimulated by morphine [115]. Opioids have an effect on steroids via the suppression of gonadotropins. Gonadal steroids, in turn, decrease the effects of opioids on LH secretion. Opioids also play an important role in the feedback inhibition of LH by gonadal steroids. In female rats, both estradiol-mediated negative feedback and the estradiol surge-induced hypersecretion of LH are upregulated by chronic opioid treatment, suggesting that opioids amplify negative and

positive feedback on gonadotropin secretion [116]. Compared with saline controls, opioid antagonists promoted an increase in the LH surge [117].

A potential relationship between long-term opioid use and reduced libido, hypogonadism, and reproductive dysfunction in women has been reported [118–123]. A study that compared 68 non-opioid-consuming women control subjects with 47 women (aged 30–75 years) who were consuming sustained action oral or transdermal opioids for control of nonmalignant pain found that testosterone, estradiol, and dehydroepiandrosterone sulfate values were 48% to 57% lower in opioid-consuming women with intact ovarian tissue than in the control subjects. LH and FSH values were on average 30% lower in premenopausal and 70% lower in postmenopausal opioid consumers. Among oophorectomized women not consuming estrogen, free testosterone levels were 39% lower in opioid consumers, indicating impaired adrenal androgen production. Additional lowering of free testosterone levels was associated independently with oral estrogen replacement and low body mass index. Menstruation had often ceased soon after beginning sustained action opioid therapy. These results document hypogonadotropic hypogonadism and decreased adrenal androgen production in most women consuming sustained action oral or transdermal opioids [124]. In support of these results, Rhodin et al. [119] found that in the opioid-treated group, the patients had signs of pituitary dysfunction affecting all axes. Significant differences were shown in hypofunction of the hypothalamic–pituitary–gonadal axis and hyperfunction of the hypothalamic–pituitary–adrenal axis, and higher prolactin levels were found in the opioid-treated group compared with the control group. The degree of pain was rated the same in both groups, but the opioid-treated group reported more side effects and a lower quality of life.

Taken together, future research into the role of endogenous opioids in reproductive disorders may lead to a better understanding of the pathophysiology of reproduction as well as to novel treatments options.

6. Drug Abuse and Addiction to Opioids

Opium, extracted from the seeds of Papaversomniferum, has been known for millennia to relieve pain, and its use for surgical analgesia has been recorded for several centuries. Most likely, opium was the first narcotic substance discovered at the dawn of humankind. The history of drug addiction is immensely rich and allows to trace the long way that humankind has had to travel to reach the contemporary level of consciousness about narcotic substances. The Sumerian clay tablet (about 2100 BC) is considered to be the world's oldest recorded list of medical prescriptions. It is believed by some scholars that the opium poppy is referred to on the tablet [125]. Some objects from the ancient Greek Minoan culture may also suggest the knowledge of the poppy. A goddess from about 1500 BC is shown with her hair adorned probably with poppy capsules and her closed eyes insinuate sedation. Additionally, juglets probably imitate the poppy capsules found in that period in both Cyprus and Egypt. The first authentic reference to the milky juice of the poppy was found by Theophrastus at the beginning of the third century BC. In the first century, the opium poppy and opium were known by Dioscorides, Pliny, and Celsus, and later on by Galen. Celsus suggests the use of opium before surgery and Dioscorides recommended patients should take mandrake (which contains scopolamine and atropine) mixed with wine before limb amputation. The Arabic physicians used opium very extensively and in the 10th century; it was recommended by Avicenna, especially for diarrhea and diseases of the eye [125,126]. Polypharmacy, including a mixture of nonsensical medications, was often used. Fortunately, for both patients and physicians, many of the preparations contained opium. The goal was a panacea for all diseases. A famous and expensive panacea was theriaca, containing up to sixty drugs including opium. Simplified preparations of opium such as *Tinctura opii* were used up to about 2000 in Denmark. In the early 1800s, science had developed and Sertürner isolated morphine from opium and was the founder of alkaloid research. A safer and more standardized effect was obtained by pure opium [127].

Medical prescriptions for opioids started to increase in the 1990s, followed closely by significant increases in nonmedical use. Opioids are highly addictive, and the use of both

synthetic and natural opioids can quickly result in dependence, which includes physical and/or psychological dependence, as well as opioid use disorder (OUD). OUD is a chronic relapsing disorder that, whilst initially driven by activation of brain reward neurocircuits, increasingly engages anti-reward neurocircuits that drive adverse emotional states and relapse, which is also related to dramatically increased rates of morbidity and mortality. An important risk factor for OUD and overdose death is the availability and volume of medical prescriptions for opioids [128,129]. Moreover, in some countries such as the USA, it is possible to buy opioids such as tramadol without a prescription on the internet [130]. A complex interplay of structural, social, developmental, and behavioral risk factors is likely to have a role in the development of OUD. An individual risk factor is male sex [131,132]; more men misuse and are addicted to opioids than women. However, clinical reports suggest that, for opioids, similar to other drugs of abuse, women progress from initial use to addiction at a faster rate than men [133]. Sex differences in the opioid system have been reported in preclinical studies, which might underlie sex differences in sensitivity to pain or addiction [134]. In addition, women have more acute pain, chronic pain such as dysmenorrhea, and chronic pelvic pain, and are prescribed opioids more often than their male counterparts. Studies have shown that there have been increased rates of use and overdose deaths in women and that significant mental health concerns are higher for them than in men [135].

Some studies have evaluated chronic opioid use and addiction in EM patients, for example, by conducting a cohort study between 2006 and 2017 comparing women aged 18–50 years with EM (N = 36,373) to those without (N = 2,172,936) in terms of risk of chronic opioid use, opioid dependence diagnosis, and opioid overdose [136]. Chronic opioid use was defined as ≥ 120 days' supply dispensed or ≥ 10 fills of an opioid during any 365-day interval. EM patients had a four times greater risk of chronic opioid use compared to women without. Multimorbidity among these patients was associated with an elevated risk of chronic opioid use [136]. In another study, a retrospective cohort studied from 2011 to 2016 included 58,472 EM patients. Women who filled an opioid prescription within 12 months of diagnosis were placed in the opioid cohort and women who did not fill an opioid prescription were placed in the nonopioid cohort. Of these, 61.7% filled out an opioid prescription during the study period. More than 95% filled prescriptions for short-acting opioids (SAOs) only, 4.1% filled prescriptions for both SAOs and extended release/long-acting opioids (LAOs), and 0.6% filled prescriptions for LAOs only. Patients who filled an opioid prescription had higher baseline comorbidities (especially gynecologic and chronic pain comorbidities) and EM-related medication use compared with patients who did not fill out an opioid prescription. Patients who filled out both LAO and SAO prescriptions had the highest total day supply of opioids, the proportion of days covered by prescriptions, and morphine equivalent daily dose. These patients also had the highest proportions of opioid switching and dose augmentation in a retrospective analysis, with the study concluding that women with EM have higher probabilities of prolonged use of opioids and concomitant use with benzodiazepines compared with women without this condition [137,138]. An additional retrospective study from 2009 to 2018 evaluated all-cause and EM-related health care resource utilization and costs among newly diagnosed patients with high risk (≥ 1 day with ≥ 90 morphine milligram equivalents per day or ≥ 1 day concomitant benzodiazepine use) versus low risk opioid use or patients with chronic (≥ 90-day supply prescribed or ≥ 10 opioid prescriptions) versus non-chronic opioid use. Out of 61,019 patients identified, 18,239 had high risk opioid use and 5001 had chronic opioid use. The analysis demonstrated significantly higher all-cause and EM-related healthcare resource utilization and total costs for high risk opioid users compared to low risk opioid users among newly diagnosed EM patients over 1 year. Similar trends were observed comparing chronic opioid users with non-chronic opioid users, with the exception of EM-related pharmacy fills and associated costs [139].

Opioid addiction involves the hijacking of the endogenous opioid system [129]. The neurocircuitry of this addiction involves three stages: the intoxication stage (opioid intoxication and incentive salience), the withdrawal affect stage (opioid tolerance and withdrawal),

and the anticipation stage (opioid craving and relapse) [129]. In the intoxication stage, preclinical studies reported that the reinforcing effects of opioids are mediated in the ventral tegmental area (VTA) and nucleus accumbens (NAc) via not only dopamine-dependent but also drug-independent mechanisms [140] (Figure 3). Neurobiological mechanisms of tolerance range from opioid receptor desensitization and downregulation to cellular and circuitry allostasis [141,142]. The anticipation stage of the addiction cycle involves dysfunction of executive function. In humans, opioid addiction has a dysregulated hypothalamic–pituitary–adrenal stress axis; this dysregulation persists during cycles of addiction [143,144]. MORs, DORs, and KORs play different roles in the mechanisms of opioid addiction: MORs promote recreational drugs and adapt to chronic activation, which also leads to tolerance and dependence; KORs enable and sustain aversive states of withdrawal and abstinence; and DORs are involved in the improvement in mood states and facilitate context learning. All of them modulate motivation. Both MOR and KOR activities drive the onset, progression, and maintenance of an addiction. Nevertheless, the contribution of DORs remains less straightforward [145].
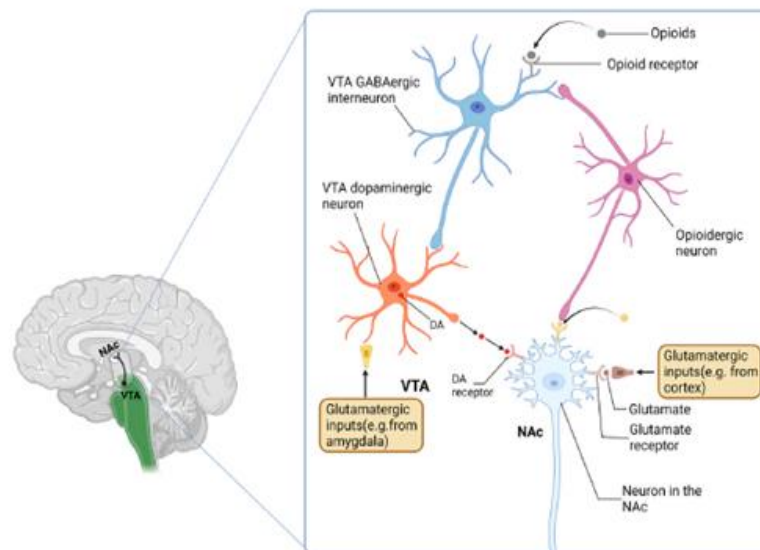

Figure 3. Rewarding actions of opioids in the VTA and NAc. Drug abuse, especially opioid abuse, has some significant effects on the area of VTA and NAc. γ -aminobutyric acid (GABA)ergic interneurons in the VTA are inhibited by opioids mainly via μ-opioid receptors but also by δ-opioid receptors. This leads to the disinhibition of VTA dopaminergic neurons and activation of reward circuitry in the Nac. Furthermore, reward circuitry is also activated directly by opioids through opioid receptors on NAc neurons. VTA: ventral tegmental area; NAc: nucleus accumbens.

More and more evidence supports the role of the N/OFQ–NOP system in addiction [146–148]. N/OFQ has a broad inhibitory effect on multiple neurotransmitter systems involved in drug reward and has been shown to decrease drug-induced dopamine levels in the nucleus accumbens [149,150]. Studies have shown that NOP agonists block the rewarding effects of morphine, cocaine, and alcohol in animal models of drug rewards such as the conditioned place preference (CPP) [148,151]. Furthermore, the fact that morphine-induced supraspinal analgesia and conditioned place preference are blocked by N/OFQ suggests that the N/OFQ–NOP system acts as an anti-opioid system for some responses. Targeting the NOP-N/OFQ system is therefore a potential approach to reduce the rewarding effects of multiple abused substances and develop pharmacotherapy to treat addiction to various drugs and possibly polydrug addiction. However, equivocal results with a few synthetic

small-molecule NOP agonists were obtained [148,151]. Furthermore, in September 2021, Grünenthal announced that the first participants have been enrolled in a randomized, placebo- and active-controlled clinical trial for its peripherally restricted NOP receptor agonist. The compound is being developed to provide a non-opioid therapy option that offers a strong analgesic effect without the side effects commonly associated with opioids. The experimental medicine trial will evaluate the extent and duration of the pharmacological effect of the oral NOP agonist in an experimental pain model. The results of the trial were expected to be available in early 2022, but we could not find them until the publication of this paper [152].

7. Conclusions

Even though the pathophysiology of EM-associated pain is not completely understood, strong pieces of evidence support that neurogenic inflammation may play a crucial role. As we discussed above, current treatment options are not the best choices for EM-associated pain as they not only interfere with ovulation but also are ineffective for all patients. Endogenous opioid peptides can be secreted from immunocytes, occupy peripheral opioid receptors on sensory nerve endings, and produce analgesia by inhibiting the excitability of these nerves or the release of pro-inflammatory neuropeptides. For several hundreds of years, opium has been used for pain relief, and nowadays, opioids remain a gold standard for the treatment of pain. In some cases, it is recognized that there is no substitute for opioids in achieving satisfactory pain relief. However, undesirable side effects following acute administration and long-term use limit their clinical effect. Additionally, the already observed risk of chronic opioid use and addiction in EM patients suggests that the pharmacological drugs available now are not the best option for these patients. More and more studies have suggested that multi-mechanistic opioids can be a valid alternative to traditional opioids for their safer profile. Particular interest has been paid to the role of the NOP receptor in terms of analgesia and improved tolerability. Emerging evidence has shown a promising functional profile of bifunctional NOP/MOP partial agonists as safe and nonaddictive analgesics [85,89,153]. While coactivation of NOP and MOP receptors may provide a viable treatment option for pain and drug abuse, caution is warranted for bifunctional NOP/MOP "full" agonists. NOP/MOP "partial" agonists provide considerable hope for the future of NOP-active compounds and the potential for opioid-type analgesics with reduced side effects and abuse liability. However, until now, the role of NOP receptors in EM has not been investigated. Therefore, this review offers a thought that still needs further investigation but may provide potential options for relieving EM-associated pain.

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Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

Publication list

1. **Guan Q**, Velho RV, Jordan A, Pommer S, Radde I, Sehouli J, Mechsner S. Nociceptin/Orphanin FQ Opioid Peptide-Receptor Expression in the Endometriosis-Associated Nerve Fibers-Possible Treatment Option? Cells. 2023 May 15;12(10):1395. doi: 10.3390/cells12101395.

2. **Guan Q,** Velho RV, Sehouli J, Mechsner S. Endometriosis and Opioid Receptors: Are Opioids a Possible/Promising Treatment for Endometriosis? Int J Mol Sci. 2023 Jan 13;24(2):1633. doi: 10.3390/ijms24021633.

3. **Guan QH,** Shi WJ, Zhou LS, Tao AL, Li L. Effect of epigallocatechin-3-gallate on the status of DNA methylation of E-cadherin promoter region on endometriosis mouse. J Obstet Gynaecol Res. 2020 Oct;46(10):2076-2083. doi: 10.1111/jog.14358. Epub 2020 Aug 24.

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